BIOLOGICAL ROLES OF A

DERMATAN SULPHATE PROTEOGLYCAN

BY

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

In Oral Biology

Faculty of Dentistry

Edmonton, Alberta

Spring, 1994

ISBN 0-612-11265-9

Canada

UNIVERSITY OF ALBERTA

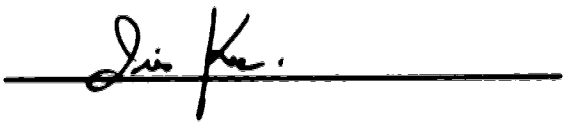
RELEASE FORM

NAME OF AUTHOR: Iris Mary Kuc

TITLE OF THESIS: Biological Roles of a Dermatan Sulphate Proteoglycan

DEGREE: Doctor of Philosophy

YEAR THIS DEGREE GRANTED: Spring, 1994



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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Biological Roles of a Dermatan Sulphate Proteoglycan submitted by Iris M. Kuc in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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To Ed, whose support and encouragment
were invaluable, as always

and

To Alanna, who made me understand
what its really all about.

ABSTRACT

Dermatan sulphate proteoglycan II (DS-PGII) is a small interstitial proteoglycan found within the extracellular matrix of connective tissues. By virtue of its ability to bind onto the surface of type I collagen fibrils, DS-PGII is thought to control the rate of collagen fibril formation, and modulate the diameter of the fibrils formed. The present study was undertaken to further define the role of DS-PGII in tissues.

Five bovine tissues: gingiva, sclera, skin, tendon and the temporomandibular (TMJ) disc composed predominantly of type I collagen, were selected for study. The investigation proceeded along three lines. 1) Fibroblasts were obtained from explant cultures and used as a source of DS-PGIIs for characterization. 2) DS-PGIIs extracted directly from the tissues were compared to those isolated in vitro and were used in an in vitro fibrillogenesis assay to examine effects on the rate of collagen fibril formation and the morphology of the fibrils. 3) Ultrastructural studies were carried out to establish any correlations between the properties of the various DS-PGIIs and the structure and organization of the tissues in which they are found.

DS-PGIIs isolated from culture media had identical core proteins and similar amino acid compositions between each other and to a previously defined bovine skin DS-PGII. The molecules varied only in the length of their dermatan sulphate (DS) glycosaminoglycan (GAG) side chain. Skin DS-PGII had the

smallest GAG chain while tendon DS-PGII had the largest. In addition, subpopulations of fibroblasts displayed GAG chain length heterogeneity within tissue types. Extracted DS-PGIIs were comparable to those isolated in vitro and all reduced the rate of collagen fibril formation, resulting in the formation of larger diameter fibrils. Ultrastructurally, collagen fibril diameters varied from tissue to tissue and DS-PGII could be demonstrated on the surfaces of fibrils with Cuprolinic Blue. Within a single tissue, TMJ disc, a correlation was found between the length of the DS chain and the interfibrillar spacing in two distinct zones. No such correlations could be established for the other tissues.

The results suggest that DS-PGIIs are modulators of extracellular matrix architecture, influencing the rate at which collagen fibrils are formed, their diameters, and possibly the packing of fibrils in the tissues.

Acknowledgement

I wish to thank the members of my advisory committee Dr. C. Cass and Dr. J. Osborn, for their useful comments and advice in this study. I am also indebted to Carole Dodd for her expert technical assistance in various aspects of this project and Colleen Murdoch for her guidance and assistance with the photography and electron microscopy. Finally, I wish to thank my supervisor, Dr. P. Scott, for the considerable time he has devoted to the completion of this study. His encouragement and advice were greatly appreciated.

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LIST OF ABBREVIATIONS

Abs	Antibodies
BSA	Bovine serum albumin
CHAPS	3(3-cholamide propyl) dimethylammonio-1-propanesulfonate
CS	Chondroitin sulphate
CS-PG	Chondroitin sulphate proteoglycans
dH_2O	Distilled water
ddH_2O	Double distilled water
DMEM-0	Dulbecco's Modified Essential Media without fetal calf serum
DMEM-10	Dulbecco's Modified Essential Media with 10% fetal calf serum
DMMB	1,9-dimethylmethylene blue
DS	Dermatan sulphate
DS-PG	Dermatan sulphate proteoglycan
DS-PGII	Dermatan sulphate proteoglycan II
E.M.	Electron microscopy
FACITS	Fibril associated collagens with interupted helixes
FCS	Fetal calf serum
GAG(s)	Glycosaminoglycan(s)
GlcA	Glucuronic acid
HA	Hyaluronic acid
HS	Heparan sulphate
HS-PG	Heparan sulphate proteoglycan
IduA	Iduronic acid

K_{av}	Partition coefficient, defined as: $K_{av} = \frac{V_e - V_o}{V_t - V_o}$
KS	Keratan sulphate
KS-PG	Keratan sulphate proteoglycan
mAbs	Monoclonal antibodies
NEM	N-ethylmaleimide
NGS	Normal goat serum
OCT	Optimum cutting temperature
PBS	Phosphate buffered saline
PG(s)	Proteoglycan(s)
PMSF	Phenylmethylsulfonyl fluoride
R_f	Relative mobility (to the front)
S.D.	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TCA	Trichloracetic acid
TGF-β	Transforming growth factor-β
TBS	Tris buffered saline
TMJ	Temporomandibular joint
V_e	Elution volume
V_o	Void volume
V_t	Total column volume

INTRODUCTION

1.1. Introduction

Connective tissue can broadly be defined as a material composed of cells, such as fibroblasts, macrophages and mast cells, enclosed within an extracellular matrix. The extracellular matrix most notably contains two major constituents: a fibrous component which includes the collagens and elastin and a ground substance containing the glycoproteins and proteoglycans.

Variation in the relative amounts of different matrix macromolecules and in their organization leads to a variety of diverse forms of tissues. Not only does connective tissue form the supporting stroma for cells, but it can also form highly specialized structures such as cartilage, bone and teeth. While in the past connective tissue has been regarded as a relatively inert framework stabilizing or forming the physical structure of an organ, in recent years it has been shown that this is not its sole role. Today, the extracellular matrix is known to play an active part in regulating the behaviour of contiguous cells, influencing their development, migration, proliferation and metabolism.

1.2. The Fibrous Component of Connective Tissues

1.2.1. The collagens

1.2.1A) structure. The collagens are a family of at least seventeen (Li et al, 1993) genetically distinct structural proteins found in the extracellular matrix of

connective tissues. All collagens are characterized by the presence of one or more triple-stranded helical ("collagenous") domains. Within these domains, the three polypeptide chains, called alpha-chains, which themselves are left-handed helices, are wrapped around one another to form a right-handed superhelix. Type I collagen is a molecule that is approximately 300nm in length and 1.5nm wide (for reviews see Chapman and Hulmes, 1984; Hay, 1981; Piez, 1984; Ramachandran, 1976; Van Der Rest and Garrone, 1991).

The alpha-chains within the collagenous domains are unique in that each consists of a repeating triplet (Gly-X-Y), where X and Y can be any amino acid but most often are proline and hydroxyproline respectively. Glycine is the only amino acid small enough to fit into the centre of the triple helix. The presence of hydroxyproline residues on the outside of the chain helps to stabilize the tertiary structure of collagen via the formation of intra- and inter-chain hydrogen bonds. Not all of the amino acids in the X and Y positions are proline or hydroxyproline. Other amino acid side chains at these positions participate in lateral interactions between collagen monomers.

1.2.1B) synthesis and assembly. The biosynthesis of collagen is best understood for type I collagen and as such it serves as a prototype for the synthesis of other collagens (for review Kivirikko and Myllylä, 1984). Individual polypeptide chains are synthesized on membrane-bound ribosomes and are transported into the lumen of the endoplasmic

reticulum as large precursor molecules called pro-alpha chains. Each pro-alpha-chain consists of three domains including a central "collagenous" domain made up of the Gly-X-Y amino acid triplets, flanked at both ends by large globular regions. In addition, at the N-terminal end there is a short hydrophobic "signal peptide". This signal peptide is a feature of all secreted proteins (Blobel et al, 1979) and directs the pro-alpha chain from the polyribosomes into the lumen of the endoplasmic reticulum. Once inside the lumen, a "signal peptidase" removes the peptide. It is also inside the lumen that susceptible lysine and proline residues are hydroxylated, and hydroxylysine subsequently glycosylated. Thereafter, three pro-alpha-chains align with one another, their juxtaposition thought to be guided by the formation of interchain disulphide bonds between the COOH-terminal globular domains. The COOH-terminal extension peptide is the most conserved sequence within the procollagen molecule (Miller, 1985) thus highlighting the significance of this region in collagen synthesis.

The assembled molecule is now referred to as procollagen and is transported out of the endoplasmic reticulum and into the Golgi apparatus where it is further modified and packaged into secretory vesicles for transport out of the cell. Once in the extracellular environment, the amino- and carboxyl-terminal peptide sequences are cleaved by specific peptidases. The collagen molecules thus produced have a marked ability to self-assemble and spontaneously form fibrils; their

association driven by electrostatic and hydrophobic interactions. Variations in the proteolytic processing of collagen and in levels of glycosylation and hydroxylation of the molecules may have important functional implications (see Van Der Rest and Garrone, 1991 for review).

Covalent cross-linkages form between collagen molecules located within the fibrils as a result of the action of lysyl oxidase, converting lysine or hydroxylysine residues to reactive aldehydes which then participate in the formation of various cross-links. Variations in cross-linkages occurs depending upon tissue type and age differences, with the proportion of reducible cross-links gradually diminishing with tissue maturation (Heathcote and Grant, 1980).

Type I collagen is the main constituent of tendon, bone, skin and blood vessels and is the most studied and best described of the collagens. Studies of the type I collagen fibril by electron microscopy and x-ray diffraction have provided information on its structure and organization. A model for the organization of collagen fibrils was initially provided by Hodge and Petruska (1963). They found that the two dimensional pattern of striated collagen molecules seen in electron micrographs is the result of a "67nm stagger". Collagen monomers assemble in parallel arrays and are mutually staggered by a distance referred to as "D", which is 67nm. D units have been shown to correspond to internally homologous zones within the collagen molecule. Since a collagen molecule is 4.4 D long, the nonintegral length results in overlaps (0.4

D) and gaps (0.6D)[fig 1.1].

Fig. 1.1. The regular staggering of molecules in a collagen fibril (Hodge and Petruska, 1963).

When collagen fibrils are stained with reagents such as metal salts, the resulting pattern under the electron microscope reveals the presence of a number of distinct bands. Five such groups of bands can be seen, denoted by the letters a to e; within each group additional bands are distinguished by a numerical suffix. In total twelve stained bands per period can be seen, the pattern being due to the uptake of heavy metal ions onto charged amino acid side groups. The asymmetrical nature of the pattern indicates the polarization of individual collagen molecules in a fibril. The banding pattern of collagen fibrils as seen in electron micrographs reflects the presence of D-staggered collagen molecules.

The three-dimensional organization of collagen fibrils is less well understood, although important differences exist in the three-dimensional packing of molecules between collagen types. It has been suggested that five collagen molecules are assembled in a 67nm stagger with each rotated by one fifth of a circle to make a closed helix. The resultant "microfibrils"

are therefore supercoiled, resulting in rope-like structure (Smith, 1968). An alternative theory suggests that the collagen fibril is a three-dimensional crystal, with individual molecules packed in a quasi-hexagonal array (Hulmes and Miller, 1979).

Fibrillar collagen comes in a variety of diameters and organizations depending upon the tissues and mechanical properties in which it occurs. An examination of the size and organization of collagen fibrils in various tissues clearly demonstrates the effects that such parameters have on the mechanical properties of the tissues (for review see Montes et al, 1984). For example, collagen fibrils in tendon may be up to several hundred nm in diameter (Parry et al, 1978) and are arranged into parallel bundles aligned along the major axis of stress. This provides the tissue with a high degree of tensile strength and enables it to transmit forces effectively in one direction. Compare this to the organization of collagen fibrils in skin; the fibrils are narrower and woven into a wickerwork pattern, allowing the tissue to withstand stress in several directions. Type I collagen fibrils in cornea are smaller yet, as little as 16 nm in the teleost cornea (Craig and Parry, 1981) and are orthogonally arranged, providing the tissue with optical transparency (Trelstad and Silver, 1981).

The formation of fibrillar collagen is a complex process that is dependent not only on intracellular events such as the oxidation of lysines and hydroxylysines to aldehydes, but is

also under the influence of extracellular factors. These include the conversion of procollagen to collagen and the subsequent influence of propeptides (Miyahara et al, 1984), the influence of the nonhelical ends of the collagen molecules (Comper and Veis, 1977a), the formation of extracellular compartments by fibroblasts to assist in matrix assembly (Birk and Trelstad, 1984; 1986; Birk et al, 1989) as well as the presence of other matrix molecules such as proteoglycans and fibronectin (Brokaw et al, 1985).

The assembly of collagen fibrils may be examined by looking at temperature-induced turbidity changes that occur when solutions of soluble collagen are heated. It has been shown that purified soluble collagen solutions in a defined solvent can reform fibrils that are identical to fibrils formed in vivo (Eikenberry and Brodsky, 1980; Williams et al, 1978). A typical turbidity-time curve (fig.1.2), illustrates the phases involved in collagen heat gelation; a lag phase, a growth phase and a plateau.

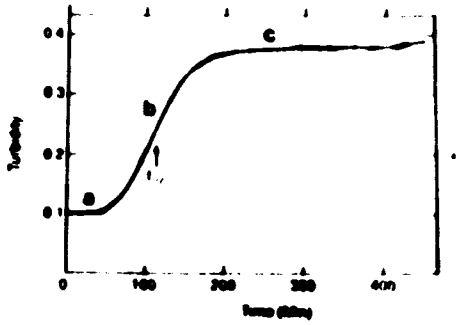

Fig. 1.2. Assembly of type I collagen in vitro to native fibrils. a) lag phase, b) growth phase, c) plateau phase. Modified from Piez, 1984.

During the lag phase, no change in turbidity is observed. Fibril nuclei or precursors are forming, with collagen monomers and the forming aggregates in a state of dynamic exchange (Wood and Keech, 1960). The aggregates have been shown to be 4-D staggered linear dimers and trimers (Silver et al, 1979; Silver and Trelstad, 1980). Once the aggregates become stable, they assemble rapidly, producing the large increase in turbidity that is seen during the growth phase. Previously it was thought that no significant aggregation occurred during the lag phase (Comper and Veis, 1977b) and thus no increases in turbidity were seen. However, it is now believed that aggregation is fairly extensive during the lag phase, and only when the forming linear oligomers undergo lateral growth do detectable changes in turbidity occur (Silver, 1981). Eventually an equilibrium is reached between monomer and the collagen fibril.

1.2.1C) collagen types. As many as seventeen collagen types have been defined to date, forming a wide range of structures. Thirteen have been characterized both at the protein and nucleotide levels, while novel collagenous sequences have been cloned for the remainder. Type I collagen is by far the most common and belongs to a group of collagens referred to as the fibrillar collagens. These collagens are all derived from a single ancestral gene (Vuorio and De Crombrugghe, 1990) and form the characteristic periodic-structured fibrils. Included in this category are Types I, II, III, V and XI. The fibrils formed from these collagens

are often heterotrophic, i.e. made up of more than one collagen type (Birk et al, 1988; Fleischmajer et al, 1990).

Types IX, XII, and XIV collagens belong to a group of collagens referred to as fibril-associated collagens with interrupted helixes (FACITs) (Gordon and Olsen, 1990). While these are non-fibrillar collagens, they are characterized by the presence of helical domains which serve as sites for the interaction and adhesion of the molecules to fibrillar collagens. These attachment sites may include chondroitin sulphate GAG chains, as described for FACITS found in cartilage (Huber et al, 1986) and vitreous humor (Yada et al, 1990). In cartilage, Type IX collagen is often cross-linked in a periodic fashion to Type II collagen (Eyre et al, 1987). Other, nonhelical domains are also present on FACITS and these are thought to serve as additional binding sites for other matrix molecules, such as proteoglycans (Vasios et al, 1988). Hence, FACITs are able to offer new functionalities to collagen fibrils via their attachment sites.

A third category of collagens are those which form sheets or protein membranes around tissues and organs, such as basement membranes and Descemet's membrane. Included here are Types IV and VII, and possibly Type X collagen. Type VI collagen forms a category of its own. Two molecules of Type VI collagen aggregate in a head-to-tail fashion forming dimers which assemble into tetramers and eventually into linear aggregates which look like beaded filaments (Mayne and Burgeson, 1987). The function of this collagen is still

obscure, even though it has been described as a minor component in most extracellular matrixes.

Lastly, Type VII collagen has been described as the collagen forming anchor fibrils. By binding to basement membranes and to anchoring plaques found close to basement membranes, it is able to form a network which entraps interstitial collagen fibrils and helps in the adherence of the epithelium and stroma.

1.2.2. Elastin

The presence of elastin fibers in the extracellular matrix of various connective tissues such as dermis, large blood vessels and lung, endow these tissues with the property of elasticity. Elastic fibers comprise two morphologically and chemically distinct components, an abundant amorphous component which accounts for up to 90% of the mature fiber and a microfibrillar component, made up of small fibrils roughly 10-12 nm in diameter and located mainly around the periphery of the amorphous material (for review see Gosline and Rosenbloom, 1984; Mecham, 1991a; Robins, 1988; Rosenbloom, 1987). Little is known about the characteristics of the microfibrillar component. The term elastin per se however, refers only to the amorphous component of the fibril which is responsible for its elastic properties.

In a survey examining the occurrence and amino acid composition of elastin throughout the animal kingdom, Sage and Gray (1979) found that virtually all vertebrates, except the jawless fish (Agnatha), contain elastin with similar

characteristics. They all contain multiple lysine-derived cross-links called desmosines and isodesmosines and are rich in glycine, proline and hydrophobic amino acids. Information concerning the structure of elastin has come about following the discovery of tropoelastin (Smith et al, 1968), a soluble protein whose amino acid composition is very similar to that of elastin, except for the absence of cross-links and a greater number of lysine residues (Sandberg et al, 1969). Tropoelastin has a molecular weight of 72,000 to 74,000 and is now considered to be the initial translation product, synthesized mainly by fibroblasts and smooth muscle cells. Following secretion into the extracellular matrix, tropoelastin is cross-linked by lysine oxidase (Siegel, 1980). Pyridine rings are formed when up to four lysine residues covalently link two to four tropoelastin molecules. The linkages are termed desmosines and isodesmosines.

The characteristic properties of elastin are due to the random orientation of hydrophobic regions held together with the desmosine/isodesmosine cross-links. The arrangement of the chains is random in an undisturbed elastic fiber and the resultant network of fibrils is considered to be in a state of maximum entropy. When stretched, the hydrophobic bonds are disturbed, water molecules cluster around the exposed hydrophobic regions and the decrease in entropy provides the energy needed for elastic recoil once the force is released (Gosline and Rosenbloom, 1984).

1.3. The Ground Substance

1.3.1. Proteoglycans

As mentioned, proteoglycans occupy a prominent part in connective tissues. One of the more striking features of these macromolecules is variability. Not only do they come in all shapes and sizes, but the tissues in which they are found can contain a variety of different populations. A number of questions have been asked regarding what roles these diverse macromolecules may play in the normal physiology of cells and tissues. While some of the functions of particular proteoglycans have been well established, others remain relatively obscure.

One of the most common physiological functions of the proteoglycans, by virtue of their interstitial location, is space-filling and they can regulate the flow and diffusion of macromolecules through the tissue. However, because they are found at other sites, such as the cell membrane, both as integral parts of cell surfaces or pericellularly as part of the glycocalyx, and as components of the basement membrane, the proteoglycans play multiple roles in cellular interactions. They are known to bind together extracellular matrix components, influence the binding of cells to the matrix and interact with other molecules, such as growth factors, both at cell surfaces and in the matrix (Ruoslahti, 1989).

Structurally, proteoglycans are macromolecules consisting of a protein core to which one or more glycosaminoglycan

chains (GAGS) are covalently bound (for review see Hascall and Hascall, 1981; Heinegård and Paulsson, 1984; Kjellén and Lindahl, 1991; Roden and Horowitz, 1979). Earlier studies by Meyer and others (for review see Roden, 1980) were mainly concerned with the GAGs and found them to be linear polymers made up of repeating disaccharide units: an amino sugar (either N-acetylglucosamine or N-acetylgalactosamine) and a uronic acid (either D-glucuronic acid or L-iduronic acid). A number of sulphate ester and carboxyl groups are further bound to the sugar residues, giving the GAG an unusually high charge density. Various combinations of the disaccharide units, as well as the type of glycosidic linkage between the residues (α- or β-configuration), and the number and location of sulphate groups (either 0, 1 or 2 in position C-4 or C-6) define the type of GAG as hyaluronic acid, chondroitin sulphate, dermatan sulphate, heparan sulphate, heparin or keratan sulphate (fig 1.3).

Fig. 1.3. The repeating disaccharide structure of glycosaminoglycans. Taken from Hardingham and Fosang (1992).

It has been well-established that all GAGs in their native state, except for hyaluronic acid, are covalently bound to a protein core. The associated core proteins of proteoglycans have been less well studied and it is only in recent years that the importance of the protein component in these macromolecules has been considered. At the present, no universal nomenclature system has been developed for proteoglycans, although common usage has been to name a proteoglycan according to the most prominent type of GAG which is bound the protein core.

Recently, a nomenclature has been suggested for interstitial proteoglycans based on the core proteins of these molecules. Using data from immunological cross-reactivities and peptide mapping, Heinegard et al (1985) suggested that the interstitial PGs be referred to as either large proteoglycans or small proteoglycans. Of the large PGs, further groupings can be made as to whether or not they are capable of binding to hyaluronic acid to form aggregates. Proteoglycans that bind to hyaluronan and form hugh multi-molecular aggregates are referred to as aggrecan (Doege et al, 1991) or versican (Zimmermann and Ruoslahti, 1989). The small PGs are further subdivided into two groups, I and II based or differences in the protein cores.

1.3.1A) chondroitin sulphate proteoglycans. By far the best studied and hence the best described proteoglycans have been the large aggregating interstitial proteoglycans of cartilage. In early work GAGs were extracted from tissues by proteolytic digestion, however this resulted in the degradation of the protein core to which they were attached. Early experiments by Malavista and Schubert (1958), using high-speed homogenization of cartilage followed by sequential centrifugation, led to the isolation of high molecular weight molecules with protein components. From these preparations, others proposed a structural model for the large PGs (Mathews and Lozaityte, 1958; Partridge et al, 1961). However, it was not until Mascall and Sajdera (1969) began to use chaotropic solvents, such as guanidine-HCl followed by density gradient

centrifugation in CsCl gradients, that intact proteoglycans were extracted from tissues, hence providing the breakthrough that was needed in examining their structure. By using associative conditions (i.e. 0.5M guanidine HCl), they were able to show that the proteoglycans formed large aggregates with hyaluronic acid. Alternatively, when dissociative conditions were used, proteoglycan monomers could be isolated. Together with classic protein chemistry and more sophisticated molecular biology techniques, a structural model for the cartilage proteoglycans has been worked out.

Following the isolation of PGs from bovine nasal cartilage, Hascall and Sajdera (1970) found the monomers to be large macromolecules with a molecular weight of 1-4 million daltons. Complete sequences have subsequently been determined for the protein cores of rat chondrosarcoma (Doege et al, 1987) and human cartilage CS-PGs (Doege et al, 1991). Partial sequences are known for bovine (Oldberg et al, 1987) and chicken (Sai et al, 1986) cartilage CS-PGs. With a molecular weight of about 210,000 daltons, the protein core contains five protein domains or three morphologically distinct regions (Fig.1.4).

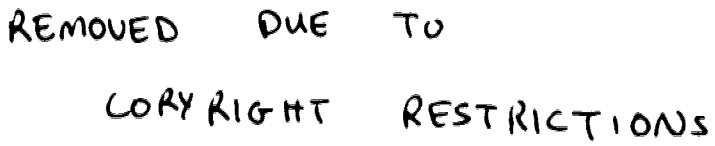

Fig. 1.4. Schematic presentation of cartilage proteoglycans and link protein. Taken from Heinegård and Oldberg, 1989.

A hyaluronic acid-binding region (Mr=65,000) is found at the amino terminal end of the protein core (Rosenberg et al, 1979; Stevens et al, 1984). It is a globular domain referred to as G1, made up of a double and single loop, containing no GAG chains and only N-glycosidically linked oligosaccharides (Heinegård and Hascall, 1974). It contains a binding site specific for a decasaccharide unit of hyaluronic acid (Christner et al, 1979), allowing for the formation of aggregates through the non-covalent interaction of the protein core and hyaluronic acid. The binding of the protein core to HA is further stabilized by a globular link protein (Baker and Caterson, 1979; Kimata et al, 1982) which has affinity for both the binding region of the proteoglycan and hyaluronic acid (Franzen et al, 1981; Hardingham, 1979). Homologous

amino acid sequences have been shown to occur in the G1 and the link protein (Goetinck et al,1987). The G1 domain is followed by an extended domain (E1) and by a second globular domain G2 (Paulsson et al, 1987); G2 is highly homologous to G1 (Doege et al, 1987, Neame et al, 1987), but does not bind to hyaluronic acid.

A keratan sulphate rich region is located adjacent to the G2 (Heinegård and Axelsson, 1977) and contains 50-60 keratan sulphate chains. These are composed of a repeating disaccharide of N-acetylglucosamine and galactose, with the sulphate groups found on position six in both residues. The chains are O-glycosidically-linked to serine or threonine residues on the protein core via N-acetylgalactosamine. This part of the protein core is made up of 23 consensus repeats of 6 amino acid residues with high amounts of glutamic acid and proline (Heinegård and Oldberg, 1989).

The major part of the core protein is made up of the chondroitin sulphate rich extended (E2) domain, accounting for more than 50% of the amino acids. This domain can be divided into two separate regions, the first containing 11 consensus repeats of 40 amino acids each and the second part made up of 7 consensus repeats of 100 amino acids (Doege et al, 1987). In each region, the serine residues in the Ser-Gly dipeptides are substituted by chondroitin sulphate chains. Approximately 100 chondroitin sulphate chains of various lengths are attached via a tetrasaccharide (D-glucuronosyl-galactosyl-galactosyl-xylosyl) which is O-glycosidically linked to serine

residues in the core (Lindahl and Roden, 1966).

An additional globular domain, G3, can be found at the carboxyl-terminal end of the molecule (Paulsson et al, 1987). This is a composite of two or three structural domains from different protein families. These include a carbohydrate-binding domain (Halberg et al, 1988) as well as a complement-regulating domain and an epidermal growth factor-like domain (Hardingham and Fosang, 1992; Zimmermann and Ruoslahti, 1989). Since more than half of the aggrecan monomers in cartilage extracts are without a G3 domain, its role in the extracellular matrix is questionable. Rather, its major function may be in intracellular processing and trafficking (O'Donnell et al, 1988).

Oligosaccharide chains are also present in the chondroitin sulphate and keratan sulphate regions of the protein core. Unlike G1, which contains N-glycosidically linked oligosaccharides, the remainder of the core protein contains a number of O-linked oligosaccharides of the type found in mucins (Lohmander et al, 1980).

As a result of their binding to hyaluronic acid, these proteoglycans are able to form supramolecular complexes which occupy very large hydrodynamic volumes relative to their molecular weight. This allows the proteoglycans to act as a "waterbed" around a network of collagen fibers, imparting a high degree of viscoelasticity to the tissues in which they are found. This property is critical for weight-bearing cartileginous tissues (Hardingham and Bayliss, 1990, Mascall

and Hascall, 1981).

In addition to being found in cartilage, similar high molecular weight aggregating chondroitin sulphate proteoglycans have been isolated from aorta (Gardell et al, 1980; Salisbury and Wagner, 1981), from cultures of aortic smooth muscle cells (Chang et al, 1983; Wight and Hascall, 1983), from tendon (Vogel and Heinegard, 1985), sclera (Rauch et al, 1986) and skin (Habuchi et al, 1986; Schmidtchen and Fransson, 1992).

The primary structure of a large CS-PG secreted by fibroblasts has been deduced from cDNA clones (Zimmermann and Ruoslahti, 1989). Although there are similarities between it and the sequence for the large CS-PG from rat chondrosarcoma (Doege et al, 1987), there are notable differences. The central GAG attachment site varies in that it contains attachment sites for only 12-15 CS chains in addition to sites for 20 N-linked oligosaccharides and a number of O-linked oligosaccharides. There are also epidermal growth factor-like repeats in the carboxyl-terminal end. Referred to as versican, the similarity of this molecule to other adhesion molecules has led to the speculation that it may participate in cell recognition, binding matrix components with cell surface glycoproteins.

1.1.1(b) heparan sulphate proteoglycans. Proteoglycans which contain heparan sulphate as their GAG are found predominantly as high molecular weight proteoglycans on cell surfaces and in basement membranes, although they may also be

present in the extracellular matrix. Several core proteins appear to represent this group of PGs (Iozzo, 1988), as such they are more aptly considered a "group" strictly on the basis of their GAG chains. Heparan sulphate consists of a repeating disaccharide unit of D-glucosamine and D-glucuronic acid or L-iduronic acid, derived from the epimerization of D-glucuronate by a specific C-5 epimerase (Lindahl et al, 1972). A unique feature of these proteoglycans is the presence of either acetyl or sulphate on the glucosamine residues.

Heparin proteoglycans differ from HS-PG in that their GAG chains are composed predominantly of repeats of D-glucosamine and L-iduronate. They also contain a larger proportion of sulphated glucosamine residues, with as many as three to four sulphate and one carboxyl group per disaccharide making them extremely anionic. Heparin sulphate PGs have been shown to be secreted by mast cells (Robinson et al, 1978), contain an antithrombin binding sequence (Lindahl, 1989) and modulate the alternative complement pathway by regulating the formation of C3 convertase (Kazatchkine et al, 1981).

Cell surface HS-PGs are common on most cells. They are bound to membrane receptors that recognize the heparan sulphate chains (Gallagher et al, 1988) or by intercalation of a transmembrane hydrophobic region of the core protein directly into the lipid bilayer (Iozzo, 1984). A great deal of structural diversity is present within this group of cell surface PGs, ranging from the relatively small HS-PG associated with the surface of hepatocytes (Kjellén et al,

1981), to the significantly larger molecule found on the surface of fibroblasts (Fransson et al, 1984) and the extremely large HS-PG synthesized by human colon carcinoma cells (Iozzo, 1984). Because of their position, it has been shown that HS-PGs can mediate cell attachment (Lark and Culp, 1984) and can bind to a variety of matrix molecules including fibronectin (Heremans et al, 1990, Laterra et al, 1983), collagen (Koda and Bernfield, 1984), laminin (Timpl, 1989) as well as fibroblast growth factors (Burgess and Maciag, 1989). In addition, heparan sulphate chains have been shown to self-associate (Fransson et al, 1983). This property is thought to play a major role in cell-cell recognition and interactions (Dietrich et al, 1983), as well as providing a matrix anchor for migrating cells (Gallagher et al, 1986). HS-PGs are also thought to influence the growth of cells (Chiarugi and Vannucchi, 1976). Together, these properties have direct implications in cancer biology, in that alterations in the proliferation, recognition, adhesion and migration of cells may contribute to the neoplastic phenotype (Iozzo, 1988).

HS-PGs are also a component of basement membranes, where they are bound by tight, non-covalent interactions of the core protein with Type IV collagen and laminin (Hassell et al, 1980; Kanwar and Farquhar, 1979). Their presence is thought to play a role in the charge-selectivity of glomerular filtration (Kanwar et al, 1980). As well, the integrity of the basement membrane appears to be essential for normal morphogenesis as illustrated in developing mammary gland

(Gordon and Bernfield, 1980).

More recently, a group of cell surface proteoglycans bearing heparan sulphate and chondroitin sulphate chains have been described and sequenced (Mali et al, 1990). These molecules, called syndecans, are characterized by a short COOH-terminal cytoplasmic domain, a transmembrane domain which shows a great deal of sequence homology between species and a large ectodomain, which bears the GAG attachment sites. The ectodomain however, varies considerably in GAG composition. It has been proposed that the binding of various matrix molecules to syndecan, such as collagen types I, III, V, fibronectin and thrombospondin, may allow for the relay of information to the cell. Syndecans may thus act as receptors for signal transduction (Hardingham and Fosang, 1992).

1.3.1C) keratan sulphate proteoglycans. Although keratan sulphate has now been identified in a number of connective tissues, it was originally thought to be restricted to the KS-rich region of the large aggregating CS-PGs (Heinegård and Axelsson, 1977) (see section 1.3.1A.) and to cornea, where one or two KS chains can be found on a core protein of Mr 40,000-50,000 (Poole, 1986). The corneal KS-PGs, referred to as lumican (Blochberger et al, 1992), were found to be unique in that the attachment of the GAGs to the core is via an N-glycosidic bond linking N-acetylglucosamine to asparagine (Nakazawa et al, 1983). This type of linkage is not known to occur in other PGs.

With the development of antibodies to the sulphated

carbohydrate moieties of keratan sulphate (Caterson *et al*, 1983), the KS was found to be widespread. Heinegård *et al* (1986) had described the occurrence of a 59 kDa protein in cartilage, which later was shown to be substituted with KS and has since been identified in sclera, tendon and cornea (Oldberg *et al*, 1989). Although this molecule shares homology to lumican (Blochberger *et al*, 1992), the molecule is nonetheless a distinct entity. It has been termed "fibromodulin" because of its ability to delay (i.e. modulate) collagen fibrillogenesis by binding to type I and type II collagen *in vitro* (Hedbom and Heinegård, 1989). In addition, the amino acid sequence of fibromodulin has been deduced from cloned cDNA and has also been found to be homologous to DS-PGI and DS-PGII (see section 1.3.1D) throughout its entirety (Oldberg *et al*, 1989). Fibromodulin has also been shown to contain 4 potential sites for KS substitution via *N*-glycosidic linkages to asparagine (Plaas *et al*, 1990), identical to those described for corneal KS-PGs.

Fibromodulin is believed to have an important role in the organisation of the extracellular matrix by virtue of its ability to bind to type I and type II collagen (Hedbom and Heinegård, 1989). The ultrastructural location of KS-PGs have been identified in the corneal matrix with Cuprolinic Blue staining. Like DS-PGII (see section 1.4.1C), they have been shown to bind to type I collagen fibrils in a periodic array, but localised to the a and c bands rather than the d and e bands (Scott and Haigh, 1988).

1.3.1D) dermatan sulphate proteoglycans. A number of tissues contain low molecular weight, non-aggregating proteoglycans in which some of the D-glucuronate residues of chondroitin sulphate have been epimerized to L-iduronate (Malmstrom et al, 1975). Disaccharide units containing L-iduronate and D-glucuronic acid are often distributed in a copolymeric fashion (Fransson and Malmstrom, 1971), hence the amount of iduronate content in the GAG chain can vary from 10 to 85% of the hexuronate (Sheehan et al, 1981). In addition, the length of the GAG chain shows considerable polydispersity, ranging from a molecular weight of 15,000 to 56,000 (Poole, 1986). The molecules carrying DS GAG side chains are often called dermatan sulphate proteoglycans (DS-PG).

These molecules have been described in a variety of fibrous connective tissues including bovine cartilage (Rosenberg et al, 1985), tendon (Vogel and Heinegard, 1985), sclera (Cöster and Fransson, 1981), bone (Sato et al, 1985), human cervix (Uldbjerg et al, 1983) and rat, calf, pig and bovine skin (Damle et al, 1982; Fujii and Nagai, 1981; Miyamoto and Nagase, 1980; Pearson et al, 1983), bovine periodontal ligament (Pearson and Gibson, 1982), temporomandibular joint disc (Scott et al, 1989), and parietal pericardium (Simionescu et al, 1988), among others. In addition to being found in adult tissues, these molecules have also been described in fetal tissues including bovine tendon (Vogel and Evanko, 1987), developing human bone (Fisher et al, 1987) and human articular cartilage (Melching and Roughley,

1989). As well, human fibroblasts in culture secrete iduronate-rich proteoglycan(s) (Carlstedt et al, 1981; Gallagher et al, 1983, Glossl et al; 1984), as do cultured bovine aortic smooth muscle and endothelial cells (Jarvelainen et al, 1991).

These proteoglycans represent a family of similar molecules. Some of their unifying features include a protein core which, after removal of the DS chain(s) with chondroitinase ABC, has a mobility on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis corresponding to a molecular weight of 45 to 48kDa. The core is rich in aspartic acid, glutamic acid and leucine but low in serine and glycine (as compared to the large cartilage-specific proteoglycans). A number of oligosaccharides have also been identified in DS-PGs, covalently bound to the core protein by N-glycoside linkages between N-acetylglucosamine and asparagine (Glössl et al, 1984; Nakamura et al, 1983; Scott and Dodd, 1990; Shinkai et al, 1983). As a result, the total molecular weight of these molecules ranges from 80,000 to 140,000.

Recently, two distinct species of dermatan sulphate proteoglycans have been isolated from mature bovine articular cartilages (Rosenberg et al, 1985). Although these molecules show similar mobilities on SDS-PAGE at low ionic strength (0.01 M phosphate), when gel electrophoresis was performed under conditions of higher ionic strength (0.375 M Tris), Rosenberg et al were able to demonstrate two distinct species

of molecules. The smaller of the species, DS-PGII/decorin, was found to have a molecular weight ranging from 87,000 to 120,000 and is representative of the widely distributed DS-PG described above. The larger molecule, DS-PGI/biglycan, was shown to have a molecular weight of 165,000 to 285,000. In addition, they were able to demonstrate self-association of DS-PGI, under conditions of high ionic strength, but not DS-PGII. The two molecules were also immunologically distinct; neither DS-PGI nor DS-PGII reacted with antibodies raised to a cartilage-specific proteoglycan monomer from bovine articular cartilage. In addition, antisera raised against a mixture of DS-PGI and DS-PGII reacted strongly in an enzyme linked immunosorbent assay with DS-PGII, but only weakly with DS-PGI, suggesting different core proteins.

It is now known that these two forms of dermatan sulphate proteoglycan are found in a number of connective tissues including fetal bovine tendon (Vogel and Evanko, 1987), bovine articular cartilage and fetal skin (Choi et al, 1989) and adult human bone (Fisher et al, 1987), although in bone the GAG chain is CS. The expression of DS-PGI and DS-PGII however is not totally overlapping. Although DS-PGII appears to be found in virtually all type I collagen rich tissues, the distribution of DS-PGI is far more specialized. DS-PGI has been identified in differentiating keratinocytes, endothelial cells and renal tubular epithelial cells (Bianco et al, 1990). These differences suggest that DS-PGI and DS-PGII may possibly be synthesized by different cell types and differ in their

functional roles.

The protein sequences for both human bone PGI and PGII have been determined (Fisher et al, 1989) as well as for bovine articular cartilage PGI (Neame et al, 1989). Fisher et al (1987) had earlier shown that the NH_2 terminal sequences of PGI and PGII were different and hence were able to use this data to probe a cDNA library. They were able to show that the sequence for PGI was unlike that of PGII, although there was 55% homology suggesting that the two proteins may be the result of a gene duplication. Neame et al (1989) were also able to show 55% homology of their PGI to the deduced cDNA sequence of PGII from human bone (Day et al, 1987). The gene for PGI has been localised to the long arm of the human X chromosome while that for PGII has been shown to be localised to the proximal short arm or long arm of chromosome 12, further confirming that these PGs are products of separate genes (McBride et al, 1990).

Neame et al (1989) found the primary structure of PGI from articular cartilage to consist of 331 amino acids, giving the unsubstitited protein a molecular mass of 37,280 Da. They describe the presence of four domains. Domain 1 is 22 amino acids in length and represents the NH_2 terminus. It contains two possible attachment sites for GAGs as determined by the consensus sequence Asp/Glu-X-X-Ser-Gly-hydrophobic (Bourdon et al, 1987), hence giving the molecule the name "biglycan". It has been shown that two GAG chains are attached to serine residues at position 5 and 11 or 10 (Choi et al, 1989; Fisher

et al, 1987). Domain 2 is made up of 28 amino acids and contains cysteine contributing to at least one disulphide bond. Domain 3 forms the majority of the molecule and contains 231 amino acids which are leucine-rich and form 10 repeating units. The COOH-terminal comprises the final domain. In addition, they identified two sites for attachment of N-linked oligosaccharides.

A similar primary structure has been described for human bone PGI (Fisher et al, 1989). In this instance, PGI was found to be made up of 338 amino acids giving the core protein a molecular weight of 37,983. They also describe a GAG attachment region containing two possible sites at the NH_2 terminal followed by a short cysteine-rich region. A large leucine-rich segment makes up the bulk of the molecule and contains 12 repeats, ending with a hydrophobic COOH-terminal.

The complete sequences of PGII from human fibroblasts (Krusius and Ruoslahti, 1986) and bovine bone (Day et al, 1987), have been described. Using cDNA clones, Day et al found the core protein of PGII to be 330 amino acids in length with a molecular weight of 36,383 kDa. Only one possible recognition consensus sequence for a GAG attachment site was found. This agrees with previous observations which place the site of GAG attachment to a serine residue at position 4 in bovine skin PGII (Chopra et al, 1985). In addition, there were three potential acceptor sites for N-linked oligosaccharides. The existence of three N-linked oligosaccharides, localised to the C-terminal end of the

protein core in bovine skin PGII, has recently been confirmed by Scott and Dodd (1990).

Krusius and Ruoslahti (1986) found that the PGII molecule had a core protein of 329 amino acids with a molecular weight of 36,319. Their molecule showed 87% homology at the nucleotide level and 90% homology at the protein level with the sequence obtained for the bovine bone molecule.

Ward et al (1987) have used the technique of rotary shadowing electron microscopy to examine the structure of DS-PGs obtained from bovine sclera. Both DS-PGI and DS-PGII have been isolated from bovine sclera (Cöster et al, 1981) and biochemical studies (Cöster and Fransson, 1981) had suggested that DS-PGI has a number of GAG chains attached to the core protein, while DS-PGII has only one or two. Rotary shadowed images of the proteoglycans illustrate globular proteins with either a single unbranched extension or "tail" in the case of DS-PGII or in the case of DS-PGI, a "branched" extension. Digestion with hydrofluoric acid removes the extensions, indicating that the "tails" are GAGs. Similar features have been described for small proteoglycans purified from bovine cartilage, sclera, tendon, aorta, cornea and bone (Mörgelin et al, 1989). In each case, the core protein was seen as a small globular particle substituted with one or two GAG chains. The major structural difference between the PGs from different tissues was in the lengths of the GAG side chains. Measurements taken from electron micrographs showed that while DS-PGI contained the longest side chains, all but in

particular samples from bone, displayed considerable polydispersity in chain length.

Electron micrographs of scleral samples (Ward et al, 1987) have also shown evidence of aggregation of PG monomers via globular region-globular region interactions for DS-PGII and both globular region and side-chain interactions for DS-PGI. Mörgelin et al (1989) described similar aggregation of the core proteins, but whether these interactions are artifacts or physiologically relevant remains to be seen.

Additional studies investigating the physical structure of DS-PGII isolated from bovine skin have shown the protein core to be a typical globular protein, with circular dichroism spectra suggesting that the molecule contains a high portion of beta-sheet and beta-turn (Scott et al, 1986). The same studies have demonstrated the presence of three disulphide bridges, two located near the N-terminal end and one near the C-terminal, which contribute to the stability of the native structure.

1.3.1E) biosynthesis of proteoglycans. A large body of information has been established regarding the biosynthesis of the large cartilage-specific proteoglycans. This information forms the basic scheme that characterizes the synthesis of all proteoglycans. In the following discussion, specifics concerning the synthesis of the DS-PGs will be highlighted.

The first step in the biosynthesis of proteoglycans involves the formation of a protein core, resulting from the transcription of the gene and the translation of resultant

mRNA on ribosomes on the rough endoplasmic reticulum of the cell (for reviews see Heinegard and Paulsson, 1984; Poole, 1986, Roden and Horowitz, 1978). Following entry into the endoplasmic reticulum, the assembly of N-linked high-mannose containing oligosaccharides begins, through the transfer of dolichol diphosphate intermediate oligosaccharides to asparagine residues on the nascent polypeptide chain.

The assembly of glycosaminoglycan chains and O-linked oligosaccharides takes place within the Golgi apparatus. The first step in the substitution of the GAG chain onto the core protein involves the formation of an O-glycosidic linkage between D-xylose and the hydroxyl group of a serine or, less often, a threonine residue (Roden, 1980). The reaction is catalyzed by xylosyltransferase; the presence of xylosyltransferase in the endoplasmic reticulum suggests that xylosylation of the protein core may also occur before the Golgi (Hoffman et al, 1984, Glössl et al, 1986). A single xylosyltransferase is involved in the synthesis of all CS-, DS-, and HS-PGs (Esko et al, 1985). As well, the specificity of the xylosyltransferase suggests that a PG core protein contains a recognition signal within it's amino acid sequence which facilitates the substitution of serine residues (Bourdon et al, 1987; Roden et al, 1985). The sequence has been defined as Ser-Gly-Xaa-Gly, where Xaa can be any amino acid (Bourdon et al, 1987), although other sequences have been known to be substituted. Mann et al (1990), using in vitro mutagenesis and synthetic peptides, have been able to show

that conservative substitutions in the sequence in decorin (such as replacment of a serine residue for a threonine residue) is still compatible with GAG attachment. This suggests that conformation of the attachment site may be more important than sequence.

Chain lengthening of the GAG then proceeds via the addition of simple sugars from uridine sugar precursors, which include UDP-glucose and UDP-N-acetylglucosamine on to a Gal-Gal-Xyl-core protein. Epimerization of these precursors at the C-4 position results in the formation of UDP-galactose and UDP-N-acetygalactosamine. Several glycosyltransferases catalyse the reactions resulting in chain lengthening. However, as with the xylosyltransferase, it has been found that a single galactosyltransferase (I) is responsible for the first step in the synthesis of CS/DS- and HS-PGs (Esko et al, 1987). Quentin et al (1990) demonstrated that a deficiency in galactosyltransferase I was responsible for a reduction in the secretion of a mature glycosaminoglycan chain-bearing molecule. Non-glycosylated PGII core protein was secreted by fibroblasts cultured from a patient who represented a progeroid variant with signs of Ehlers-Danlos syndrome.

The GAG chain is completed following the transfer of sulphate groups from 3'-phosphoadenosine-5'phosphosulphate (PAPS). The transfer is catalysed by sulphotransferases specific for both 4- and 6-sulphates.

An additional epimerization step, catalysed by a uronosyl C-5 epimerase, converts glucuronic acid to iduronic acid in

tissues that contain dermatan sulphate (Malmström et al, 1975; Malmström, 1984). The amount of conversion is highly variable (Fransson et al, 1970; Shinomura et al, 1983). It has been shown both with cultured skin fibroblasts (Silbert et al, 1986; Silbert et al, 1991) and microsomal systems (Malmström, 1984) that the degree of epimerization is dependent upon the degree of 4-sulphation of glucuronate. For example, when human fibroblasts were grown in a sulphate-rich media, all of the uronic acid in the GAG chain was epimerized to iduronic acid (Silbert et al, 1986).

Recently Cöster et al (1991) demonstrated the importance that the protein core may have in regulating the activity of uronosyl C-5 epimerase and 4-sulphotransferase. When endogenous primers, such as β-D-xyloside, were added to cultured fibroblasts, a decrease in the content of L-iduronic acid and 4-sulphated N-acetylgalactosamine residues on both the β-D-xyloside and protein core of large and small proteoglycans was seen. Because overall protein synthesis did not change with increasing levels of xyloside, it is thought that the PG core protein itself may be modulating the activity of the enzymes.

In the case of DS-PGII, either the presence of GAG chains or asparagine-bound oligosaccharides, but not both, is necessary for secretion once the molecule is synthesized (Glössl et al, 1984). Treatment of human fibroblast cultures with p-nitrophenol-β-D-xyloside alone resulted in the secretion of core protein devoid of dermatan sulphate chains.

When tunicamycin only was added to the cultures, protein cores devoid of N-linked oligosaccharides were secreted. However both inhibitors together greatly reduced the amount of secreted DS-PGII.

It has been suggested that phosphorylation may act as a signal for the intracellular routing of PGs from the endoplasmic reticulum, through the Golgi apparatus into the extracellular space (Glössl et al, 1986). They showed that both the core protein and the GAG chain of DS-PGII secreted by cultured human skin fibroblasts became phosphorylated as an early post translational modification. They suggest that phosphorylation may play a role in the intracellular routing of PGs in much the same way as the mannose 6-phosphate system (Brown et al, 1986). Phosphorylation of other PGs (PG from Swarm rat chondrosarcoma) has been also been shown (Oegema et al, 1984).

It is becoming evident that glycosaminoglycan chains are not randomly sulphated, epimerized or polymerized. Although the structure of GAG chains are all based on a repeating disaccharide unit, selective sulphation, epimerization and polymerization may produce chains with specific properties. For example, the aggrecan expressed in experimental osteoarthritic cartilage contains CS chains that are consistently longer than controls (Caterson et al, 1990). The biological functions of these synthetic changes has yet to be defined, but suggests that the expression of GAGs is not static.

1.3.1F) degradation of proteoglycans. Limited data are available regarding the catabolism of matrix proteoglycans. However, it appears that PGs can initially be degraded in the extracellular matrix by cleavage of the protein core by a variety of proteolytic enzymes, including metalloproteases and various cathepsins (Heinegård and Paulsson, 1984; Kresse and Glossl, 1987; Poole, 1986). GAGs are not thought to be cleaved extracellularly, but rather pinocytosed by the cells and degraded in the lysosomes.

In addition to degradation in the extracellular matrix, intact proteoglycans may also be transferred directly to lysosomal compartments for degradation by cathepsins and endoglycosidases. Earlier studies showed that arterial and skin fibroblasts could specifically bind and internalize DS-PGs via receptor-mediated pinocytosis (Truppe and Kresse, 1978). Receptor-mediated endocytosis of DS-PGs depends on a recognition marker residing on the protein core of DS-PGII, which could interact with a endocytosis receptor (Glössl et al, 1983; Hausser et al, 1989). The importance of endocytosis in the metabolism of DS-PGII has been shown quantitatively. Interference with endocytosis by interaction with core-directed antibodies or by binding to collagen fibrils results in excess accumulation of DS-PGII (Schmidt et al, 1990), suggesting that biosynthesis is not coupled to degradation. However, the physiologic significance of this "secretion-recapture" pathway in vivo has yet to be defined.

1.3.2 Glycoproteins

1.3.2A) fibronectin. Fibronectin is a 540,000 kDa, widely-distributed, adhesive glycoprotein found in the extracellular matrix (reviewed by Dufor et al, 1988; Hynes, 1985; Pierschbachter et al, 1985; Ruoslahti and Pierschbachter, 1987; Yamada and Kleinman, 1992; Yamada et al, 1985). It is formed by two subunits, each about 2500 amino acids long, which are linked with a pair of disulphide bonds close to the carboxyl end. Each chain is folded into multiple globular domains which are separated by flexible domains. Various forms of fibronectin are produced depending on the secreting cell type; however, they are all encoded by a single large gene containing at least 48 exons. The different forms are produced by differential RNA splicing. Fibronectins can be found as soluble dimeric forms in body fluids, as oligomers which are transiently attached to cell surfaces or as insoluble fibrils within the extracellular matrix.

The different domains of fibronectin mediate binding to cell surfaces, collagen or heparin. The recognition sequences, Arg-Gly-Asp (RGD) or Leu-Asp-Val (LDV) are important for binding to cell surface receptors. However, the RGD motif is not specific to fibronectin and is found in more than 100 other proteins. The multiple domains of fibronectin are thought to contribute to the organization of the extracellular matrix and help mediate adherence between the matrix and cells. In embryogenesis, fibronectin appears to be important in cell migration.

1.3.2B) laminin. Laminin is an adhesive glycoprotein composed of 1 heavy and 2 light polypeptide chains (designated A, B1 and B2 respectively) which are arranged to form a 900 kDa cross-like structure stabilized by disulphides (reviewed by Beck et al, 1990; Engel, 1992; Engvall, 1993; Kleinman et al, 1985; Mecham, 1991). Variant laminin chains result in at least 4 alternative laminin forms which are expressed in different tissues and at different times in development. Laminin is the first extracellular matrix glycoprotein to appear in development; it is present as early as the 2 cell stage in the mouse embryo. In mature tissues, laminin is predominantly localized in the basement membrane.

The 3 short arms and 1 long arm of laminin's cross-like structure contain several functional domains which mediate binding to Type IV collagen, entactin (nidogen), heparin sulphate and self-association into large aggregates. In addition, binding to cells is mediated by an extensive list of cell surface laminin receptors which includes several integrins and a variety of non-integrin proteins. Different cell receptors can recognise different domains on the laminin molecule. The resultant complex interactions between the multiple laminin domains with extracellular matrix components (primarily in the basement membrane) and cells influence a variety of biologic activities which include cell attachment, movement, differentiation and growth, neurite outgrowth and stabilization of the basement membrane.

1.4. Physiological Functions Attributed to the Small Interstitial Proteoglycans.

1.4.1. Collagen-proteoglycan interactions

1.4.1A) evidence from age and development studies.

Changes in collagen fibril diameters have been shown to correlate with changes in the amount and types of GAGs in tissues as they age. A number of authors have noted an increase in the amount of DS in tissues, such as skin and tendon, as the collagen content of the tissue increases and matures (Breen et al, 1970; Hoffman et al, 1957; Kawamoto and Nagai, 1976; Loewi and Meyer, 1958; Nakamura and Nagai, 1980, Scott et al, 1981). Concomitantly, there appears to be a decrease in the amount of HA and CS. It has been speculated that the high concentrations of hyaluronic acid and CS-PGs surrounding the immature fibrils may control their radial expansion and fusion, the effects of which are diminished as the concentration of DS-PGs increase in the tissue (Scott et al, 1981).

Parry et al (1982) did an extensive study on the GAG composition and mass-average diameters of collagen fibrils from a wide variety of connective tissues at different ages. When they examined individual GAGs as a fraction of total, they found that tissues with small diameter fibrils (<60 nm) had high concentrations of HA, tissues with intermediate sized fibrils (60-150nm) had a high portion of CS, while the largest diameter fibrils (>150 nm) were found in tissues that had the highest concentrations of DS. They too hypothesized

that the lateral growth of fibrils may be inhibited by excess amounts of HA. This is further supported by data that show elevated levels of HA in fetal tissues and healing wounds in which small diameter fibrils are forming (Mathews, 1975). The inhibitory effect of hyaluronic acid on fibril formation may be removed by increasing the concentration of CS and/or DS during tissue maturation, allowing for the development of larger diameter fibrils.

1.4.1B) evidence from functional and morphologic studies. Parry et al (1982) suggested that tissues subjected to increasing tensile loads during their development show further increases in the amount of DS, which in turn allows for additional lateral aggregation of collagen fibrils. They speculated that the cells may be synthesizing increasing amounts of DS in response to the changing mechanical environment. Support for their hypothesis came from the rabbit flexor digitorium profundus tendon, a tendon which is subjected to contiguous and simultaneous tensile and compressive forces during normal function (Gillard et al, 1979). The compressive zone of the tendon contains a GAG content of 2.3-3.5% of which 60% is CS. The tensile zone however, has a GAG content of only 0.2%, of which 70% is DS. Ultrastructural studies have found collagen fibril diameters of 150nm in the compressive zones, but 200nm in the tensile zones (Merrilees and Flint, 1980), consistent with the idea that collagen fibril diameters may be related to the GAG composition and functional characteristics of a tissue.

Additional studies have also demonstrated a correlation between increasing fibril diameters and decreasing GAG content (Flint et al, 1984; Merrilees et al, 1987; Scott et al, 1981).

Vogel et al (1986) initiated fibroblast cultures from two regions of the bovine deep flexor tendon. They isolated fibroblasts from areas that were subjected to tensile forces (proximal zone) and compressive forces (distal zone). Fibroblasts from the proximal/tensional zone secreted predominantly DS-PGs, while fibroblasts obtained from the distal/compressed regions secreted predominantly CS-PGs and much smaller amounts of DS-PGs. Failure to provide compressive loads in vitro resulted in a decrease in the synthesis of CS-PGs (Koob and Vogel, 1987). Their results appear to suggest that cells subjected to different mechanical forces may be stimulated to secrete one product over another. As well, collagen fibril diameters in the CS-PG rich distal region were smaller than those in the proximal zones (Evanko and Vogel, 1990).

1.4.1C) evidence of binding to collagen from ultrastructural studies. Evidence to suggest that DS-PGs can interact with collagen came originally from ultrastructural studies. Proteoglycans lack any distinct morphological characteristics in tissue sections, but because they are polyanionic, they can be localized by cationic probes. Heavy metal cations, basic dyestuffs, and polycations all react with the GAG chain of the PG via a simple ion-exchange process. The phenomenon is unspecific as any polyanionic substrate can

react with a cationic stain to form a coloured complex. A number of cationic substances have been used in electron microscopic studies to demonstrate proteoglycans, including Alcian Blue (Ruggeri et al 1975), Ruthenium Red (Luft 1971), Toluidine Blue O (Shepard and Mitchell 1976), vital dyes such as lysozyme (Eisenstein et al, 1970) and heavy metals such as colloidal iron (Curran et al, 1965).

This basic principal can be further manipulated to give considerable specificity by using the critical electrolyte concentration method (CEC) (Scott, 1973). When dyes are used in conjunction with an electrolyte, such as Mg^{2+}, differences in the relative affinities of carboxylates ($-COO^-$), phosphate esters ($-PO_4^-$) and sulphate esters ($-SO_4^-$) for the electrolyte enables discrimination between the anions. It is particularly useful that sulphate esters are the last to give up dye in competition with Mg^{2+} so that they remain stained after polyanions containing only carboxylate and phosphate esters have lost their stain. This allows the distinction between sulphated compounds, such as the GAG chains of PGs, and polycarboxylates, such as hyaluronic acid.

The copper-containing dyes, including cinchomeronic acid and quinolonic acid (Scott, 1980) have been used extensively to demonstrate the distribution of PGs in the extracellular matrix. A wide variety of patterns of electron dense filaments are produced when the extracellular matrix is stained, reflecting different species of sulphated GAGs bound to various PG protein cores. The pattern of their staining

appears to depend upon differences in the chemical composition and macromolecular size of the GAGs (van Kuppevelt et al, 1984b, Tawara et al, 1988). Depending upon the tissue location, size and sensitivity or resistance of the various electron dense filaments to enzymatic digestions, various authors have been able to distinguish between DS-, HS- and CS-PGs in a number of tissues including the guinea pig prostate (Chan and Wong, 1989), mouse interphotoreceptor matrix (Tawara et al, 1988), mouse lung alveoli (van Kuppevelt et al, 1984a), human trabecular tissue (Tawara et al, 1989) and human Bruch's membrane (Call and Hollyfield, 1990). In all cases three distinct types of filaments were described; those lying within the basement membrane and being representative of HS-PGs, those lying within interstitial spaces of the extracellular matrix and thought to be representative of the CS-PGs, and lastly those associated with the surfaces of collagen fibrils, thought to represent DS-PGs.

DS-PGs have specifically been localized to the d and e bands of collagen fibrils in vivo. By using the Cuprolinic Blue-critical electrolyte concentration method, their association has been described in a number of tissues, including rat tail tendon (Scott et al, 1981), bovine tendon, sclera, periodontal ligament, cornea and skin (Evanko and Vogel, 1990; Scott and Haigh, 1988; van Kuppevelt et al, 1987) and human and rabbit sclera (Young, 1985), among others.

The histochemical localisation of DS-PGs has further been confirmed by immunoelectronmicroscopic studies. Using

antisera against synthetic peptides monospecific to either DS-PGI or DS-PGII, Fleischmajer et al (1991) were able to demonstrate the binding of DS-PGII onto the surface of both embryonic and adult human skin collagen. Positive staining often followed an approximately 60 nm periodicity, with the majority of the staining occurring between the d and e bands. Antibodies to DS-PGI did not stain the collagen fibrils. Pringle and Dodd (1990), using mAbs to the core protein of bovine skin DS-PII, were also able to specifically demonstrate its association to the d and e bands of bovine tendon collagen.

In addition to electron micrographic studies demonstrating the binding of GAG containing material to the surface of collagen fibrils, Brown and Vogel (1989) directly demonstrated and quantified the nature of the binding of DS-PGII to type I collagen in an in vitro study using a fibril-forming assay. Radiolabelled proteoglycans isolated from cultured bovine tendon fibroblasts were allowed to bind with acid extracted bovine tendon collagen; the amount of bound and free proteoglycan was then used in Scatchard plot analysis. They were able to demonstrate substantial binding of either intact or core protein DS-PGII, but not free GAG chains, DS-PGI, or large PGs, to collagen. They speculate that the interaction between DS-PGII and collagen is complex, involving non-covalent bonds as well as conformational changes, in the core protein or the collagen molecule, once binding has begun.

1.4.2. Inhibition of collagen fibrillogenesis.

As discussed, collagen fibrillogenesis remains a complex process, sensitive to a number of factors. Due to their coexistence in connective tissues, proteoglycans and/or glycosaminoglycans may play a role in the assembly of collagen fibrils. It was first suggested by Gross (1956) that glycosaminoglycans may play a role in the organization of collagen fibrils. Following the development of a procedure to examine the rate of collagen fibrillogenesis under the influence of various additives, Gross and Kirk (1958) found that HA and CS did not affect the rate or extent of collagen fibrillogenesis. Wood and Keech (1960) went on to define fibrillogenesis as a process involving a nucleation (or lag) phase and a growth phase and illustrated that the rate of precipitation of collagen could vary with pH, ionic strength and temperature. As well, they were able to show that CS and KS shortened the lag phase, and as such accelerated fibril formation when added to a collagen solution <u>in vitro</u>, while the addition of HS retarded fibrillogenesis. However, subsequent studies suggested that glycosaminoglycans such as DS and HS, by virtue of their ionic interaction with collagen, may accelerate nucleation (Obrink, 1973). This is in contrast to the additional studies by Wood (1960) and Keech (1961) which found that while CS and KS could very slightly accelerate fibril formation, DS and HA had little effect on collagen nucleation or growth.

A number of studies have also looked at the effects of proteoglycans on the assembly of collagen fibrils. Earlier

work by Toole and Lowther (1968) demonstrated a delay in collagen fibrillogenesis following the addition of very small amounts of DS-PG monomers; this was not the case when larger aggregates of PGs were used in the assay (Lowther and Natarajan, 1972). Oegema et al (1975) demonstrated a marked retardation in collagen fibrillogenesis in the presence of either proteoglycan monomers or aggregates (from nasal bovine cartilage) at physiologic ionic strength and pH. They suggested that inhibition was a result of electrostatic interactions and was related to the size of the proteoglycans, and not to the binding of core protein to collagen fibrils. Others have found that aggregated CS-PGs do not inhibit collagen fibrillogenesis (Chandrasekhar et al, 1984). Results are contradictory, and illustrate the sensitivity of the kinetics of in vitro fibrillogenesis. In addition to the early work of Wood and Keech (1960), further studies have demonstrated the sensitivity of in vitro matrix assembly to the pH, temperature and ionic strength of solutions used, to differences in the source and preparation of collagen or to the preparation of proteoglycans (Comper and Veis, 1977b; Williams et al, 1978). This makes comparison between studies difficult, and probably accounts for some of the contradictory results from study to study. However, these investigations all serve to illustrate the influence that a component of the extracellular matrix may have on the assembly of collagen molecules.

More recently, a number of investigations have looked

specifically at the influence of DS-PGs on collagen fibrillogenesis. Vogel et al (1984) showed that small DS-PGs isolated from bovine tendon were able to inhibit the fibrillogenesis of both type I and type II collagen in a turbidity assay. The effect was dependent upon the core protein, as the GAGs alone had no effect on fibrillogenesis. Their results were confirmed by the work of Scott et al (1986), in which they demonstrated a decrease in the rate of collagen fibrillogenesis by bovine skin DS-PGII. The effect was also found to be specific for the core protein. Reduced protein core did not affect fibrillogenesis, illustrating the importance of disulphide bonds in the binding of the molecule to collagen. Uldbjerg and Danielsen (1988) however, were unable to demonstrate a decrease in the rate of collagen fibrillogenesis by a small DS-PG isolated from human uterine cervix. They suggest that they may be looking at the effects of different populations of PGs.

1.4.3. Interactions with other molecules.

DS-PGII is able to bind transforming growth factor-β (TGF-β) through its core protein. By so doing, it neutralises the activity of the growth factor (Yamaguchi et al, 1990). TGF-β also stimulates the expression of DS-PGI and DS-PGII core proteins and may affect the biosynthesis of the GAG side chains (Bassols and Massagué, 1988). These PGs may therefore be effector molecules in a negative feedback loop that regulates TGF-β activity (Ruoslahti and Yamaguchi, 1991). However, TGF-β stimulates the synthesis of DS-PGI to a greater

extent than that shown for DS-PGII or fibromodulin (Vogel and Hernandez, 1992; Westergren-Thorsson et al, 1991), consistent with the different functional properties ascribed to these molecules in the extracellular matrix. It is also thought that since the binding of DS-PGII to TGF-β is reversible, DS-PGII may act as a reservoir of TGF-β in tissues.

The core protein of DS-PGII has also been shown to co-localise with fibronectin fibrils on the surface of cultured fibroblasts (Schmidt et al, 1987). Subsequently, an interaction between the core protein and a cell-binding domain of fibronectin was established (Winnemöller et al, 1991), possibly explaining the inhibitory effect of decorin on fibroblast adhesion to a fibronectin surface (Lewandowska et al, 1987). Schmidt et al (1991) have identified a pentapeptide sequence (NKISK) between positions 85-89 which may function in the interaction between decorin and fibronectin. However, they suggest that additional binding sites probably exist on the core, since complete inhibition of binding with the pentapeptide was not achieved.

1.8. Purpose of the study

The precise role of DS-PGII in connective tissues has yet to be defined. In the preceding discussion, it has been shown that DS-PGII may influence collagen fibril diameters, bind to type I collagen and affect the rate of collagen fibrillogenesis as well as bind to other components of the extracellular matrix. The present study will further investigate the functions of DS-PGII.

First, DS-PGIIs secreted by fibroblasts isolated from various bovine fibrous tissues and including gingiva, sclera, skin, tendon and TMJ disc tissues, were characterized. Their characterisation helped to define whether possible differences in structure correlate with the ultrastructural features of the corresponding tissues, namely collagen fibril diameters and interfibrillar distances. It is hypothesized that the packing of collagen fibrils in tissues may be related to the length of the GAG chain of various DS-PGIIs.

Secondly, DS-PGIIs were isolated directly from the various tissues and compared to those secreted *in vitro*. Knowing whether or not the molecules *in vivo* are identical to those *in vitro*, assisted in further defining the influences of environmental stimuli on the characteristics of DS-PGII in tissues.

Third, the role of DS-PGII in collagen fibrillogenesis was investigated. By examining the effects of the various tissue-specific DS-PGII molecules on the rate of fibrillogenesis and the morphology of the fibrils thus formed, a further role for DS-PGII in the organization of the extracellular matrix was defined. The advantage of the present study was that the conditions under which fibrillogenesis occurs were standardized, thus making comparisons between the various tissue-specific molecules relevant.

Lastly, ultrastructural studies of the tissues were used to confirm the matrix location of DS-PGII.

MATERIALS

2.1. Tissue Culture

Dulbecco's Modified Essential Medium (#430-2100), the antibiotic/antimycotic solution (#600-5340PE), fetal calf serum (#200-6140AJ), and the crude trypsin (Difco 1:250) were all obtained from Gibco Life Technologies Inc. (Grand Island, N.Y.). Sterivex GS 0.22μm filters were from Millipore Filter Co. (Bedford, MA).

Tissue culture dishes (60 x 15 mm) were obtained from Falcon-Becton Dickinson Labware (Lincoln Park, N.J.). Culture flasks (75cm^2 and 150cm^2) and modified polystyrene centrifuge tubes (10mL and 50mL) were from Corning Glassworks (Corning, N.Y.). Round 24-well culture plates were from Costar (Cambridge, MA). The Nunclon cell factories were obtained from Nunc InterMed (Denmark).

Hematall was from Fischer Scientific (Fair Lawn, N.J.).

2.2. Extraction of Proteoglycans From Tissues

All reagents used in the extraction buffer, excluding urea and EDTA and including the proteinase inhibitors were analytical grade or better and purchased from Sigma Chemical Corp. (St. Louis, MO). The urea and EDTA were purchased from Fisher Scientific.

2.3. Chromatography

DEAE-cellulose was obtained from Whatman Ltd. (Maidstone, Kent, U.K.). Columns, molecular weight standards, Sephacryl-

400 SF, and Octyl-Sepharose CL-4B were from Pharmacia LKB Biotechnologies (Piscataway, N.J.). CHAPS was from Pierce Chemical Co.(Rockford, IL.) Guanidine HCl and sodium acetate were from Sigma Chemical Corp.

2.4. Gel Electrophoresis

The acrylamide, bisacrylamide, SDS, and TEMED were obtained from BioRad Lab. (Richmond, CA) as were the Coomasie Blue and Stains-All. 2-mercaptoethanol and the bromphenol blue were from Sigma Chemical Corp.

2.5. Immunoblots and Dot Blots

The nitrocellulose paper (BA85) was from Schleicher and Schuell (Dassel, F.R.G.). The peroxidase-conjugated rabbit anti-mouse IgG was obtained from Cappell (Organon Teknika Inc., Scarborough, Ont.). The KS antibodies and bovine nasal cartilage monomer were from ICN ImmunoBiologicals (St. Laurent, Que.). The Amido black and the Pyronin Y were purchased from BioRad Lab.

Bovine serum albumin, cobalt chloride, diaminobenzidine tetrahydrochloride, Concanavalin A, horseradish peroxidase (Sigma Type VI), CS antibodies (CS-56) and the peroxidase-conjugated goat anti-mouse IgM were all from Sigma Chemical Corp. Tween 20 was from Fisher Scientific.

2.6. Cellulose-Acetate Electrophoresis

The cellulose-acetate membranes and Sepra Clear II were

obtained from Gelman Sciences Inc. (Ann Arbor, MI). CS-B was from ICN ImmunoBiologicals (Lisle, IL), while the CS-A and CS-C standards were from Seikagaku Kogyo Co. (Tokyo, Japan). Hyaluronic acid (97-071) was from Miles Scientific (Naperville, IL). Alcian Blue 8GX (12020) was from Terochem (Edmonton, Alta.). The pyridine and glacial acetic acid were purchased from Fisher Scientific.

2.7. Enzymatic Methods

Chondroitinase ABC, ACII, and B were obtained from Seikagaku (Rockville, MD). The sodium acetate and TCA were purchased from Fischer Scientific. Tris, papain, cysteine HCl, cathepsin C, cacodylate, and leupeptin were purchased from Sigma Chemical Corp. The glycopeptidase F was from Boehringer Mannheim Canada (Dorval, Que.).

2.8. Electron Microscopy

The glutaraldehyde, OsO_4, and uranyl acetate were all E.M. grade and purchased from J.B. EM Services Inc. (Pointe-Claire, Que.), as were the copper grids. The lead citrate was from Eastman Kodak Co. (Rochester, NY). The LX 112 resin and formvar were obtained from Ladd Research Industries Inc. (Burlington, Vermont). Cuprolinic Blue was from BDH Inc. (associate of E. Merck, Darmstadt, West Germany). The OCT compound was from Tissuetek (Miles Scientific). The GS 0.22μm filters were from Millipore Filter Co. (Bedford, Mass.). The crossed lined grating replica was purchased from Ernest F.

Fullman Inc. (NY).

All other reagents used for EM including sodium acetate, $MgCl_2$, sodium tungstate and propylene oxide were analytical grade or better and were from Sigma Chemical Corp.

2.9. Collagen Fibrillogenesis *in vitro*

The DS was from Miles Scientific and the bovine nasal cartilage monomer was from ICN ImmunoBiologicals. All other reagents used in the buffers were analytical grade or better and purchased from either Sigma Chemical Corp. or Fischer Scientific.

2.10. Analytical Methods

The DMMB was purchased from Serva (Hauppauge, N.Y.). Immulon 96 well plates, sodium formate, formic acid, Na_2CO_3, $CuSO_4$, Na tartrate and the Phenol reagent were all obtained from Fischer Scientific.

METHODS

3.1. Tissue Culture

3.1.1. Establishment of explant cultures

Young adult bovine tissue (between the ages of 3-4 years, as determined by dentition) was used to establish explant cultures. In all cases, representative tissue was obtained from freshly slaughtered animals (Gainers; Edmonton), placed on ice and transported to the laboratory. Within a period of approximately one hour, the tissue to be used was further dissected clean, cut into a manageable size, placed in cold phosphate buffered saline (PBS; 0.15M pH 7.2) and kept on ice until transport to the tissue culture room.

The following tissues were collected:

1) sclera- once eyes were dissected free from their sockets, strips of sclera, approximately 10mm x 30mm were taken from the mid-posterior section of the eyeball. Using a #10 scalpel blade, the strips were scraped on both the exterior and interior surfaces to remove adherent muscle tissue and vitreous humour and then placed in cold saline.

2) skin- samples of skin were taken from any available area of the animal. Hair was shaved off the exterior surface and all visible subcutaneous fat in the hypodermis removed with a #10 blade. No attempts were made to dissect the papillary layer from the reticular layer of the dermis, nor to remove the epithelial layer. Approximately 10mm x 10mm squares of tissue were isolated.

3) gingiva- 10mm x 50mm strips of gingiva were dissected

from the lingual aspect of mandibles along the mylohyoid ridge. A periosteal elevator was used to lift the gingiva away from underlying periosteum. Epithelium was not dissected from the underlying dermis.

4) tendon- the deep flexor tendon was isolated from bovine feet. Samples of tissue were taken from the proximal area of the tendon, representing an area of the tendon that normally functions under tension (fig 3.1). The isolation method has been described by Vogel et al (1986). After adhering fascia was dissected free, a strip of tissue measuring 20mm x 40mm was removed from the tendon and placed in saline.

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Fig. 3.1. Drawing of the superior aspect of the bovine flexor tendon for explant culture. Taken from Vogel et al (1986).

5) temporomandibular joint (TMJ) disc- three areas of the disc were defined according to anatomical features; a thin central zone, a thick, "cushiony" zone located lateral to the central zone, and an outermost peripheral zone (fig 3.2). Ten mm x 10mm squares of tissue was removed from each area and

placed into cold saline.

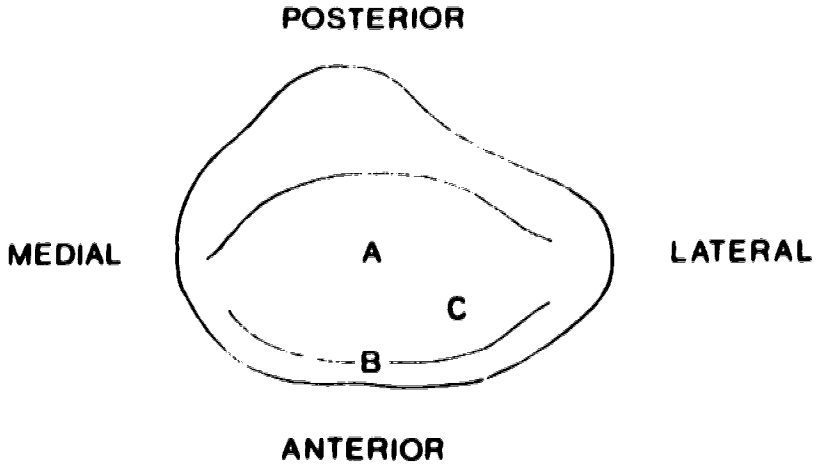


Fig. 3.2. Drawing of the superior aspect of the bovine TMJ disc showing the areas taken for explant culture. A) thin, central zone B) peripheral zone C) "cushiony" zone.

Once the tissues were taken to the culture room, all subsequent manipulations were done inside a horizontal flow laminar hood using sterile technique. All solutions used were sterile, either by heat (in the case of PBS) or by ultrafiltration using a Sterivex GS 0.22µm filter. All petri dishes, forceps, scalpel blades, etc. were also sterile.

Samples of tissue to be cultured were removed from the saline and placed into a 60 x 15 mm petri dish containing 2ml of Dulbecco's Modified Essential Medium (DMEM) with antibiotics-antimycotic (100 units/mL penicillin G, 100 µg/mL streptomycin and 0.025 µg/mL amphotericin B). DMEM always contained the antibiotics-antimycotic unless otherwise specified. The tissue was finely minced into at 1-0.5 mm^3 pieces, although attempts were made to obtain samples of

tissue as small as possible. The tissue was then placed in a modified polystyrene centrifuge tube containing 10 mL of sterile media and centrifuged for 5 min at 2000 rpm. The media was poured off, fresh media added, the tube vortexed and placed back into the centrifuge. The tissue was rinsed this way for a minimum of six times.

Following the final rinse, the samples were placed back into a petri dish containing DMEM with 10% fetal calf serum (FCS) (referred to as DMEM-10). Four pieces of tissue were transferred to a 60 x 15mm tissue culture dish and held in place by a sterile piece of glass cover slip tacked down in one corner with sterile silicone grease. Five mL of medium was added to each plate; the plates were then maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The medium was changed every 5-7 days until a substantial outgrowth of cells could be observed; in most cases 3-4 weeks. The cultures were examined by phase contrast microscopy. By the third to fourth cell passage (see 3.1.1A), a uniform fibroblastic phenotype was evident. A minimum of 12 plates were cultured from each of the isolated tissue samples.

3.1.1A) Subculture When a sufficient number of cells were observed in each plate radiating outwards from the explants, the cells were subcultured using standard passaging techniques (Freshney, 1983). The explants were first physically removed from each plate and the plates then rinsed three times with sterile PBS. Three mL of 0.25% crude trypsin in PBS was added to each plate. When the cells began to

detach (between 5-10 minutes), 3 mL of DMEM-10 were added to the plates to stop the action of the trypsin, and cells were further dispersed mechanically by repeated pipetting. Cell suspensions from each of the plates were pooled and pelleted at 2000 rpm, rinsed twice with DMEM-10 and then seeded at a concentration of 2-3 x 10^5 cells per mL with 12 mL per 75 cm^2 flask. Cell numbers were determined using a haemocytometer. Fresh medium was added to the flasks once cell attachment took place, in about 2-3 days, and was changed thereafter every 5-7 days. Passaging was continued on confluent monolayers at a 1:3 split until cells were in passage three or four. All further experiments were done on cells in passage three or four unless specified otherwise.

1.1.2. Cell factories

In order to obtain a large volume of medium from which PGs could be extracted, a Nunclon Cell Factory (total surface area of 6,500 cm^2) was used. Factories were seeded with cells in passage three or four at a concentration of 2-3 x 10^4 cells per mL to a total volume of 1000 mL per factory. The factory was gassed for 5 minutes with 5% CO_2 in air prior to placement in the incubator.

Medium was changed on a weekly basis until cell confluence was reached, at which time the collection of media began. To collect media, the cell factory was first rinsed 3 times with 500 mL of PBS. One thousand mL of fresh DMEM without FBC (referred to as DMEM-0) was then added, the factory gassed and placed in the incubator for a period of 24

hours. At the end of the 24 hours, the medium was collected, centrifuged at 10,000 rpm for 30 min. at 4°C. Sodium azide (0.02% w/v) was added to the supernatant which was then kept frozen at -22°C until use. DMEM-0 was added for a second 24hr period, media collected and stored as before. Fresh DMEM-10 was then added to the factory, the cells allowed to recover for anywhere from 3 to 7 days, after which the cycle would begin again. As many as 10 cycles, collecting a total of 2000 ml per cycle, were carried out for each cell factory.

3.1.3. Additional cell strains

Explant cultures were also initiated from two additional animals, so that a total of three cell strains from each of the five tissues were available for the isolation of DS-PGII. However, instead of seeding cell factories, 150 cm^2 culture flasks were seeded with cells in passage three or four at a concentration of 2-3 x 10^4 cells per mL using 40 mL per flask at a time. Confluent flasks were rinsed with PBS, and cycled through two changes of DMEM-O as described above. Usually five flasks were seeded at a time, and cycled five times so that a total of 2000 mL of media was collected from each cell strain.

3.1.4. Determination of doubling times

Growth curves of cultured cells were generated in order to determine their growth cycle and doubling times (the time taken for a culture to increase 2-fold in the middle of the exponential phase of growth). The method followed was essentially that described by Freshney (1983) and is as

follows. Confluent cells were trypsinized as for subculture. Cell numbers were determined in a haemocytometer, and the cell suspension was diluted to 1 x 10^4 cells per mL of DMEM-10. Two 24-well tissue culture plates were seeded with 1 mL of the cell suspension per well. The plates were then placed back into the incubator and maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2. After a period of 24 hours, each of three wells was rinsed three times with PBS, following which 1 mL of 0.25% trypsin in PBS was added to each well. When the cells were detached, they were transferred to a microfuge tube and each well was rinsed with an additional 0.5 mL of PBS. Following centrifugation, the trypsin/PBS mixture was aspirated off and the cells were resuspended in 1 mL of Hematall®. One half mL of the sample was then added to 9.5 mL of Hematall® and the cell numbers in each 10 mL of solution determined with a Coulter counter following manufacturers instructions. The number of cells per mL of culture media were determined every other day until cell confluence was reached. In all cases, this was within two weeks. Fresh medium was added to the plates as required, usually every 4-5 days.

The number of cells per mL of medium was plotted on a log scale against time on a linear scale and a growth curve generated. From the curve, a population doubling time was calculated during the exponential phase of growth.

3.1.5. Determination of cell volumes

Once cultured cells in each passage number had reached

confluence, 9-10 wells from the 24-well plates were trypsinized, rinsed and the cells pooled and centrifuged. The cells were then resuspended in 100mL of PBS, and using the Coulter counter, the number of cells in each volume range of cell size (preset on the counter and previously calibrated with various sizes of latex beads) was determined.

3.2. Extraction of Proteoglycans from Tissue

Proteoglycans were isolated directly from tissues following a method developed in the laboratory of C.H. Pearson and P.G. Scott. Tissues to be used were obtained as described for the initiation of explant cultures. Samples of gingiva, skin, sclera, proximal zone of the flexor tendon, and peripheral zone of the TMJ disc were finally chopped over ice and then placed into an extraction buffer that consisted of 7M urea, 0.05M Tris-HCl, 0.15M NaCl with proteinase inhibitors (10mM EDTA, 100mM 6-aminohexanoic acid, 5mM benzamidine HCl, 0.5mM N-ethylmaleimide (NEM), 1mM phenylmethylsulfonyl fluoride (PMSF), 0.02% NaN_3, 5mg/L leupeptin, and 5mg/L pepstatin), pH 6.5. Ten mL of extraction buffer were used for every gram of wet weight of tissue.

The sample, together with buffer, was then stirred for 40-48 hrs in the cold room (4°C). Following this period, any remaining tissue and extraction buffer were filtered through glass wool lined with cheesecloth. The undissolved tissue was discarded. The supernatant was centrifuged under refrigeration at 1300 rpm for 30 minutes and then immediately

applied to a DEAE-cellulose column (section 3.3.1).

3.3. Isolation of DS-PGII

The methods described below were used to isolate DS-PGII either from culture media (obtained from the cell factories or 150cm² flasks), or from tissue extracts.

3.3.1. Ion-exchange chromatography

Sulphated proteoglycans present in tissue culture media were separated from hyaluronic acid and other proteins by passage down a 1.6 x 8 cm DEAE-cellulose column at 4°C. The column was equilibrated with a 25mM phosphate buffer (pH 7.4) containing proteinase inhibitors (10mM EDTA, 5mM benzamidine hydrochloride, 1mM PMSF, 0.5mM NEM and 0.02% NaN_3). To isolate DS-PGII secreted by cultured fibroblasts in either cell factories or flasks, 2000 mL of media was applied overnight to the column at a flow rate of 80 mL/min. The column was then rinsed for a minimum of 3 column volumes of cold starting buffer. Charged molecules were eluted with a 1M NaCl gradient in 25mM phosphate buffer containing proteinase inhibitors at a flow rate of 40mL/hr. Two mL fractions were collected.

Fractions from the peak (read at 280nm) were further examined for the presence of sulphated PGs by a dimethylmethylene blue (DMMB) binding assay (Farndale et al, 1986) and by dot blotting onto nitrocellulose paper and subsequent staining with a mAb (6D6) to DS-PGII (see 3.8.3.). Fractions which were positive for both sulphated PGs and DS-

PGII were pooled, dialysed against dH_2O at 4°C and lyophilised.

Sulphated PGs present in tissue extracts were also initially isolated by ion-exchange chromatography. In this case, DEAE-cellulose was equilibrated in extraction buffer (7M urea/Tris buffer with proteinase inhibitors) and then poured into a 1.6 x 8 cm column. The column was rinsed with a minimum of three bed volumes of buffer, following which the supernatant was applied to the column at a rate of 40-50 mL/hr. Once the sample was completely bound to the column, the column was rinsed a second time with buffer to reestablish 0 absorbancy at 280 nm. At least two bed volumes were required to remove unbound molecules. Bound sulphated PGs were subsequently eluted with a linear gradient of 0-1M NaCl in extraction buffer, at a rate of 50 mL per hour. Positive fractions, corresponding to a single broad peak, were confirmed with the DMMB assay and dot blots stained with antibodies to the core protein of DS-PGII. They were pooled together, dialysed against dH_2O at 4°C and lyophilised.

1.3.2. Gel filtration chromatography

The positive peak obtained from ion-exchange chromatography was further purified by gel filtration chromatography. Anywhere from 1 to 10mg of lyophilised sample, together with 30µl of ^2H_2O was dissolved in 2 mL of a 25mM phosphate, 0.5M NaCl buffer containing proteinase inhibitors, pH 7.4. The sample was applied to a 1.6 x 135 cm Sephacryl-400 SF column equilibrated with the same buffer and

allowed to run for 20 hours at a rate of 14mL per hour. Three mL fractions were collected. The column fractions were once again monitored by absorbency at 280nm, with the DMMB assay and dot blots stained with antibodies to DS-PGII and CS.

Columns were previously equilibrated with a mixture of molecular weight standards, which included blue dextran, ferritin, thyroglobulin and ribonuclease. The relative mobility of a DS-PGII standard, purified from bovine skin (Zangrado et al, 1989), was also determined for each column prior to the application of unknowns.

Fractions were pooled into three separate peaks. Fractions from peak 1 represented the V_o and were thought to contain CS positive material (as determined by immunoblotting), while the K_{av} of fractions from peak 2 corresponded to that of DS-PGII as determined by a column run of the DS-PGII standard. In most cases, fractions obtained from peak 3 were not positive for sulphated material or reactive to antibodies to DS-PGII or CS and were discarded. Fractions which were thought to contain small PGs secreted by each of the various fibroblast strains were pooled together according to tissue of origin and either dialysed against dH_2O at 4°C, or concentrated with an Amicon ultrafiltration cell using a Diaflo PM10 ultrafiltration membrane, and subjected to a second column run. After the second run, often a small peak representing the V_o was evident, however the majority of the sample eluted as the DS-PGII containing peak. Fractions from

this peak were further dialysed against water, lyophilized and the purity of the fractions established by SDS-PAGE, immunoblotting and amino acid analysis. The identity and characteristics of each DS-PG molecule secreted by the tissue-specific cell strains was further analysed by cellulose acetate electrophoresis, sequential glycopeptidase-F digestion, cathepsin C and papain digestion.

In addition, fractions positive for DS-PGII from Octyl-Sepharose chromatography (3.3.3) were also subjected to gel-filtration chromatography.

3.3.3. Hydrophobic chromatography

Differences in the hydrophobic properties of the core proteins of DS-PGI and DS-PGII allows the molecules to be separated by chromatography on Octyl-Sepharose. The method followed was that of Choi et al (1989) and was used to purify material isolated from tissue extracts subsequent to passage down a Sephacryl-400 SF column. Octyl-Sepharose CL-4B was initially equilibrated in 4M guanidine HCl, 0.15M sodium acetate and 0.2% NaN_3, pH 6.3 at room temperature and then poured into a 1.6 x 4.5 cm column. The column was rinsed with a minimum of 4 column volumes with buffer, following which 2-3 mg of sulphated PGs, previously isolated from bovine gingiva, sclera, skin, tendon and TMJ disc by ion-exchange chromatography, were dissolved in 2 mL of buffer and applied. Each sample, together with an additional mL of buffer used as a wash, was allowed to run into the column. The sample was

then left to bind to the Octyl-Sepharose for 2 hours, following which elution began. Initially, the column was washed with the 4M HCl guanidine buffer for one hour at a flow rate of 0.4 mL/hr. The column was subsequently eluted with a linear gradient of 0-1% CHAPS in buffer, at a flow rate of 24 mL/hr for 3 hrs. Two mL fractions were collected and absorption monitored at 240nm.

The DMMB assay was used to determine the relative amounts of sulphated GAG in each of the fractions. Six separate groups of fractions were pooled, dialyzed against dH_2O, lyophilized and run on SDS-PAGE to confirm that the separation of DS-PGI from DS-PGII was complete. Pooled fractions which corresponded to the molecular weight of DS-PGII were further purified by gel chromatography as described above.

3.4. Confirmation of Molecules as DS-PGII

3.4.1. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Acrylamide gel electrophoresis was used to demonstrate the presence of various proteoglycans in the fractions separated by chromatography. The method used was that of Mechanic (1979) and is as follows. Seven percent slab gels were used at all times and were made by combining 12.2 mL of 20% acrylamide, 3.5 mL of 154.17 gm M.W. bisacrylamide, 3.5 mL of 10x concentrated 1M Tris buffer containing 1% SDS, and 12.3 mL dH_2O. The solution was catalyzed by the addition of 9µL

TEMED poured between 16 x 18 cm glass plates in a gel casting stand and allowed to polymerize overnight at room temperature. Samples for electrophoresis were dissolved in sample buffer (40 μM Tris borate, 2% SDS, 6M urea, pH 8.6) containing 10% 2-mercaptoethanol and 0.001% bromophenol blue, boiled for 5 minutes and centrifuged for 60 seconds in a microcentrifuge. Aliquots (25-80 μL) of sample were then applied to lanes of the gel and electrophoresed at 100 volts for 4-5 hours, or until the dye front was approximately 1.5 cm from the bottom of the gel. The running buffer used was a 1M Tris buffer containing 1% SDS, adjusted to pH 8.6 with NaOH.

When gels were to be stained with Coomasie Blue R250, 15-20μg protein weight of sample was dissolved in sample buffer and applied to each lane. At the completion of electrophoresis, gels were removed from the glass slabs, the dye front marked with India ink, and submerged in Coomasie Blue (0.125% in 7.5% acetic acid and 50% methanol) for 1-2 hours. Gels were destained in 7.5% acetic acid in 50% methanol, photographed and dried.

When gels were to be stained with the cationic carbocyanine dye Stains-All, 10-15μg protein weight of sample was dissolved in sample buffer and applied to each lane. At the completion of electrophoresis, gels were removed from the glass slabs, the dye front marked with India ink and the gels submerged in 0.005% Stains-All in 50% formamide (made from a 0.1% Stains-All in 100% formamide stock solution). Gels were

destained in H_2O, photographed and dried.

3.4.2. Immunoblotting

Samples of DS-PG isolated from either culture media or from tissue extracts were immunoblotted and stained with a number of antibodies to confirm the purity of the preparation and the identity of the molecule isolated. Essentially the method of Towbin et al (1979) was followed. Three μg protein weight of each sample were dissolved in sample buffer, applied to a 7% acrylamide gel and electrophoresed as described above. When electrophoresis was complete, the dye front was marked with Pyronin Y and the gel transferred to nitrocellulose paper previously soaked in running buffer. The gel together with the nitrocellulose paper were then placed in a Trans-Blot cell apparatus filled with 1M Tris buffer, pH 8.6. Transfer was allowed to proceed for 18-20 hrs using 6 volts and 50-100 milliampere current.

Transfer of antigen was complete when blotted gels stained with Coomassie Blue R250 showed no evidence of any remaining protein. Lanes of nitrocellulose paper containing molecular weight standards were cut away and stained with Amido Black for 1-2 hours. The remaining nitrocellulose paper was stained with a variety of antibodies. The nitrocellulose paper was first incubated in Tris buffered saline (TBS; 0.15M NaCl, 0.05M Tris HCl, pH 7.6) containing 2% (w/v) bovine serum albumin (BSA) for 45 mins at 37°C. The paper was then rinsed with TBS (without BSA) and each lane cut into strips as

required. Each strip was then incubated with appropriate controls and antibodies. A variety of mAbs to DS-PGII (Pringle et al, 1985) were used; 6D6, 3B3, 7B1 and 5D1, representing different epitopes on the protein core of DS-PGII. Recently, the location of the epitopes of the mAbs have been identified (Scott et al, 1993). It was shown that mAbs 7B1, 3B3, 5D1 and 6D6 all recognize different epitopes. The epitope for 7B1 was localized towards the N-terminal of the protein core and was thought to involve residues 5-10 or 6-11. The epitope for 5D1 was localized to residues 120-147, with octapeptides 121-128 showing the strongest antigenicity. One hexapeptide, residues 146-151, was significantly antigenic towards 3B3, while the epitope for 6D6 was located towards the C-terminal of the protein core. The minimum sequence for its recognition appeared to be the tetrapeptide 241-244.

Monoclonal ascites fluid was diluted 1:1000 in TBS with 0.1% Tween. Strips were incubated in the primary antibody for 120 mins at room temperature. Four mL of solution was used per lane. The strips were then rinsed four x 5 minutes in TBS and incubated for an additional 90 minutes with secondary antibody. In the case of the mAbs to DS-PGII, a 1:1000 to 1:2000 dilution of peroxidase conjugated rabbit anti-mouse IgG in TBS/Tween was used. Following an additional four x 5 minute rinses with TBS, the strips were placed into a solution of 0.5% (w/v) diaminobenzidine tetrahydrochloride, 0.1% H_2O_2 and 1% $CoCl_2$ (aq) in TBS for 5-10 minutes. Once sufficient

color had formed, the reaction was stopped by soaking the strips in H_2O for 30-60 minutes, followed by air drying.

Antibodies to CS and KS were also used for staining. Polyclonal KS Abs were used at a dilution of 1:2000 in TBS/Tween and stained with secondary antibody as described above. For mAb to CS (CS-56), staining was essentially done as described above, except PBS was used in place of TBS. The primary antibody was diluted 1:1000 in PBS/Tween, and the secondary antibody was a 1:500 dilution of peroxidase conjugated goat anti-mouse IgM.

Positive controls for immunoblotting were used and included samples of a DS-PGII standard and CS-PGs from bovine nasal cartilage.

3.4.2A) affinity staining with Concanavalin A

Occasionally, blotted gels were also affinity-stained with concanavalin A. The method, from transfer of the slab gels to nitrocellulose paper and initial blocking of the nitrocellulose paper with 2% BSA in TBS, was as described above. Following rinsing in TBS, the nitrocellulose was incubated with concanavalin A (0.005% w/v in TBS containing 0.0005M $CaCl_2$, 0.5% w/v BSA) for 2 hours at room temperature, washed four x 5 min in TBS and incubated for an additional 90 mins in 0.005% (w/v) horse-radish peroxidase (in TBS containing 0.0005M $CaCl_2$, 0.5% BSA). Following four x 5 min washes in TBS, the colour was developed with diaminobensidine tetrahydrochloride as described.

3.4.1. Cellulose-acetate electrophoresis

DS-PGII molecules isolated from culture media were digested with papain (section 3.5.2) and the resultant GAGs subjected to cellulose acetate electrophoresis. In order to determine the composition of a preparation of GAGs, cellulose acetate membranes (1" x 6" strips) were soaked in a dH_2O:glacial acetic acid:pyridine (460:36:4 ml v/v) buffer, pH 3.4 for a minimum of 15 minutes prior to the application of sample (Habuchi et al, 1973). A dual applicator was used to load 3µl each of sample and standards onto what would be the cathode side of the strips. Each 3µl was equivalent to 10µg of standard or sample. Standards were a mixture of DS, C-6S, and hyaluronic acid. Loaded strips were then placed into the electrophoresis tank filled with buffer, and electrophoresed at 1.25 mA per strip for one hour at room temperature.

Upon completion of electrophoresis, strips were stained in 0.2% (aq) Alcian Blue 8GX in 0.05M $MgCl_2$, 0.025M NaAc in 50% ethanol, pH 5.8 for one hour. The strips were destained in several washes of 0.05M $MgCl_2$, 0.025M NaAc in 50% ethanol, pH 5.8 until the bands could be clearly distinguished. The strips were then soaked in Sepra Clear II for 5 minutes, mounted on a glass microscope slide and cleared in a 100°C oven for 10 minutes, or until the background became transparent. The slides were then scanned at 600nm.

Cellulose acetate electrophoresis was also used to separate GAGs on the basis of the charge density of ester

sulphate groups (Wessler, 1971). The technique was essentially as described above, except for a few minor alterations. Samples and standards (DS and C-6S) were loaded centrally on strips which had been previously soaking in a 0.1M HCl buffer, pH 1.2. They were then electrophoresed in the same buffer at 6.25 V per strip for 3 hours at room temperature. Following completion of the run, strips were stained, destained, cleared and scanned as described above.

3.5. Enzymatic Methods

3.5.1. Chondroitinase ABC and Chondroitinase B digestion

Chondroitinase ABC specifically removes GAG chains containing N-acetylgalactosamine and glucuronate or iduronate (Oike *et al*, 1980). Samples of DS-PGs were dissolved in digestion buffer (0.1M sodium acetate, 0.01M Tris, pH to 7.3 with NaOH) at a concentration of 1μg/μL. To 20μg of sample, 2μL of a 1U/100μL solution of chondroitinase ABC was added (0.1U of enzyme is required to digest 1mg of PGs). The solution was incubated in a 37°C water bath for 2 hours. Following digestion, sample buffer was added, the solution boiled and applied to lanes of acrylamide gels. Appropriate amounts of digested material were used depending upon whether the gels were to be blotted, or stained with Coomasie blue or Stains-All (see sections 3.4.1 and 3.4.2.).

Samples were occasionally digested with chondroitinase B, which specifically digests GAGs containing iduronate as part

of the disaccharide unit (Michelacci and Dietrich, 1975). Samples to be digested were treated as above; to 20μg of sample in digestion buffer, 2μL of a 0.1U/100μL solution was added (0.003U of enzyme is required to digest 1mg of PGs) and the samples incubated for 2 hours at 37°C. Various concentrations of the digestions as needed were then subjected to SDS-PAGE, following the appropriate preparatory steps.

3.5.2. Papain digestion

Samples of DS-PGII purified directly from tissues or from culture media were digested with papain in order to hydrolyse the protein core. Samples were dissolved in acetate buffer (0.1M sodium acetate, 0.005M EDTA, pH adjusted to 6.0 with NaOH) at a concentration of 1.5μg/μL. To 20μL of dissolved sample, 20μL of activated papain suspension (200μL 5 x [acetate buffer], 700μL ddH_2O, 0.88 mg cysteine HCl and 50μL papain) was added. The mixture was then placed in a 65°C water bath for 18 hours. Following digestion, the samples were treated with trichloroacetic acid (TCA) to precipitate the papain. A sufficient volume of 100% (w/v) TCA was added to the digestion mixture to make the final concentration of the mixture 6% (w/v) TCA. Precipitation was allowed to proceed at room temperature for 30 minutes, after which the samples were centrifuged at maximum speed in a microcentrifuge for 10 minutes. The supernatant was removed, the precipitate washed with 6% TCA, centrifuged and the supernatants pooled. The supernatants were dialysed against dH_2O at 4°C and

lyophilized. Papain digested samples were then subjected to SDS-PAGE and/or cellulose acetate electrophoresis.

3.5.3. Cathepsin C digestion

The sequential removal of dipeptides from the NH_2-terminal of the protein core of PGs can be achieved by digestion with cathepsin C (Chopra et al, 1985). Samples to be digested were dissolved in dH_2O at a concentration of 2μg/μL. Cathepsin C was previously dissolved in 1% NaCl, 50mM EDTA and kept at -22°C until just prior to use. To 16μg of sample to be digested, 52μL of digestion buffer (10mM cacodylate, 0.1M EDTA, 0.014% 2-mercaptoethanol (v/v), and 2.4% leupeptin (w/v), pH 4.5 adjusted with HCl) was added along with 7μL of cathepsin C. The solution was incubated at room temperature for 30 minutes, following which sample buffer was added, the solution boiled and the samples subjected to gel electrophoresis as described.

3.5.4. Glycopeptidase F digestion

In order to hydrolyse the N-glycoside linkage of asparagine-linked oligosaccharides from the core protein of proteoglycans (Tarentino et al, 1985), samples were digested with an endoglycosidase. Samples of DS-PGII were dissolved in 0.1M Tris HCl, 0.1M sodium acetate buffer, pH 7.3 at a concentration of 1μg/1μL. Samples were initially incubated with chondroitinase ABC to obtain core proteins (as described) and then incubated with varying concentrations of glycopeptidase-F (0.005, 0.025, 0.05, 0.25 and 0.5 units) in

buffer (0.1% (w/v) SDS, 0.5% (v/v) Triton X, 1% (v/v) 2-mercaptoethanol, 0.02M EDTA and 0.025M sodium phosphate pH 7.0) at 37°C for 18-20 hours.

Following incubation, 20-40μL of SDS-PAGE sample buffer was added to each sample dilution and then treated as described for SDS-PAGE; loading 2-4μg of sample to each well of a 7% acrylamide gel. Two identical gels were run for each sample. Following electrophoresis, both gels were electroblotted onto nitrocellulose paper; one blot was stained with mAbs to DS-PGII (6D6) and the other was affinity-stained with concanavalin A.

3.6. Electron Microscopy

3.6.1. Electron microscopy to determine collagen fibril diameters and percent area occupied by collagen

Tissue samples taken from young adult cows were essentially dissected as described for the establishment of explant cultures. Samples of gingiva, sclera, skin, the proximal section of the deep flexor tendon and the peripheral and central zones of the TMJ disc were collected from freshly slaughtered animals. They were initially chopped into 1-2 mm^3 pieces in cold iso-osmotic saline (0.9% NaCl) and then placed into 2.5% glutaraldehyde in 0.1M phosphate buffer overnight at 4°C. The samples were rinsed three x 9 minutes in 0.1M phosphate buffer and then transferred into 1% OsO_4 in phosphate buffer for 1 hour at room temperature. Following

rinsing with dH_2O, the samples were dehydrated through a series of alcohols (50%, 70%, 80%, 90%, 95% and 98%) for 20 minutes each. The final dehydration step was in propylene oxide for 1 hour, from which the samples were transferred to a 1:1 propylene oxide/LX 112 resin mix and left uncapped in a fume hood overnight. The samples were further penetrated with fresh LX-112 resin for an additional 24 hours, embedded in Beem capsules with fresh resin, vacuumed for 1/2 hour and then allowed to cure in a dry oven for 48 hours at 60°C.

Thin sections (50-90nm) were cut on a Sorvall microtome, using a diamond knife, and then transferred onto 200 mesh copper grids. The grids were then stained for 8-9 mins in a solution of saturated uranyl acetate in absolute ethanol followed by staining in lead citrate (0.25 gm in 50 ml of boiled and cooled dH_2O to which 5 pellets of NaOH had been added) for an additional 7 minutes. All solutions were previously filtered through 0.22μ GS filter. Stained grids were examined with a Hitachi 7000 electron microscope and photographed at a number of magnifications ranging from 5,000 to 21,000x. Electron micrographs of representative areas were printed, from which collagen fibril diameters were determined using a Zeiss Mop3 digitizer. Diameters were measured at the narrowest point of fibrils cut in cross-section. For each tissue, collagen fibril diameters and percentage of area occupied by collagen were determined from micrographs obtained from the samples of three separate animals.

Magnifications were calibrated at each photography session using a crossed line grating replica (2160 lines/mm). The digitizer was also used to determine the proportion of the surface area of each photograph occupied by collagen fibrils. The mean interfibrillar distance (i.e. spacing between fibrils) for each micrograph was determined using the formula: $F=\sqrt{xy}/\sqrt{n}-D$, where xy is the surface area from which the collagen fibril diameters were measured, n is the number of fibrils measured and D is the mean fibril diameter.

3.6.2. Cuprolinic Blue staining

Samples from each of the tissues described above were also stained with the heavy metal cationic dye, Cuprolinic Blue, to illustrate sulphated proteoglycans. The method of Scott et al (1981) was used with slight modification and is as follows. Samples were stained and fixed overnight at 4°C with 2.5% glutaraldehyde in 25mM sodium acetate buffer containing 0.05% Cuprolinic Blue and 0.3M $MgCl_2$, pH 5.7. The samples were then rinsed three x 5 minutes in the above solution without Cuprolinic Blue, and post-stained in 0.5% sodium tungstate (aq) for 30 minutes. Dehydration was begun by submersing the samples in 0.5% aqueous sodium tungstate in 50% ethanol (v/v) and then continued to embedding and sectioning as described above. Grids were stained with 2% aqueous uranyl acetate prior to being photographed.

3.6.3. Enzyme digestions

Five x 5 mm samples from the peripheral and central zones

of the disc, and skin were obtained as described. The samples were then immediately embedded in OCT compound and kept at -70°C until use. When required, 40-60μm thick sections were cut in a cryostat and placed into single wells of an ice-cold 24 well tissue culture plate. The sections were rinsed with Tris buffer (0.025M Tris, 0.18M NaCl, 0.05% BSA with 5mM benzamidine HCl, 0.1M 6-amino-n-caproic acid, pH 8.0) to remove OCT compound. Enzyme (1U of chondroitinase ABC or 1U of chondroitinase AC) in 1mL of Tris buffer was then added to each of the wells containing the various samples. Tris buffer without enzyme was added to control wells. Following incubation at 37°C for 2 hours, wells were rinsed again with buffer, and then the tissues were fixed and stained with Cuprolinic Blue, embedded, sectioned and stained with 2% aqueous uranyl acetate as described above.

3.6.4. Collagen fibrillogenesis in vitro for electron microscopy

Previously prepared bovine skin collagen (see section 3.7.1) was dissolved at a concentration of 2mg/mL in 0.01M acetic acid for 24-48 hours at 4°C. Following centrifugation at 16,000 rpm for 60 minutes, the clear supernatant was kept on ice until ready to use. Samples of each of the various tissue-specific DS-PGII molecules (isolated directly from tissues) were dissolved in cold dH_2O at a concentration of 0.25μg/μL. In vitro fibrillogenesis was carried out by adding 25μL of solubilized collagen to 100μL of dH_2O, 125μL of 2x

[PBS], and 50μL of each DS-PGII sample in glass test tubes. For controls, the DS-PGII sample was replaced with 50μL of dH_2O. The solutions were vortexed and incubated overnight in a 37°C waterbath.

When fibrillogenesis was complete, the dense "clot" evident in each tube was fixed and stained overnight at room temperature with 2.5% glutaraldehyde in 25mM sodium acetate containing 0.3M $MgCl_2$ and 0.05% Cuprolinic Blue, pH 5.7. The clot was then rinsed three x 5 minutes with 2.5% glutaraldehyde in 25mM sodium acetate containing 0.3M $MgCl_2$, pH 5.7, and post-stained in 0.5% (aq) sodium tungstate overnight at room temperature. The clot was then rinsed three x 5 minutes with dH_2O and teased onto 200 mesh copper grids previously coated with formvar. Random areas of each grid were photographed, magnifications were calibrated using a crossed line grating replica (2160 lines/mm), and collagen fibril diameters were measured from each micrograph using a graded 7x magnification eyepiece.

3.7. Collagen Fibrillogenesis in vitro

3.7.1. Preparation of collagen

Type I collagen was previously prepared from fetal bovine skin and purified by repeated salt-precipitation using the method of Volpin and Veis (1971).

3.7.2. Establishment of fibrillogenesis turbidity curves

Collagen was solubilised in 0.1M acetic acid at a

concentration of 2mg/mL as described above. Samples of each tissue-specific DS-PGII (isolated directly from tissues) were dissolved in cold dH_2O at a concentration of 1μg/μL. *In vitro* fibrillogenesis proceeded by mixing 150μL of solubilized collagen with 320μL dH_2O, 500μL 2x [PBS] and 30μL of DS-PGII in a glass test tube. All solutions were ice cold. The mixture was vortexed, placed under vacuum for 30-60 seconds and then carefully transferred to a cuvette. The samples were placed into a Varian DMS 200 UV/visible spectrophotometer, whose inner-chamber temperature was maintained at 29°C by means of a circulating water bath. Fibrillogenesis was allowed to proceed, and monitored at a UV wavelength of 330 nm, plotting absorbency against time. Fibrillogenesis continued until a plateau was reached; in the case of samples containing DS-PGII this was within an hour. For control solutions, in which the DS-PGII containing solution was substituted with dH_2O, fibrillogenesis was complete within 20 minutes.

Varying concentrations of DS-PGII (45μg and 60μg) were used in the fibrillogenesis assay, always adjusting the final volume of solutions added to the cuvette to 1mL. DS and CS-PGs standards were also used as controls for fibrillogenesis. They were dissolved in cold dH_2O at a concentration of 1μg/μL and used in concentrations ranging from 15, 30, 60, 90, and 100 μg.

3.8. Analytical Methods

3.8.1. Dimethylmethylene blue assay for sulphated GAGs

The presence and quantity of sulphated GAGs in various column fractions was determined using the dimethymethylene blue assay as described by Farndale et al (1986). A DS standard was dissolved in 25mM phosphate buffer with 1M NaCl, pH 7.4, at a concentration of 0.1μg/μL. Standards were subsequently diluted in additional volumes of buffer so that final concentrations ranged between 100 to 500 ng of DS per 50μL. Fifty μL of each dilution were then pipetted (in triplicate) into individual wells of Immulon 96 well-plates. Fifty μL of each fraction sample were also pipetted into individual wells. To each well (standards and samples), 150 μL of working dye solution was added. The working dye solution was prepared immediately prior to use and consisted of a 3:2 mixture of a stock dye solution (0.002% DMMB (w/v), 0.2% sodium formate (w/v), and 0.2% formic acid (v/v) in dH_2O) and a stock buffer solution (0.2% sodium formate (w/v) and 0.2% formic acid (v/v)). The wells were gently mixed and allowed to stand at room temperature for approximately 2 minutes. The colour reaction was read in a plate reader at 595nm. The absorbency of the standards was plotted against the concentration of each standard. The amount of sulphated GAG present in each fraction was then determined directly from the graph.

3.8.2. Dot blots

The presence of specific GAGs and PGs in various column

fractions was confirmed by [illegible] blotting each fraction onto nitrocellulose paper and immunostaining with antibodies either to DS-PGII (mAb 6D6) or CS [illegible]. Five µL of each fraction were spotted on a dot [illegible] and applied onto nitrocellulose paper (BA85) by suction. The nitrocellulose paper was previously moistened with 10-20 µL of distilled H_2O. The vacuum suction was maintained for 5-10 minutes, following which the paper was removed, air dried and stored at 4°C until staining. Staining of selected fractions with the various antibodies was as described in section 3.4.2.

3.8.3. Lowry's protein determination

The protein content of samples was determined by the method of Lowry et al (1951), with slight modification. Bovine skin DS-PGII was used as the standard and was dissolved in dH_2O at a concentration of 1mg/mL. Varying dilutions of the standards were then made in order to obtain final concentrations ranging from 5-100µg per mL. Fifty to 100µg of unknown sample were also dissolved in 1mL of dH_2O. To 500 µL of the various dilutions of the standard and samples, 250 µL of the alkaline copper reagent (100:1:1 mixture of 5% (w/v) Na_2CO_3 in 0.25N NaOH, 1% (w/v) $CuSo_4$, and 2% (w/v) NaK tartrate) was added, the solutions vortexed and allowed to stand at room temperature for 10 minutes. Two hundred and fifty µL of an 8x dilution of the Lowry reagent (Phenol Reagent; 2N Folin-Ciocalteau) were then added, the standards and samples once again vortexed and then left to stand for an

additional 45 mins. The resulting colour change was read in a spectrophotometer at 740nm. The absorbency of the standards was plotted against the concentration of each standard. The amount of protein in each sample (as determined by absorbance) was obtained directly from the graph.

3.8.4. Amino acid determination

Amino acid analyses were done by the Walters PICO TAG™ method after hydrolysis with 6N NCl in evacuated and sealed tubes, for 22-24 hours at 110°C. The derivatives were separated using Beckman 110A pumps and the Walters PICO TAG™ columns (3.9mm x 15cm).

3.9 Others

3.9.4. Immunohistochemistry

Fibroblasts from TMJ disc explant cultures were grown to confluency on sterile glass coverslips. The coverslips were rinsed in TBS and then fixed in methanol with 2% H_2O_2 for 30 minutes. The coverslips were then placed in acetone for 15 minutes and dehydrated through a series of alcohols ending with dH_2O. They were first blocked in 5% NGS in TBS for 30 minutes and then placed in the primary Ab- either a 1:5 dilution of 6D6 ascites supernatant in TBS or in a control solution- 5% NGS in TBS overnight at 4°C. The next day the coverslips were rinsed 4 x five minutes in TBS and exposed to the secondary Ab- a 1:50 dilution of goat anti-mouse serum. Incubation proceeded for 30 minutes at room temperature

following which the coverslips were rinsed again 4 x five minutes with TBS, then incubated with a 1:300 dilution of mouse conjugated peroxidase-antiperoxidase for an additional 30 minutes. The coverslips were then exposed to a solution of DAB as described for the immunoblots (section 3.4.2), counterstained with hematoxylin, and mounted.

RESULTS

4.1. Morphology of Cultured Cells

Numerous fibroblast-like cells, as judged by their spindle shaped morphology, could be seen radiating outwards from tissue explants within a period of 1-2 weeks. In some cases (cultures established from skin and gingiva), sheets of epithelial-like cells were also evident, but these were soon overgrown by the fibroblasts. By passage 2 to 3, a relatively homogenous population of cells was established from each cell strain, although differences in the morphology of the cells could be seen from tissue type to tissue type. These differences remained consistent between the various tissues even when additional cell strains were established from separate animals. Plate 4.1 demonstrates these differences. Cultures established from skin and gingiva were more spindle-shaped in appearance, in contrast to cultures from sclera and tendon. Cells from the peripheral zone of the TMJ disc were characteristically polygonal.

4.1.1. Immunohistochemistry of cultured TMJ disc fibroblasts. In order to confirm that the cells isolated from the TMJ disc were fibroblasts, capable of secreting substances characteristic of mesenchymal cells, the cells were fixed and immuno-stained with mAbs to DS-PGII (Pringle et al, 1985). The presence of "punctate" immunoreactive material could be demonstrated intracellularly, lying in a supranuclear position consistent with the cellular location of the rough endoplasmic reticulum (Plate 4.2). Hence, it appears that these cells

were capable of secreting an extracellular matrix material, irrespective of their uncharacteristic morphology.

4.2. Purification of DS-PGII From Cell Cultures

4.2.1. Ion-exchange chromatography.

Sulphated molecules secreted into the culture media by fibroblasts grown either in cell factories or 150 cm^2 flasks were initially isolated by ion-exchange chromatography. For each cycle of media collection and regardless of fibroblast type, the elution profile was similar and revealed the presence of a single broad peak which eluted between 0.4 and 0.7 M NaCl (fig.4.1). Fractions contained within the peak were shown to contain dimethylmethylene blue (DMMB) reactive material and were concluded to be a mixture of sulphated glycosaminoglycans, including DS, CS, and HS. The presence of DS-PGII in the mixture was confirmed by dot blotting fractions onto nitrocellulose paper, followed by staining with a mAb (6D6) to bovine skin DS-PGII (Plate 4.3).

Although each fibroblast cell strain established from the different tissues secreted DMMB positive material into the culture media, the amount of this material varied from tissue type to tissue type (Table 4.1).

TABLE 4.1: Weight (mg/100 mL) of sulphated GAGs in culture media.

GINGIVA[2]	0.075[1]
SCLERA	0.013
SKIN	0.055
TENDON	0.010
TMJ DISC	0.011

[1] expressed as mg/100mL, averaged from a minimum of 10 column runs.
[2] source of cultured cells

4.2.2. Gel-filtration chromatography

Fractions positive for sulphated GAGs obtained by ion-exchange chromatography were subsequently pooled and dialysed against water. Lyophilized material applied to a calibrated Sephacryl-400 SF column demonstrated elution profiles which were similar for material collected from the culture media of all five tissues. For each run, fractions were pooled into four peaks based on absorbance at 280nm. Fractions forming peak 1 represented the V_o, fractions containing DS-PGII and sulphated GAGs (on the basis of the DMMB assay and dot blots) were found in peak 2, fractions in peak 3 contained no DMMB reactive material and all later fractions, collected beyond the V_t, and forming a fourth peak were also nonreactive to DMMB. Fractions forming peaks 3 and 4 were not investigated further. Fractions from peak 2 were considered to contain DS-PGII on the basis of the dot blots. They also had a K_{av} which corresponded to a DS-PGII standard (Zangrando et al, 1989) (Table 4.2).

TABLE 4.2: K_{av}s of DS-PGII positive fractions obtained from Sephacryl 400-SF gel chromatography of media from cultured fibroblasts.

DS-PGII standard	0.66 (± 0.02)[1]
Gingiva[2]	0.65 (± 0.03)
Sclera	0.53 (± 0.05)
Skin	0.63 (± 0.01)
Tendon	0.62 (± 0.09)
TMJ Disc	0.60 (± 0.07)

[1] K_{av}(S.D.) taken as an average of 5 column runs
[2] source of the sample

For samples from tendon (fig 4.2A), TMJ disc (fig 4.3A) and sclera (fig 4.4A), fractions collected from peak 1 contained DMMB positive material which was reactive to CS antibodies. A dot blot illustrating the positive staining of earlier fraction I (31-48) to CS antibodies and fraction II (46-60) to DS-PGII antibodies (6D6), is shown in Plate 4.4. The sample shown is that from the TMJ disc and corresponds to the gel chromatograph illustrated in Fig. 4.3A. No such reactive material was present in fractions representing peak 1 for samples obtained from skin (fig 4.5A) and gingiva (fig 4.6A).

Fractions positive for sulphated GAGs and DS-PGII were pooled according to the specific tissues from which they were isolated and subjected to an additional run on Sephacryl-400 SF. Chromatographs of these final runs illustrates the presence of only two peaks based on absorbance at 290nm; a small V_o peak, which in the case of fractions collected from

the media of TMJ disc fibroblasts (fig. 4.3B) contained a reduced amount of DMMB reactive material, and a large peak 2. Fractions pooled from peak 2 of sclera (fig. 4.4B), skin (fig. 4.5B), gingiva (fig. 4.6B) and tendon (not shown) were DMMB reactive and contained DS-PGIIs as judged by dot blots. Fractions I and (II for TMJ disc material) collected from the peak 2s of these final column runs were subsequently used to characterise each tissue-specific DS-PGII molecule.

4.3. Characterisation of DS-PGII Isolated From Cell Cultures

4.3.1. SDS-PAGE

The purity of each tissue-specific DS-PGII molecule was judged by a number of criteria. Initially SDS-PAGE was done on the fractions I (II for TMJ disc material) obtained by gel filtration chromatography. The protein content of the pooled fractions was previously determined by Lowry's protein determination. For each run, the intact molecule together with molecular weight standards and the DS-PGII standard were electrophoresed and stained with Coomassie Blue to rule out any contaminating proteins. In all cases, electrophoresis of the intact molecule revealed the presence of only a single band of material which varied in apparent molecular weight (Mr from 93,000 to 120,000) depending upon the cells from which it was isolated (Plate 4.5). Skin fibroblasts secreted the smallest molecule, while larger molecular weight material was secreted by gingival, TMJ disc and tendon fibroblasts.

Due to very limited sample sizes, the chondroitinase ABC

core of each molecule was not electrophoresed along with the intact molecule during the initial characterization steps (i.e. in gels stained with Coomasie blue and Stains-All). However, the molecular weight of the chondroitinase ABC core of each molecule was determined by Western blotting (see section 4.3.3).

Gels were also stained with Stains-All to rule out the presence of any contaminating acidic polysaccharides or nucleic acids. Only intact molecules were run and again in all cases, only a single band of material was visible on stained gels. The size of the band varied, reflecting the tissue from which it was isolated. The results were identical to those illustrated in Plate 4.5 and are not shown.

Fibroblasts in culture are able to secrete DS-PGI (Fransson et al,1991; Schmidtchen et al, 1990). While the media collected from each of the fibroblast cultures was shown to contain a molecule corresponding in size to DS-PGII, in two tissues, particularly the TMJ disc and to a much lesser extent tendon, SDS-PAGE of the DS-PGII positive fractions from initial gel-filtration chromatography revealed the presence of a higher molecular weight material (Plate 4.6). Rosenberg et al (1985), described the occurrence of two DS-PG molecules on SDS-PAGE when electrophoresis was done under conditions of high ionic strength. These conditions allow for the self-aggregation of DS-PGI molecules, which then appear as higher molecular weight material (Mr=200,000). Since all slab gel electrophoresis was run in a Tris/Borate buffer, of high pH

(8.6) and high ionic strength, the aggregation of DS-PGI could occur, if this were present in our samples. In fractions taken from later column runs, no such material was evident on gel electrophoresis, presumably because the amount of contaminating DS-PGI in the final preparation was very small. Since gel chromatography does not successfully separate DS-PGI from DS-PGII, the possibility exists that extremely small amounts of the DS-PGI could still be present in the final preparation.

4.3.2. Protein determination

Although the protein content of each sample was low (Table 4.3), the purity of the samples as judged by SDS-slab gel electrophoresis was good, suggesting that whatever was increasing the weight of the samples was neither protein, polysaccharide nor nucleic acid. Attempts to increase the purity of the samples by ethanol precipitation or redialysis/lyophilization were unsatisfactory. As such, subsequent weights of the samples used in all experimental protocol take this discrepancy into account and weight values are expressed as weight of protein, relative to the DS-PGII standard, rather than the actual weight of the sample.

TABLE 4.3: Protein content of samples purified from culture media.

Gingiva[1]	7%[2]
Sclera	18%
Skin	16%
Tendon	5%
TMJ Disc	7%

[1] source of sample
[2] percentage protein as determined by Lowry's and relative to a bovine skin DS-PGII standard.

4.3.3. Immunoblotting

Western blots were carried out on the pooled fractions from Sephacryl 400SF to further confirm the purity of the fractions and their identity. For each of the tissue-specific DS-PGII molecules, both intact and chondroitinase ABC cores were electrophoresed, blotted onto nitrocellulose paper and stained with mAbs developed to the protein core of bovine skin DS-PGII (Pringle et al, 1985).

All intact and core tissue-specific molecules were reactive to all four mAbs (6D6, 3B3, 5D1 and 7B1). Electrophoresis of the protein cores resulted in a single or doublet band corresponding to the DS-PGII standard, and with an apparent Mr of 45,000 to 48,000. The size of the protein core of DS-PGII secreted by the various tissue-specific fibroblasts was identical. The only variation between tissue types was in the size of the intact molecules. Plate 4.7 illustrates the staining pattern of the molecules isolated from the various cultured cells, on Western blots.

In order to confirm that the preparations were not contaminated with any CS- or KS-PGs, samples blotted onto nitrocellulose paper were also stained with Abs to CS and KS. Bovine nasal cartilage proteoglycan monomer was used as a positive control. Because samples were run on 7% acrylamide gels, the resolution of the high molecular weight cartilage PG (Mr > 1,000,000) was not very good. However a band of positive staining material could be detected at the origin when samples of nasal cartilage PG were immunoblotted and stained with CS-56. No such positive material was detected in any of the DS-PGII preparations (Plate 4.8).

4.3.4. Cellulose acetate electrophoresis

Experiments were carried out to determine the composition of the GAG side chain of the isolated PGs. Each PG molecule isolated from the various fibroblasts was degraded with papain and the resulting products separated by cellulose acetate electrophoresis. Electrophoresis of the GAGs was carried out alongside a mixture of GAG standards, including HA, DS and CS. On the basis of their relative mobilities, the GAG chains from each digested PG molecule corresponded to the DS standard and hence was concluded to be DS (fig. 4.7).

In order to ensure that electrophoretic mobility of the samples was not related to any variations in charge distribution, each PG sample was also run in buffer conditions used to separate molecules on the basis of charge density of their sulphate groups (Wessler, 1971). Once again, each sample ran with, or between the CS and DS standards (fig.

4.8), illustrating sulphation, and hence charge, is close to or at 1.0. Differences in the separation of CS and DS standards may be due to slight differences in the degree of sulphation between the molecules; the sulphate content of the standards were not characterized fully and therefore the influence of additional sulphate residues on IduA cannot be ruled out.

4.3.5. Digestion with cathepsin C

Enzymatic digestion of PGs with cathepsin C results in the sequential removal of dipeptides from the NH_2-terminus of the protein core. The enzyme is able to remove only the first 6 amino acids from DS-PGII and can therefore remove the DS chain, which has been shown to be attached to residue 4 of the protein core (Chopra et al, 1985). The method can be used to determine the number of GAG chains on a PG molecule; if more than one chain is present, then the molecular weight of the digested molecule on SDS-PAGE would remain greater than that of the core protein alone.

Fractions I (II for TMJ disc fibroblasts) collected from the final Sephacryl column runs (figs. 4.3 to 4.6) of cultured material from the various fibroblasts, were digested with cathepsin C in order to show that the molecular constituents of the fractions were predominately DS-PGII and not PGI. Because DS-PGI is believed to contain two GAG chains, at position 5 and 11 or 10 (Choi et al, 1989 and Fisher et al, 1987), electrophoresis would demonstrate a molecule with a molecular weight greater than its chondroitinase ABC core;

cathepsin C removing only the first GAG chain. However, electrophoresis of each of the digested samples from the various tissues showed the presence a single protein band with a Mr of 45,000-48,000 on Coomassie-stained gels. Stains-All stained gels of the same digested material demonstrated the presence of a single broad band which varied in molecular weight depending upon the fibroblasts from which the media was isolated. Plate 4.9 illustrates the differences in the size of the single GAG chain from DS-PGII secreted by skin fibroblasts and DS-PGII secreted by gingival fibroblasts. The results establish the presence of only one GAG chain on each DS-PG molecule, thus confirming its identity as DS-PGII. Similar results were obtained for material from scleral, TMJ disc and tendon fibroblasts and are not shown.

4.3.6. Glycopeptidase F digestions

Sequential removal of N-linked oligosaccharides from the protein core of proteoglycans is possible when the cores are digested with varying concentrations of an endoglycosidase (Scott and Dodd, 1990). Intermediates formed during deglycosylation help to establish the number of N-linked oligosaccharides. The presence of 3 oligosaccharide has been suggested for DS-PGII from human fibroblasts (Krusius and Ruoslahti, 1986) and shown for bovine skin DS-PGII by Scott and Dodd (1990). Digestion of intact DS-PGII molecules with chondroitinase ABC yields a core protein with an apparent Mr of about 45,000, a slightly higher value than has been predicted from protein sequencing studies (Mr=36,000). The

difference in weight reflects the presence of oligosaccharides which remain following the removal of the single GAG chain.

Previous studies have shown that deglycosylation of the protein core of bovine skin DS-PGII, with varying concentrations of glycopeptidase F, yields two glycosylated intermediates in addition to a fully-glycosylated and a completely deglycosylated molecule (Scott and Dodd 1990). In the present study, each tissue-specific DS-PGII from fraction I (II from TMJ disc fibroblasts) from Sephacryl 400SF was digested with varying concentrations of glycopeptidase F in order to demonstrate the presence of three N-linked oligosaccharides. As shown on Western blots stained with 6D6, digestion of each molecule yielded four bands, thus establishing the presence of three N-linked oligosaccharides (Plate 4.10). When the electroblots were stained with concanavalin A, both the undigested intact molecules and the chondroitinase ABC digested molecules demonstrated a strong reaction with the lectin, indicating the presence of oligosaccharides. However, no staining was evident when the samples were incubated with glycopeptidase F, suggesting the removal of all oligosaccharides by the enzyme. Results are shown for only one sample, in this instance from cultured tendon fibroblasts. However, identical results were obtained for each of the additional tissue-specific DS-PGIIs.

4.3.7. Size of the GAG chain

Samples of DS-PGII purified from fraction I (II from TMJ disc fibroblasts) from Sephacryl 400SF were digested with

papain to remove the protein core and the resultant products subjected to gel electrophoresis. The gels were then stained with Stains-All (Plate 4.11), and the relative size of each tissue-specific GAG chain was determined (Table 4.4).

TABLE 4.4: Rf values of papain digested DS-PGII purified from culture media.

DS-PGII Standard	0.66
Skin[1]	0.66
Sclera	0.63
Gingiva	0.58
TMJ Disc	0.57
Tendon	0.57

[1] source of sample

Actual molecular weights of polysaccharides cannot be determined by gel electrophoresis because of their extended shape, polydispersity and charge, however a comparison of their sizes can be made. The weight-average molecular weight for the DS chain of bovine skin DS-PGII has been determined by light scattering studies (Zangrando et al, 1989) and was shown to be 24,000. Results from the papain digests show that the relative mobility of DS-PGII isolated from cultured skin bovine fibroblasts and that of the standard DS-PGII are identical. The largest chains were isolated from tendon and TMJ disc DS-PGII.

4.3.8. Amino acid composition

The amino acid composition of each of the preparations

was determined in order to further confirm their purity and ensure that their composition is consistent with data published for other DS-PGII molecules. As can be seen from the data, all five preparations are high in aspartic acid, glutamic acid and leucine. However, DS-PGII prepared from the flexor tendon was also high in glycine suggesting the sample may not be as pure as the other preparations.

TABLE 4.5: Amino acid compositions of DS-PGII preparations extracted from culture media (average of two determinations).

Amino Acids	Skin[1] DS-PGII	Skin[2]	Tendon	Sclera	TMJ Disc	Ging.
Asx	122[3]	130	101	129	115	130
Thr	38	38	29	39	36	36
Ser	62	61	86	61	88	62
Glx	109	120	138	129	132	129
Pro	70	76	74	75	68	69
Gly	74	70	120	81	105	85
Ala	50	50	58	60	62	51
Val	62	64	65	60	62	59
Met	9	12	19	11	1/	4
Ileu	65	50	39	47	40	49
Leu	124	120	95	113	102	110
Tyr	29	34	35	30	31	31
Phe	34	33	26	37	30	32
Lys	83	84	78	69	61	83
His	28	26	17	27	20	28
Arg	28	34	31	32	32	38
Cys	13	ND[4]	ND	ND	ND	ND

[1] Pearson and Gibson, 1982
[2] source of sample
[3] expressed as residues/1000
[4] ND- not determined

4.3.9. Conclusions from cell factory studies

Fibroblasts cultured from five different bovine tissues were shown to be capable of secreting proteoglycans, amongst which one was identified as DS-PGII. Each tissue-specific DS-PGII molecule was shown to react with a panel of mAbs developed to the DS-PGII core protein, each contained a single GAG chain that was DS in composition, and three N-linked oligosaccharides. The molecular mass of each DS-PGII varied depending upon the tissue from which the fibroblasts were cultured. However, it was shown that variations in the size of the intact molecule were due to differences in the length of the GAG chain. In all cases, the molecular weight of the protein core, when determined with or without oligosaccharides, was the same regardless of the tissue from which it was cultured.

4.4. Establishment of Additional Cell Strains

We have shown that differences in the size of DS-PGII molecules from tissue to tissue are the result of differences in the size of the GAG chain. In order to determine whether or not these variations are the result of peculiarities of each established cell strain, or are truly specific to the tissue in question, additional cell strains from each tissue

were established. Explant cultures for each of the five tissues were established from two animals as described (section 3.1.1). From the collected culture media, proteoglycans were isolated using ion-exchange chromatography. Chromatographic profiles were identical to those previously described and are not shown.

Polyacrylamide gels electrophoresed with fractions positive for sulphated proteoglycans, as judged by the DMMB assay, were immunoblotted and stained with 6D6, 7B1, 3B3 and 5D1. Fractions were also digested with chondroitinase ABC and electrophoresed along side undigested fractions. Each fraction from the various cultured tissues contained sulphated proteoglycans which reacted to the mAbs, confirming the secretion of DS-PGII by the fibroblasts. However, when intact DS-PGIIs secreted by each of the three cell strains of gingival, scleral, skin, tendon and TMJ disc fibroblasts, were run alongside one another and stained with 6D6, variations in the molecular mass within each tissue type were noted (Plate 4.12, Table 4.6). In all cases, cell strain 1 represents the DS-PGIIs isolated from cell factories and is the strain that was used for further study. However, an overall difference in the size of DS-PGII derived from tissue to tissue was demonstrated; DS-PGII secreted by skin fibroblasts having on average a smaller molecular mass than DS-PGII secreted by tendon fibroblasts, which had the greatest molecular mass.

TABLE 4.6: Apparent molecular mass of DS-PGII produced by fibroblast cell strains from different tissues.

Cell Strain	Ging.[1]	Sclera	Skin	Tendon	TMJ Disc
1	101,000	93,000	89,000	101,000	93,000
2	101,000	96,000	96,000	115,000	109,000
3	110,000	101,000	103,000	101,000	107,000
mean	104,000	97,000	96,000	106,000	103,000
S.D.	± 4,242	± 3,299	± 5,715	± 6,599	± 7,118

[1] source of sample

4.5. Determination of Doubling Times and Cell Size of Continuously Cultured Cells.

4.5.1. Doubling times and its effect on molecular mass of secreted DS-PGII.

To what extent possible inherent differences between fibroblasts from one tissue to another can affect the size of DS-PGII manufactured by the cell remains unclear. Even less clear is what controls the size of the GAG chain secreted by subpopulations of fibroblasts isolated from the same tissue type. As the results in section 4.4 suggest, there may be factors other than the "tissue of origin" which would control the polymerization of the DS chain secreted by the various fibroblasts. The addition of GAG chains onto a protein core is a post-translation process that occurs within the Golgi apparatus. However, the factors that determine the final length of the GAG chain remain unclear. A number of ideas have

been put forward (see Heinegård and Paulsson, 1984 for review). Included is the suggestion that chain elongation stops once the nonreducing terminal galactosamine is sulphated, the possibility that steric factors may determine the length of the chains or that the level of UDP-xylose within the cell may regulate the biosynthesis of GAG chains by inhibiting UDP-D-glucose dehydrogenase, which is necessary for the formation of UDP-glucuronic acid. In addition one can raise the possibility that as cells age or if they demonstrate differences in growth rates, there may be an effect on the time that the core protein remains in the Golgi compartment, thus controlling the time available for the addition of sugar precursors onto the growing chain.

The possibility that age or the growth rate of cultured cells may determine the length of the GAG chain and hence the molecular mass of secreted DS-PGII, was considered when our results displayed differences in the molecular mass of DS-PGII secreted by the various subpopulations of tissue-specific fibroblasts. In order to demonstrate whether or not this may be a possibility, two cell strains from two different connective tissues were selected for further investigation. For each cell strain, the molecular mass of intact DS-PGII secreted at different passages (passage 4, 6 and 9), as well as doubling times and cell size at these passages, was investigated.

Bovine flexor tendon fibroblasts from cell strains 1 and 2 were selected on the basis of the initial differences seen

in the size of DS-PGII they secreted (101 Kd for cell strain 1 verses 115 Kd for cell strain 2 -see Table 4.6). Media collected from the cell strains at each particular passage were subjected to ion-exchange chromatography. Fractions containing sulphated proteoglycans were dialysed and lyophilised. Both intact and chondroitinase ABC digested fractions were subjected to polyacrylamide gel electrophoresis. Gels were then transferred to nitrocellulose and stained with 6D6 (Plate 4.13). The immunoblots demonstrated the presence of DS-PGII in the fractions, and were used to calculate the molecular mass, which varied according to cell strain and passage (Table 4.7). Cell strain 1, on average, maintained its lower molecular mass, while strain 2 continued to secrete a larger DS-PGII. Chondroitinase ABC digested fractions also contained a 6D6 positive core, which in all cases had a molecular mass of 45-48 Kd (Plate 4.13).

Doubling times also varied between the two strains, with cell strain 2 in all passages maintaining a slower doubling time when compared to cell strain 1 (Table 4.7, figs. 4.9 and 4.10).

TABLE 4.7: Apparent molecular mass of secreted DS-PGII and doubling times for two cell strains of bovine tendon fibroblasts.

Cell strain (tendon)	Apparent molecular mass	doubling time (days)
cell strain 1- p4[1]	101,000	2.0
cell strain 1- p6	112,000	1.7
cell strain 1- p9	103,000	1.7
cell strain 2- p4	112,000	2.5
cell strain 2- p6	115,000	3.7
cell strain 2- p9	112,000	4.3

[1] p= passage number

Bovine gingival fibroblasts were also selected on the basis of initial differences in the size of DS-PGII secreted between cell strain 1 (101,000 kd) and cell strain 3 (110,000 kd) (see Table 4.6). Media collected from the cell strains at each particular passage were processed as described above (Plate 4.14). Results illustrating differences in the apparent molecular mass between the cell strains and various passages, and doubling times are shown in Table 4.8 and Figs. 4.11 and 4.12. and 11.

TABLE 4.8: Apparent molecular mass of secreted DS-PGII and doubling times for two cell strains of bovine gingival fibroblasts.

Cell strain (gingiva)	Apparent molecular mass	Doubling time (days)
cell strain 1- p4[1]	105,000	2.7
cell strain 1- p6	95,000	2.6
cell strain 1- p9	102,000	1.5
cell strain 3- p4	110,000	3.0
cell strain 3- p6	110,000	3.9
cell strain 3- p9	112,000	3.0

[1] p= passage number

As with the results for tendon fibroblasts, cell strain 1 of gingival fibroblasts maintained, on average, its lower molecular mass as detected by initial electrophoresis. Strain 3 continued to secrete a larger DS-PGII. Doubling times once again could be shown to vary between the two strains, with cell strain 3 in all passages maintaining a slower doubling time when compared to cell strain 1.

The results show that passage number does not appear to influence the size of DS-PGII secreted within each cell strain. It is possible that this relationship may change as the cells are maintained longer in culture. Rather, a correlation was found between overall growth rates within a particular cell strain and molecular mass. On average, cells which had a faster doubling time (cell strains 1 of tendon and gingival fibroblasts), secreted a DS-PGII molecule that was

smaller. As shown from the preceding culture studies, this difference in size can be attributed to differences in the length of the GAG chain. The converse is true, slower growing cells (cell strain 2 of tendon fibroblasts and cell strain 3 of gingival fibroblasts) secreted a larger DS-PGII molecule.

4.5.2. Cell size (volume) and its relationship to doubling times

In vitro cellular aging has been shown to be accompanied by an increase in cell volume, particularly in slow or nonreplicating cell populations (Mitsui and Schneider 1976). The most likely interpretation of such data is that cell volume increases as a result of continued accumulation of cellular macromolecules without the mechanism of cell division to restore the macromolecular contents to normal levels. This is supported by data showing that rapidly replicating cells tend to maintain a constant nuclear size and cell size regardless of age (Mitsui and Schneider 1976).

Cell volume was followed as a function of passage number, although the various cell strains described above were not maintained to senescence. The results for bovine tendon fibroblasts are shown in Table 4.9 and Fig. 4.13. Those for bovine gingival fibroblasts can be found in Table 4.10 and Fig. 4.14. Interestingly, tendon fibroblasts appeared to become smaller with increasing passage number, for both cell lines 1 and 2. Conversely, gingival fibroblasts became larger with aging.

TABLE 4.9: Cell volumes for cell strain 1 verses cell strain 2 of cultured bovine tendon fibroblasts.

	cell strain 1			cell strain 2		
cell vol.[1]	p4[2]	p6	p9	p4	p6	p9
.5-1	10.23[3]	11.2	27.6	5.9	3.5	7.5
1-2	49.4	46.1	64.3	13.2	29.2	50.7
2-3	27.6	22.8	23.3	27.9	33.0	29.4
3-4	6.7	5.3	7.3	22.7	10.6	7.2
4-5	1.4	1.7	2.1	12.9	3.7	2.2
5-6	0.5	0	0.9	7.8	1.6	0.8
6-7	0	0	0	4.8	0.7	0
7-8	0	0	0	2.9	0	0

[1] in microns^3 X 10^3
[2] p= passage number
[3] percentage of total number of cells counted

TABLE 4.10: Cell volumes for cell strain 1 verses cell strain 3 of cultured bovine gingival fibroblasts.

	cell strain 1			cell strain 3		
cell vol.[1]	p4[2]	p6	p9	p4	p6	p9
.5-1	11.4[3]	20.9	5.7	10.8	6.9	2.5
1-2	52.7	55.2	34.7	56.8	33.3	4.6
2-3	24.7	16.7	33.6	50.2	34.3	20.3
3-4	7.6	4.1	15.5	13.7	19.3	28.5
4-5	2.4	1.2	6.5	3.9	7.9	19.7
5-6	0.9	0.3	2.5	1.3	2.4	10.7
6-7	0	0	0.9	0	0	5.4
7-8	0	0	0	0	0	2.4

[1] in microns^3 X 10^3
[2] p= passage number
[3] percentage of total number of cells counted

4.6. Purification of DS-PGII from Bovine Tissues

4.6.1. Ion-exchange chromatography

Sulphated proteoglycans present in extraction buffers obtained from various bovine tissues were initially isolated by ion-exchange chromatography. The elution profiles for each of the tissue extracts were identical to those described for the separation of sulphated PGs from culture media and are not shown. Material reactive to DMMB eluted as a single broad peak between 0.4 and 0.7M NaCl; the amount of material varied from tissue to tissue (Table 4.11).

TABLE 4.11: Weight (mg/gm) of sulphated GAGs from tissue extracts.

Gingiva[1]	0.23[2]
Sclera	0.40
Skin	0.30
Tendon	0.55
TMJ Disc	0.37

[1] source of tissue
[2] expressed in mg/gm, averaged from a minimum of 2 digestions.

4.6.2. Hydrophobic chromatography

Pooled, positive fractions obtained from ion-exchange chromatography were dialyzed, lyophilized applied to Octyl-Sepharose columns in order to further separate PGs. The method has been shown to be effective in separating DS-PGI from DS-PGII (Choi et al., 1989). Fig. 4.15 shows the

separation profile of DS-PGs, and other PGs, from bovine gingiva, sclera, flexor tendon and TMJ disc. Fractions I represent material which did not bind to the column, eluting early during the wash phase and therefore thought to represent DS-PGII (Choi et al, 1989). The remaining material eluted from the column during the 0-1% CHAPS gradient (between 0.3-0.4%) and is thought to contain DS-PGI. Although the presence of a second absorbance peak at 240nm was not always obvious on the chromatogram, both absorbance peaks 1 and 2 are reactive to DMMB, suggesting the presence of sulphated material. Only a single absorbance peak was evident for material obtained from skin (fig. 4.16), suggesting the absence of DS-PGI in this particular tissue.

When the eluted material was pooled into six groups, and subjected to gel electrophoresis, three different populations of polysaccharide-containing material could be resolved on Stains-All stained gels (Plate 4.15). Fractions I contained DS-PGII, as judged by its lack of binding to Octyl-Sepharose, the correspondence of its molecular weight to a DS-PGII standard, and other characteristic features to be described. Later fractions (II,III,IV, and V) revealed the presence of a higher molecular weight material, thought to represent the self-associated oligomers of DS-PGI. As well, each of the fractions I through VI were contaminated with a very high molecular weight material, which did not migrate into the gel, and is thought to represent the large CS-PGs. Gel electrophoresis of material obtained from tendon, TMJ disc,

sclera and gingiva all had similar patterns on stained gels. However, fractions obtained from samples of skin were pooled into only five groups and revealed the presence of a single Stains-All positive band throughout fractions I to V. Fraction V contained a "smear" of positive material, possibly representing breakdown products of material containing GAGs. No evidence of either a higher molecular weight material representing CS-PGs or DS-PGI could be found on material isolated from skin.

4.6.3. Gel-filtration chromatography

When fractions I (from Octyl-Sepharose chromatography) were dialyzed, lyophilized and applied to a calibrated Sephacryl-400 SF column, the elution profiles were slightly different, depending upon the tissue from which the fractions were obtained. For fractions I from sclera, skin, tendon and TMJ disc, 4 absorbance peaks could be detected on the chromatograph (fig. 4.17). Fractions I (absorbance peak 1 from Sephacryl 400SF) represented the V_o, and in the case of sclera and tendon, contained some DMMB reactive material. The majority of DMMB positive material however, was detected in fractions III collected from the second absorbance peak for sclera, skin and tendon and in the third absorbance peak for material from the disc. In all tissues, no DMMB positive material was detected beyond fraction number 70 (corresponding to absorbance peak 4).

Material extracted from bovine gingiva posed difficulty in isolation. On two separate attempts at purifying PGs

following ion exchange, hydrophobic and gel chromatography, Stains-All stained gels of fractions obtained from the final purification step (gel chromatography) showed smears of material, suggesting proteolytic degradation. An additional attempt at isolation was run in buffer systems using protease inhibitors. The inhibitors substantially reduced the resolution of absorbance peaks at 290nm , and only two peaks, with peak 1 representing the V_o could be discerned. However, as with samples obtained from sclera and tendon, DSHB material was identified in both absorbance peaks (fig. 4.18).

The K_{av}s for the second absorbance peaks from gingival, scleral, skin and tendon tissues and for peak 3 of the TMJ disc tissue are listed in Table 4.12. On the basis of the K_{av}s, which correspond to a DS-PGII standard previously run on a Sephacryl 400-SF column (K_{av} = 0.66 ± 0.02), fractions III from these peaks were concluded to contain DS-PGII. Further characterisation, to be described, confirmed their identity.

TABLE 4.12: K_{av}s of DS-PGII positive fractions obtained from Sephacryl 400-SF gel chromatography of tissue extracts.

Gingiva[1]	0.69[2]
Sclera	0.71
Skin	0.62
Tendon	0.69
TMJ Disc	0.51

[1] source of sample
[2] K_{av}

4.7. Characterisation of DS-PGII From Bovine Tissues

4.7.1. SDS-PAGE

The identity and the purity of each of the tissue-specific DS-PGII molecules extracted from tissues was initially confirmed on Stains-All stained polyacrylamide gels (Plate 4.16). Fractions obtained from gel chromatography were pooled, dialyzed and lyophilized and then run on the polyacrylamide gels. As seen on Plate 4.16, each of the fractions I through IV from skin-specific material contained only a single band, whose molecular weight corresponded to the DS-PGII standard. Fractions I through IV of each of the remaining tissue extracts revealed the presence of two bands of Stains-All staining material; a higher molecular weight material seen fractions I and II and a band of material once again corresponding to the approximate molecular weight of the DS-PGII standard in fractions III. Fractions III from the tendon-specific material, however, continued to contain a band of high molecular weight material. It was thought that rather than containing CS-PGs, this band may be an aggregate of DS-PG. When fractions I through IV of the tendon extracts were digested with chondroitinase B and run on polyacrylamide gels, fractions I and II continued to contain a distinct band of high molecular weight material, while the material in fractions III and IV appeared to have undergone some digestion (Plate 4.17). These results confirm that the high molecular weight material seen in the fractions III and IV contained a portion of DS-PG, although may still have been contaminated

with a small amount CS-PGs.

4.7.2. Protein determination.

Lowry's protein determination was carried out on each of the DS-PGII samples obtained from gel chromatography and the results are shown in Table 4.13. Each tissue-specific material displays good purity, except for material isolated from gingival tissue (shown are the results for material obtained from the SA-67 column run). As mentioned, difficulties were encountered in the purification of this material on several attempts, and although only a single band of Stains-All staining material could be seen on gel electrophoresis (Plate 4.16) and immunoblots (Plate 4.18) suggested the presence of DS-PGII, the results here suggest contamination. As with material purified from culture media, all subsequent weight determinations of each DS-PGII sample for electrophoresis and fibrillogenesis experiments are expressed as weight of protein relative to a DS-PGII standard rather than actual weights.

TABLE 4.13: Protein content of samples purified from tissue extracts.

Gingiva[1]	8%[2]
Sclera	52%
Skin	93%
Tendon	64%
TMJ Disc	59%

[1] source of sample
[2] percentage protein as determined by Lowry's and relative to a bovine skin DS-PGII standard.

4.7.3. Immunoblotting

As with the material purified from culture media, DS-PGIIs isolated from the various tissues were electrophoresed, blotted onto nitrocellulose paper and stained with a battery of antibodies to confirm identity and purity. The mAbs 6D6, 3B3 and 7B1 are specific to different epitopes on the core protein of bovine skin DS-PGII. Stained intact DS-PGII and deglycosylated core proteins were positive (Plate 4.18). Each intact molecule was also immunostained with antibodies to KS and CS; giving a negative response in each case (Plate 4.19).

4.7.4. Size of the GAG chain

Samples of DS-PGII purified from the tissue extracts were digested with papain. The resultant products were electrophoresed on polyacrylamide gels and stained with Stains-All to demonstrate the relative size of each tissue-specific GAG chain (Plate 4.20, Table 4.14). As with material obtained from culture media, the size of each GAG chain varied from tissue to tissue. DS-PGII isolated from bovine skin was found to be the smallest in size compared to DS-PGII from bovine tendon, which appeared to be the largest. Unlike the material from culture media, the differences in size from tissue to tissue were more pronounced. Because the size of the core proteins of each molecule are similar from one to another (Plate 4.18), the differences in the molecular weight of the intact molecules can be directly attributed to differences in the size of the attached GAG chain.

TABLE 4.14: Rf values of papain digested DS-PGII purified from tissue extracts.

DS-PGII standard	0.69
Skin[1]	0.69
Sclera	0.58
TMJ Disc	0.54
Gingiva	0.49
Tendon	0.46

[1] source of sample

4.7.5. Amino acid composition

The amino acid composition of each tissue-specific preparation of DS-PGII was determined in order to further confirm the identity and purity of the material. DS-PGII purified from bovine skin, sclera, tendon and TMJ disc all displayed an amino acid composition consistent with the DS-PGII standard; high in aspartic acid, glutamic acid and leucine. Material purified from gingival tissues was high in serine and glycine, but low in leucine. This may be the result of a contamination of the preparation, and is reflective of the difficulties that were encountered in purifying gingival DS-PGII.

TABLE 4.15: Amino Acid composition of DS-PGII preparations from tissue extracts (average of two determinations).

Amino Acids	Skin[1] DS-PGII	Skin[2]	Tendon	Sclera	TMJ Disc	Ging.
Asx	122[2]	136	140	132	131	103
Thr	38	42	42	41	44	39
Ser	62	63	67	75	88	105
Glx	109	119	117	128	138	136
Pro	70	80	77	72	69	65
Gly	74	71	66	77	78	104
Ala	50	51	51	54	50	62
Val	62	67	64	64	63	74
Met	9	7	8	10	18	ND
Ileu	65	48	51	46	44	37
Leu	124	118	121	110	101	88
Tyr	29	31	36	33	32	33
Phe	34	29	30	28	26	27
Lys	83	77	73	70	65	67
His	28	27	27	30	25	33
Arg	28	34	34	31	28	27
Cys	13	ND[3]	ND	ND	ND	ND

[1] Pearson and Gibson, 1982
[2] expressed as residues/1000
[3] not determined

4.8. Effects of DS-PGII Extracted from Bovine Tissues on Collagen Formation *in vitro*.

4.8.1. Turbidity assays

Previous studies have shown that bovine skin DS-PGII reduced the rate of collagen fibrillogenesis *in vitro* (Scott

et al, 1986). In addition, it has been shown by Scott *et al* (1986) and others (Brown and Vogel, 1989; Vogel *et al*, 1984) that the protein core is responsible for the effect; inhibition can be achieved with either intact DS-PGII or core protein only, but not with DS alone. Fibrillogenesis studies, using a variety of tissue-specific DS-PGII molecules, were done with intact DS-PGII only.

The results show that when DS-PGII, extracted from the various tissues are used in a turbidity assay, the rate of collagen fibril formation was decreased. In all cases the reduction was at least two-fold, and the effect was not altered by varying the concentrations of DS-PGII used (Table 4.16, fig. 4.19). Although the rate of fibrillogenesis was reduced, when precipitation was allowed to continue to completion (i.e. when a plateau was reached suggesting that fibril formation was complete), final optical densities were similar to those obtained when collagen alone was used in the assay. Varying concentrations of either bovine nasal cartilage PGs or a DS standard did not reduce the rate of fibrillogenesis.

The problems encountered in purifying DS-PGII from gingival tissues have been discussed and although the purity of the samples was questionable, the preparations were used in the turbidity assay nonetheless. As shown in Table 4.16, two preparations of gingival DS-PGII extracted from tissues (SA-67 and SA-71) did not affect the rate of collagen fibrillogenesis *in vitro*. However, when gingival DS-PGII isolated from

culture media was used in the assay, the rate of collagen fibrillogenesis was reduced to a rate comparable with the other tissue preparations of DS-PGII.

TABLE 4.16: Effect of various DS-PGII preparations and other molecules on the rate of collagen fibril formation *in vitro*.

Effector	[Conc.] (μg/ml)	Relative Rate [1]
Skin DS-PGII	30	0.41 ± 0.06[2]
Skin DS-PGII	45	0.45
Skin DS-PGII	60	0.40
Scleral DS-PGII	30	0.29 ± 0.015
Gingival (SA-67) DS-PGII	30	1.3
Gingival (SA-67) DS-PGII	150	1.26 ± 0.047
Gingival (SA-71) DS-PGII	30	0.97
Gingival (SA-71) DS-PGII	100	1.0
Gingival(culture) DS-PGII	30	0.54 ± 0.01
TMJ Disc DS-PGII	30	0.42 ± 0.015
Tendon DS-PGII	30	0.38 ± 0.015
Tendon DS-PGII	45	0.44
Bovine nasal cartilage PGs	15	1.14
Bovine nasal cartilage PGs	30	1.57 ± 0.105
Bovine nasal cartilage PGs	90	1.2
Dermatan sulphate	30	1.3
Dermatan sulphate	100	1.4

[1] compared to the rate for collagen alone
[2] standard deviation for at least two assays

4.8.2. Effects of DS-PGII on the size of collagen fibrils formed in vitro.

The mean width of collagen fibrils formed during fibrillogenesis, following the addition of each of the tissue-specific DS-PGII preparations, was examined on electron micrographs. The results show that when each DS-PGII molecule was added to solubilised collagen, the mean width of fibrils formed was always greater than when collagen alone was allowed to undergo fibrillogenesis (Table 4.17, fig. 4.20).

Each formed collagen "clot" was fixed in glutaraldehyde containing 0.5% Cuprolinic Blue, to allow for the visualization of the DS chain of DS-PGII. Micrographs taken of collagen formed with DS-PGII illustrate electron dense filaments radiating from the surface of the collagen fibrils (Plate 4.21). No such filaments are evident when collagen fibrils are formed without added PG. These results show that during fibrillogenesis, DS-PGII becomes bound onto the surface of the forming collagen fibrils.

TABLE 4.17: Collagen fibril diameters for collagen formed in vitro in the presence of various tissue-specific DS-PGII molecules.

Control[1]	40.59 ±	10.4[2]	(149)[3]
Gingiva[4]	*67.87 ±	18.63	(98)
Sclera	*60.88 ±	13.59	(133)
Skin	*67.19 ±	17.13	(152)
Tendon	*51.37 ±	11.72	(122)
TMJ Disc	*63.49 ±	17.23	(178)

[1] refers to diameters obtained when fibrillogenesis proceeded without the addition of any PGs.
[2] mean fibril diameters (± S.D.) in nm
[3] number of fibrils measured
[4] source of sample
*p <.01 when compared to collagen alone.

4.9. Electron Microscopic Studies on Tissues

4.9.1. Collagen fibril diameters

The results obtained following the isolation of each tissue-specific DS-PGII molecule have shown that the molecules vary by virtue of the length of their DS side chains. It has also been shown that DS-PGII is able to affect the rate of collagen fibrillogenesis and influence the final diameter of collagen fibrils formed in vitro. It is known that collagen fibril diameters vary from tissue to tissue depending upon the age of the animal, the mechanical role of the tissue, and the composition of its extracellular matrix (see Parry and Craig, 1984 for review). Electron micrographs were taken of each of the tissues from which DS-PGIIs were isolated, and collagen fibril diameters were measured from transverse sections in

order to determine whether or not the diameter of the fibrils could be related to the biochemical composition of DS-PGII (Plate 4.22). The results are shown in Table 4.18. The data presented here represents the amalgamation of collagen fibril diameters obtained from the various tissues of at least three individual animals.

TABLE 4.18: Collagen fibril diameters taken from electron micrographs of various bovine tissues.

Gingiva[1]		51.1 ± 11.8[2] (930)[3]
Sclera		214.7 ± 121.1 (997)
Skin		106.9 ± 26.8 (1489)
Tendon		171.3 ± 98.2 (754)
TMJ Disc	centre	67.9 ± 31.7 (4408)
	periphery	91.8 ± 44.4 (3396)

[1] source of sample
[2] mean fibril diameters (± S.D.) in nm
[3] number of fibrils measured

The large standard deviations evident for fibril diameters obtained from sclera and tendon are reflective of a bimodal distribution, if not trimodal for fibrils measured from tendon. The distributions of each tissue type are clearly evident on histograms (Fig. 4.21).

A combined histogram of collagen fibril diameters from the peripheral and central zones of the TMJ disc is shown in fig. 4.22. It illustrates the differences in the size of the fibrils between the two zones. Fibrils from the central zone are significantly ($p<0.05$) smaller than fibrils found in the periphery of the disc.

4.2.3. Interfibrillar distances

Mean interfibrillar distances were calculated in order to determine whether or not the spacing between collagen fibrils could be related to the length of the GAG chain of the various tissue-specific DS-PGII molecules. The results, together with the percentage of area occupied by collagen in the matrix of each tissue, are shown in Table 4.19. The data have been collected from three individual animals.

TABLE 4.19: Mean interfibrillar distances and percentage of area occupied by collagen fibrils determined from electron micrographs of various bovine tissues.

Gingiva[1]	35.5 ± 3.7[2] (4)[3]	28.7 ± 6.5[4]
Sclera	105.7 ± 21.5 (9)	51.3 ± 6.7
Skin	38.2 ± 3.3 (6)	48.4 ± 5.9
Tendon	93.3 ± 32.9 (5)	54.6 ± 3.3
TMJ disc center periphery	43.8 ± 5.8 (5) 38.0 ± 7.8 (5)	37.6 ± 4.9 49.7 ± 5.8

[1] source of sample
[2] mean interfibrillar distance (± S.D.) in nm
[3] number of micrographs from which data was collected
[4] mean % area occupied by collagen (± S.D.)

A correlation can be found between the interfibrillar distances, percentage area occupied by collagen and collagen fibril diameters. For example, gingival tissues have the smallest collagen fibril diameters, are more closely packed together and yet the collagen accounts for the smallest percentage of the tissues extracellular matrix. Conversely, tendon and scleral tissues, whose collagen fibril diameters

follow a bimodal distribution, are comprised of much larger diameter fibrils which are further spaced apart, but overall occupy over half of the extracellular matrix.

4.9.3. Cuprolinic Blue staining

When sections of each of the various bovine tissues were stained with Cuprolinic Blue, an array of electron dense filaments could be seen radiating from the surface of, and between, collagen fibrils (Plate 4.23). In most cases, particularly in E.M. of skin, the electron dense filaments were aligned along the surface of the fibrils in a periodic fashion, often associated with the d or e band of the fibril. In tissues which contained a bimodal distribution of collagen fibril diameters (i.e. sclera and tendon), filaments could be found associated with both large and small diameter fibrils. In micrographs taken of gingival tissues, where the fibrils are more closely packed than in the other tissues, it was difficult to see whether or not the electron dense filaments were actually radiating from the surface of the fibrils. However, a periodic array was still evident.

Comparing the central and peripheral zones of the TMJ disc, the central zones were seen to contain an increased proportion of larger, broad filaments. Unlike the peripheral zone, these filaments appear to be lying in an interfibrillar position. Filaments in the peripheral zone however, appear to be regularly spaced along the surface of the collagen fibrils at a distance equal to the D-period of the fibrils.

4.9.4. Enzyme digestions

In order to confirm the GAG nature of the Cuprolinic Blue staining filaments, samples were digested with enzymes prior to staining. Samples of skin and the center of the TMJ disc were used because of the representation of thin, periodically aligned and collagen associated filaments in the skin, and the larger, interfibrillar filaments prominent in the center of the disc.

In both tissues, all Cuprolinic Blue positive filaments, either those associated with the surface of collagen fibrils or lying between the fibrils, were removed with chondroitinase ABC (Plate 4.23). The Cuprolinic Blue positive filaments were only partially affected by digestion with chondroitinase AC. Both enzymes are capable of specifically digesting CS-4, CS-6, and DS, although chondroitinase AC will digest only at a glucuronic acid residue, explaining the partial digestion. When both tissues were digested with chondroitinase AC, the Cuprolinic Blue positive filaments were still evident, although greatly reduced in number and much shorter length. The data confirms that the electron dense filaments are GAG.

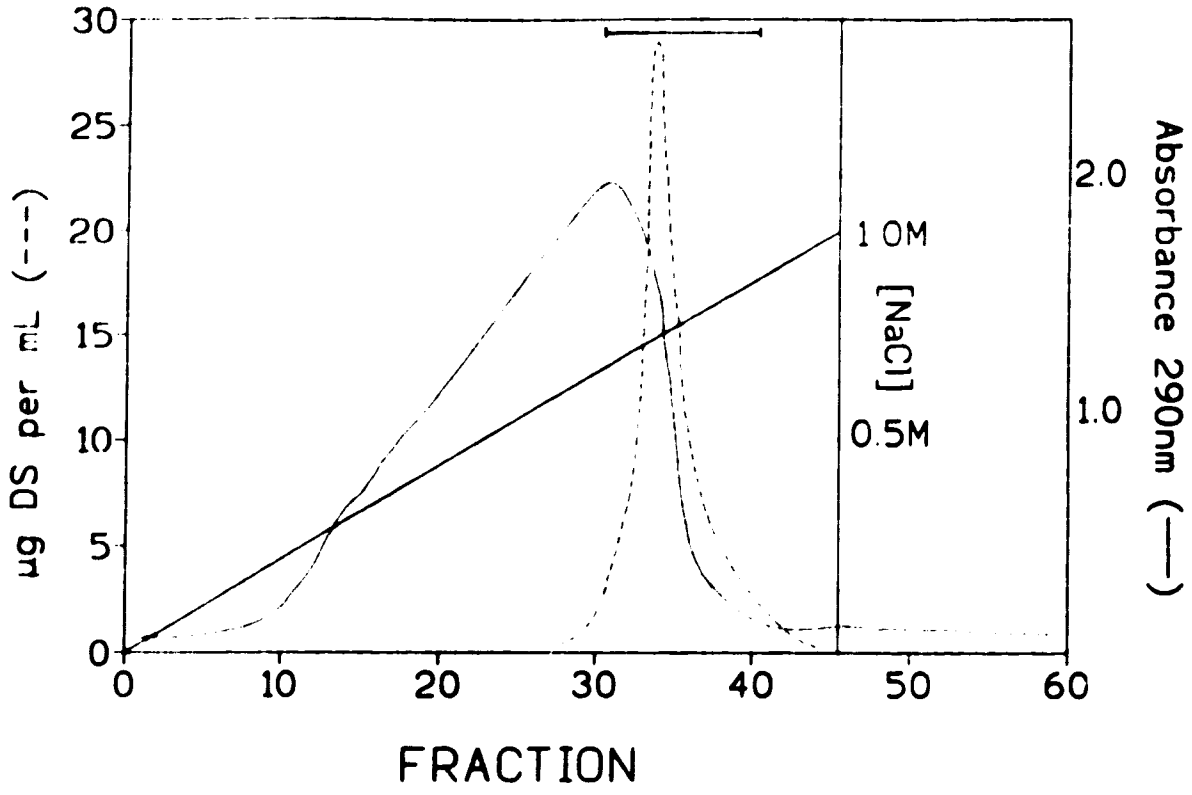


Fig. 4.1. Purification of culture media from bovine TMJ disc fibroblasts by chromatography on DEAE-cellulose. 2000mL of culture media was applied to a 1.6 x 8 cm column equilibrated in 25mM phosphate buffer with proteinase inhibitors and eluted with a 1M NaCl gradient. 2mL fractions were collected, and pooled as shown by bar. (---) is result of a dye binding assay for GAGs.

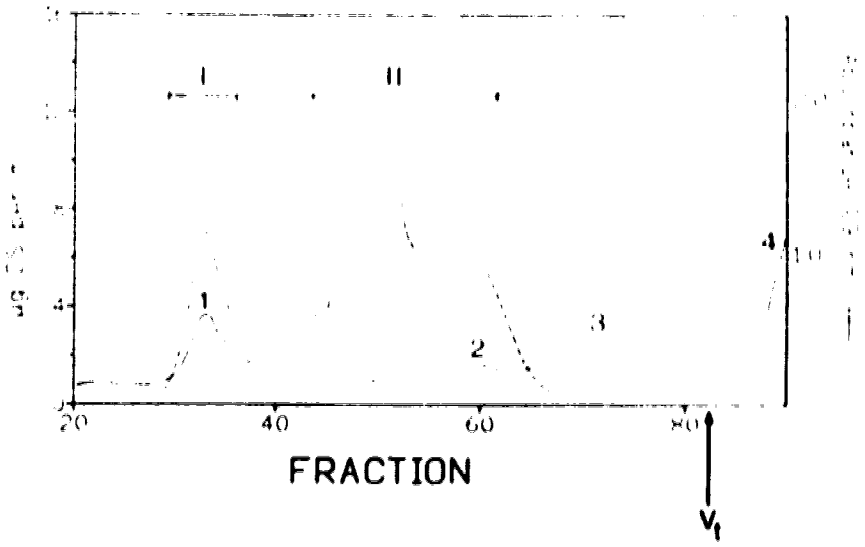


Fig. 4.2. Purification of sulphated material secreted by bovine tendon fibroblasts on Sephacryl-400SF. A) a sample (1.5 mg) of GAG positive material pooled from ion-exchange chromatography was applied to a 1.6 x 135 cm column equilibrated and eluted with 25mM phosphate/0.5M NaCl. 3mL fractions were collected, and pooled as shown by bars. (---) is result of a dye binding assay for GAGs.

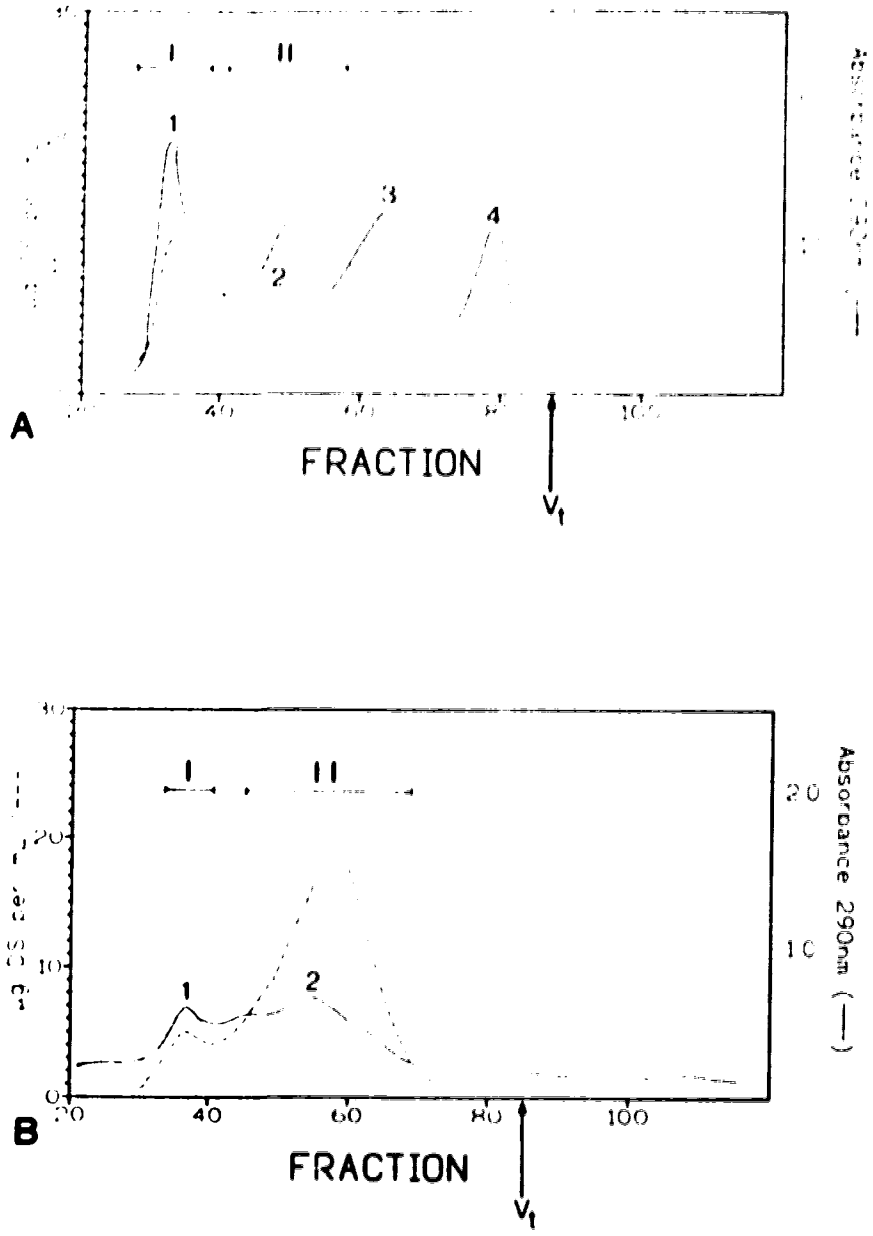


Fig. 4.3. Purification of sulphated material secreted by bovine TMJ disc fibroblasts on Sephacryl-400SF.
A) a sample (3.2 mg) of GAG positive material pooled from ion-exchange chromatography was applied to a 1.6 x 135 cm column equilibrated and eluted with 25mM phosphate/0.5M NaCl. 3mL fractions were collected, and pooled as shown by bars. (---) is result of a dye binding assay for GAGs.
B) fractions pooled from II of A) were concentrated and 1.6 mg applied to the column as above. 3mL fractions were collected and pooled as shown by bar.

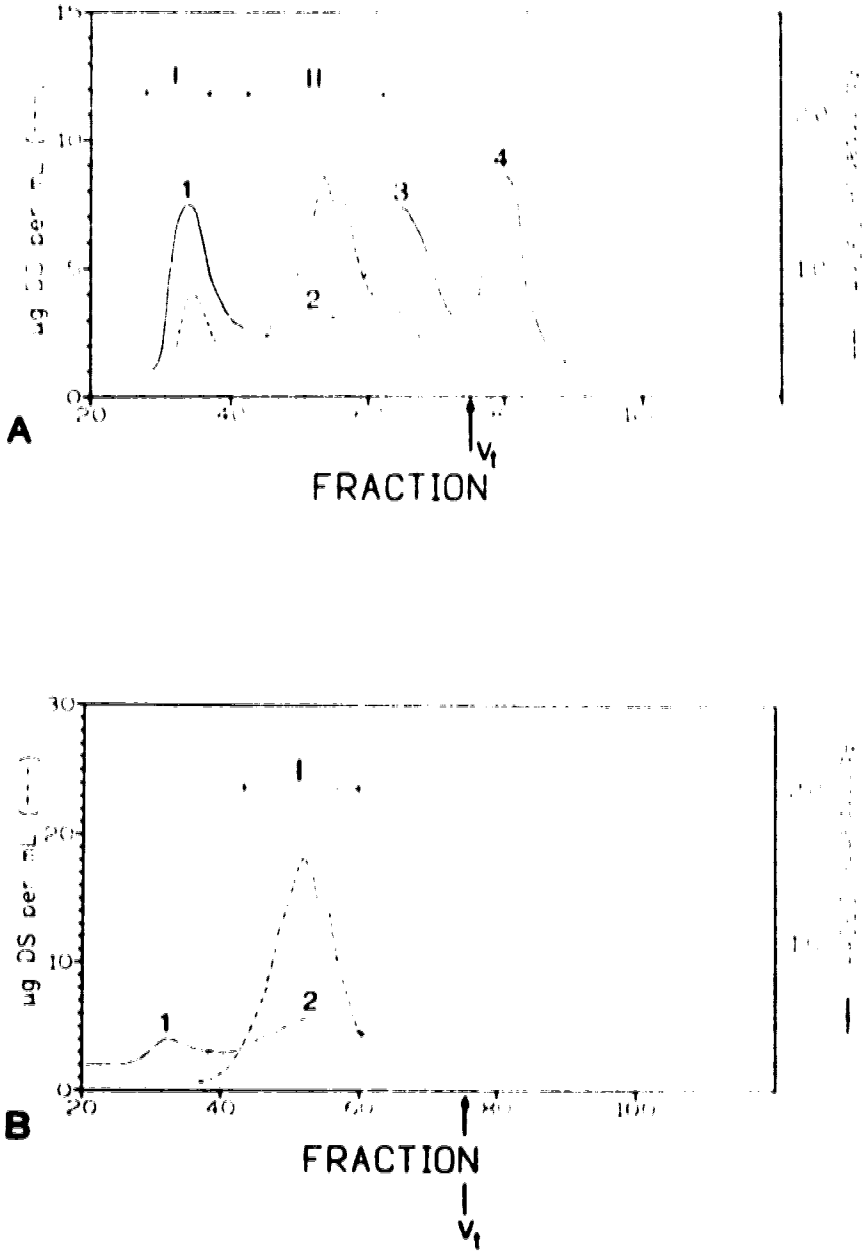


Fig. 4.4. Purification of sulphated material secreted by bovine sclera fibroblasts on Sephacryl-400SF.
A) a sample (2.8 mg) of GAG positive material pooled from ion-exchange chromatography was applied to a 1.6 x 135 cm column equilibrated and eluted with 25mM phosphate/0.5M NaCl. 3mL fractions were collected, and pooled as shown by bars. (---) is result of a dye binding assay for GAGs.
B) fractions pooled from II of A) were concentrated and 1.2 mg applied to the column as above. 3mL fractions were collected and pooled as shown by bar.

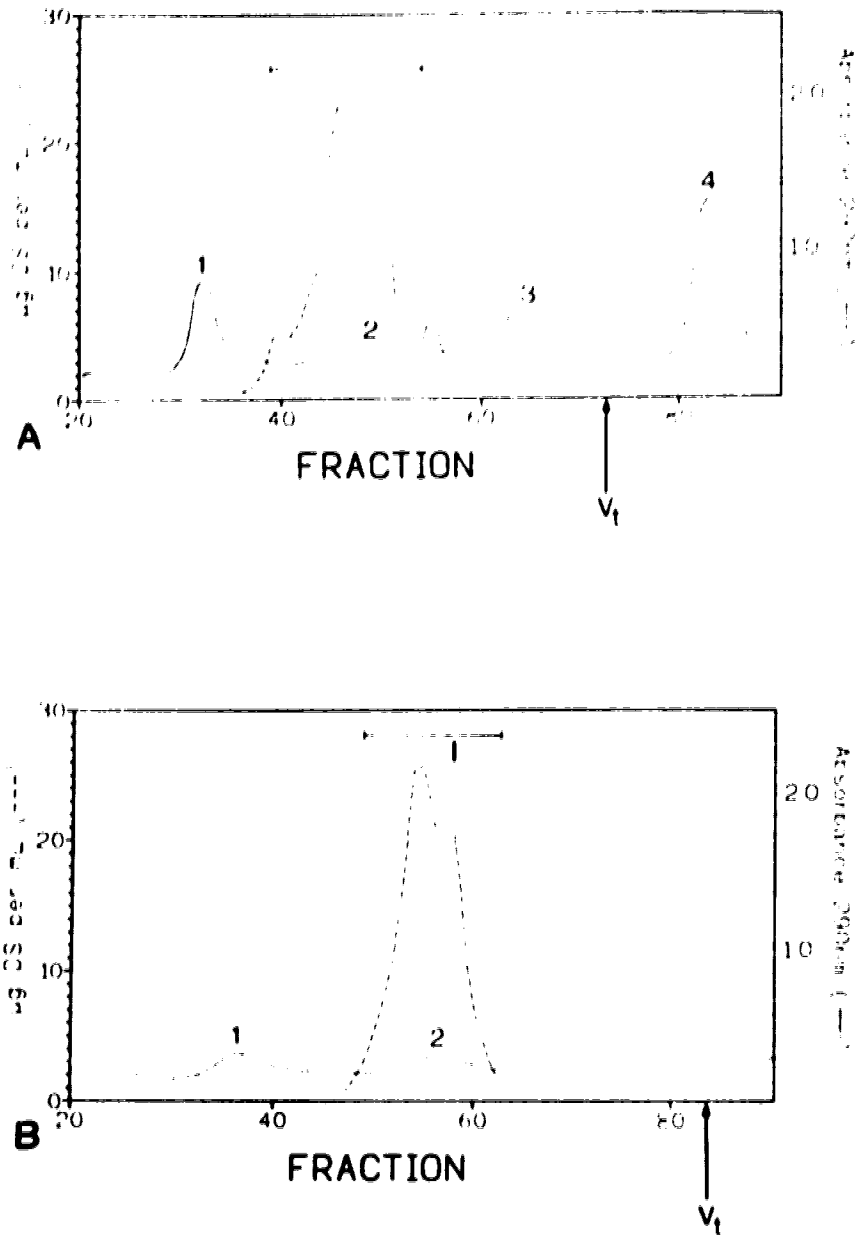


Fig. 4.5. Purification of sulphated material secreted by bovine skin fibroblasts on Sephacryl-400SF.
A) a sample (8.1 mg) of GAG positive material pooled from ion-exchange chromatography was applied to a 1.6 x 135 cm column equilibrated and eluted with 25mM phosphate/0.5M NaCl. 3mL fractions were collected, and pooled as shown by bar. (---) is result of a dye binding assay for GAGs.
B) fractions pooled from A) were concentrated and 1.9 mg applied to the column as above. 3mL fractions were collected and pooled as shown by bar.

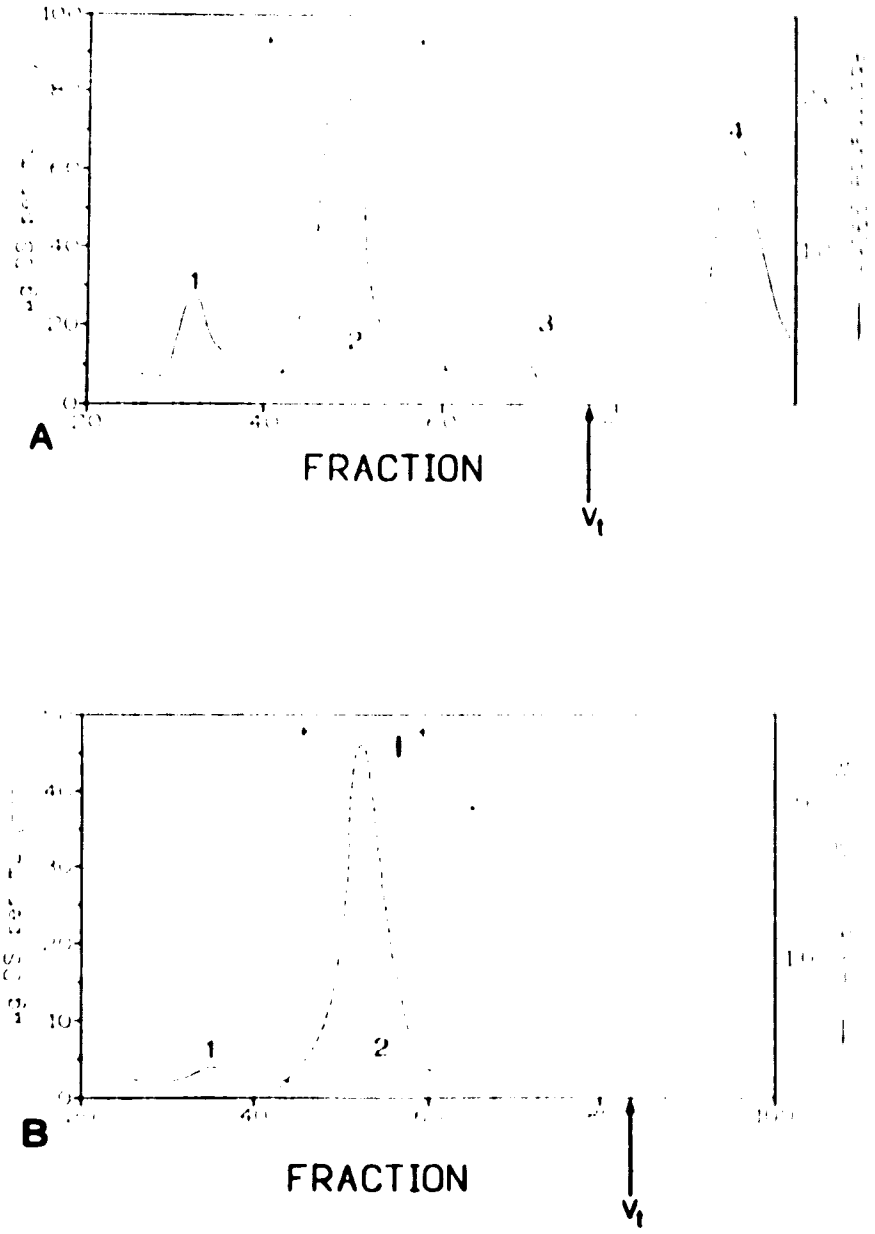


Fig. 4.6. Purification of sulphated material secreted by bovine gingiva fibroblasts on Sephacryl-400SF.
A) a sample (9.0 mg) of GAG positive material pooled from ion-exchange chromatography was applied to a 1.6 x 135 cm column equilibrated and eluted with 25mM phosphate/0.5M NaCl. 3mL fractions were collected, and pooled as shown by bar. (---) is result of a dye binding assay for GAGs.
B) fractions pooled from A) were concentrated and 4.6 mg applied to the column as above. 3mL fractions were collected and pooled as shown by bar.

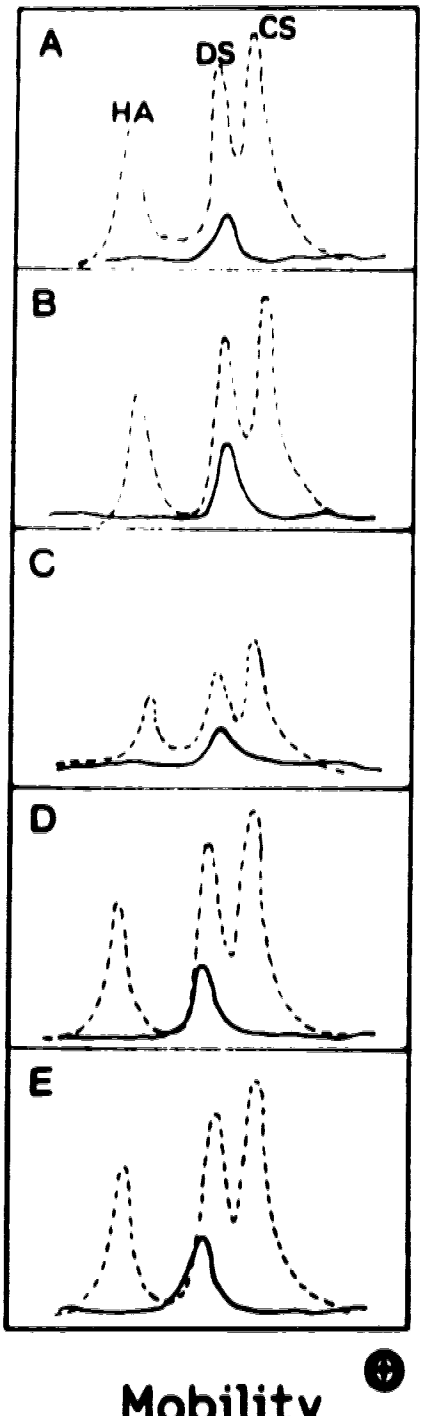


Fig. 4.7. Cellulose acetate electrophoresis of GAGs obtained by papain digestion of fractions I (II for TMJ disc material) from Sephacryl 400SF chromatography (figs. 4.3 to 4.6). (---) represents GAG standards. A) from gingival fibroblasts B) from skin fibroblasts C) from scleral fibroblasts D) from TMJ disc fibroblasts E) from tendon fibroblasts. Electrophoresis was run in acetic acid/pyridine buffer.

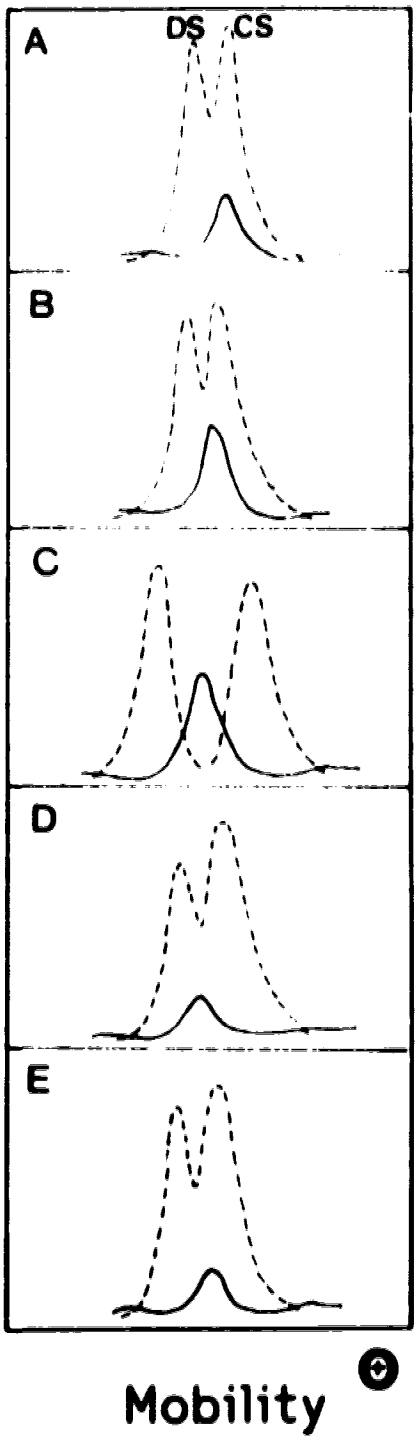


Fig. 4.8. Cellulose acetate electrophoresis of GAGs obtained by papain digestion of fractions I (II for TMJ disc material) from Sephacryl 400SF chromatography (figs. 4.3 to 4.6). (---) represents GAG standards. A) from gingival fibroblasts B) from skin fibroblasts C) from scleral fibroblasts D) from TMJ disc fibroblasts E) from tendon fibroblasts. Electrophoresis was run in 0.1N HCl buffer.

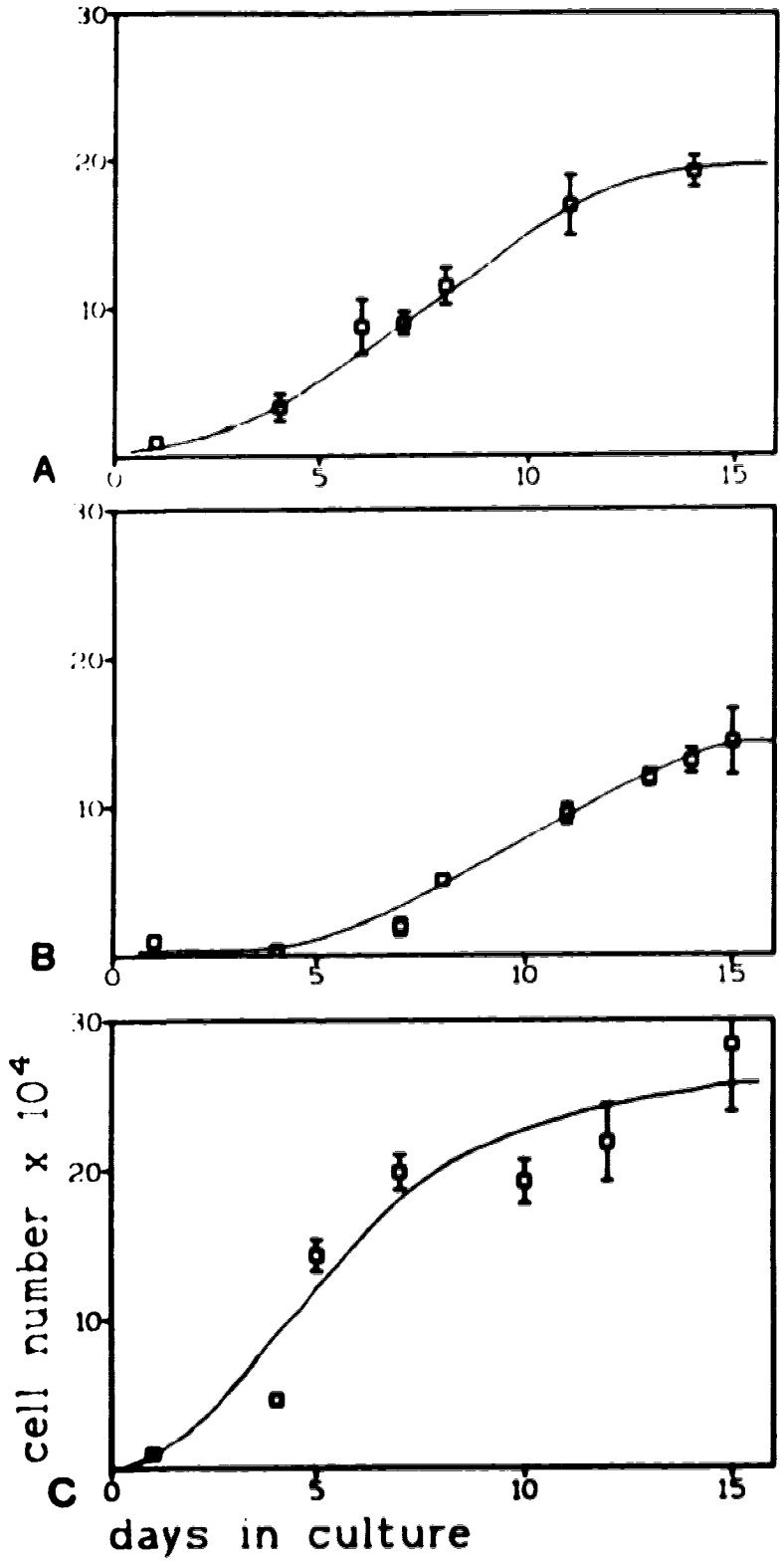


Fig. 4.9. Growth curves generated for cell strain 1 of bovine tendon fibroblasts in various passages. A) passage 4, t_d= 2.0 days B) passage 6, t_d= 1.7 days, C) passage 9, t_d= 1.7 days.

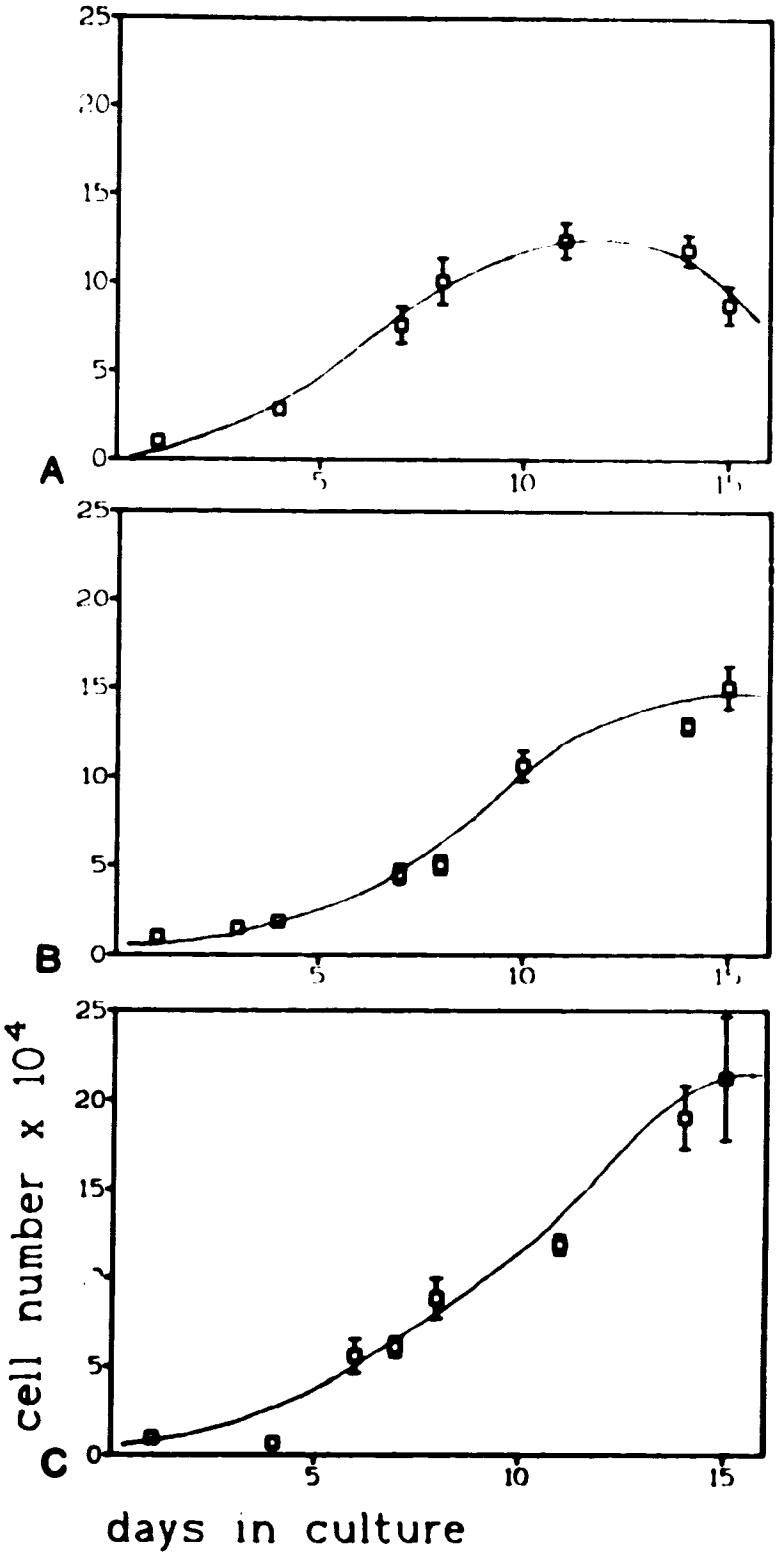


Fig. 4.10. Growth curves generated for cell strain 2 of bovine tendon fibroblasts in various passages. A) passage 4, t_d= 2.5 days B) passage 6, t_d= 3.7 days C) passage 9, t_d= 4.3 days.

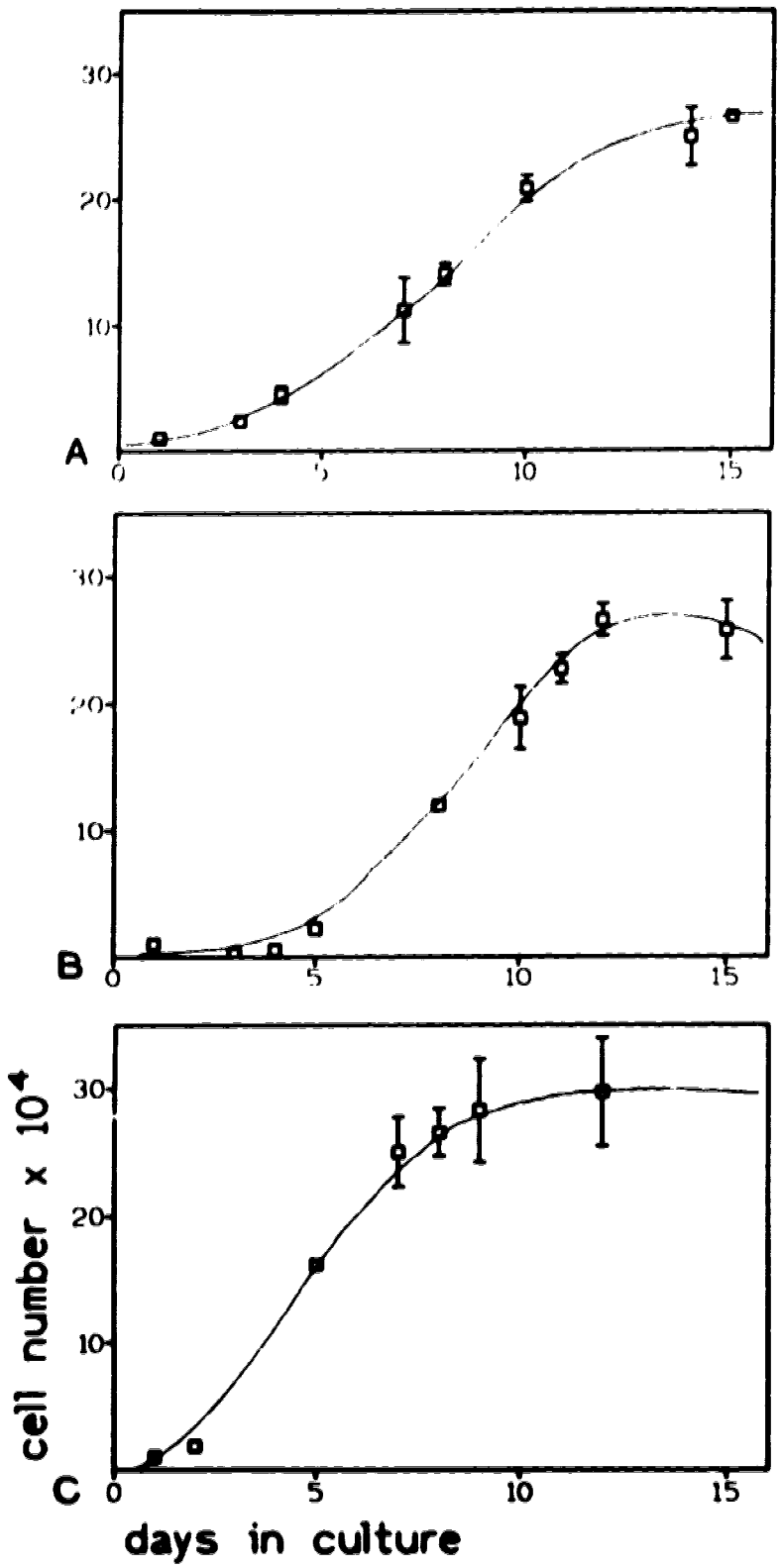


Fig. 4.11. Growth curves generated for cell strain 1 of bovine gingival fibroblasts in various passages. A) passage 4, t_d= 2.7 days B) passage 6, t_d= 2.6 days C) passage 9, t_d= 1.5 days.

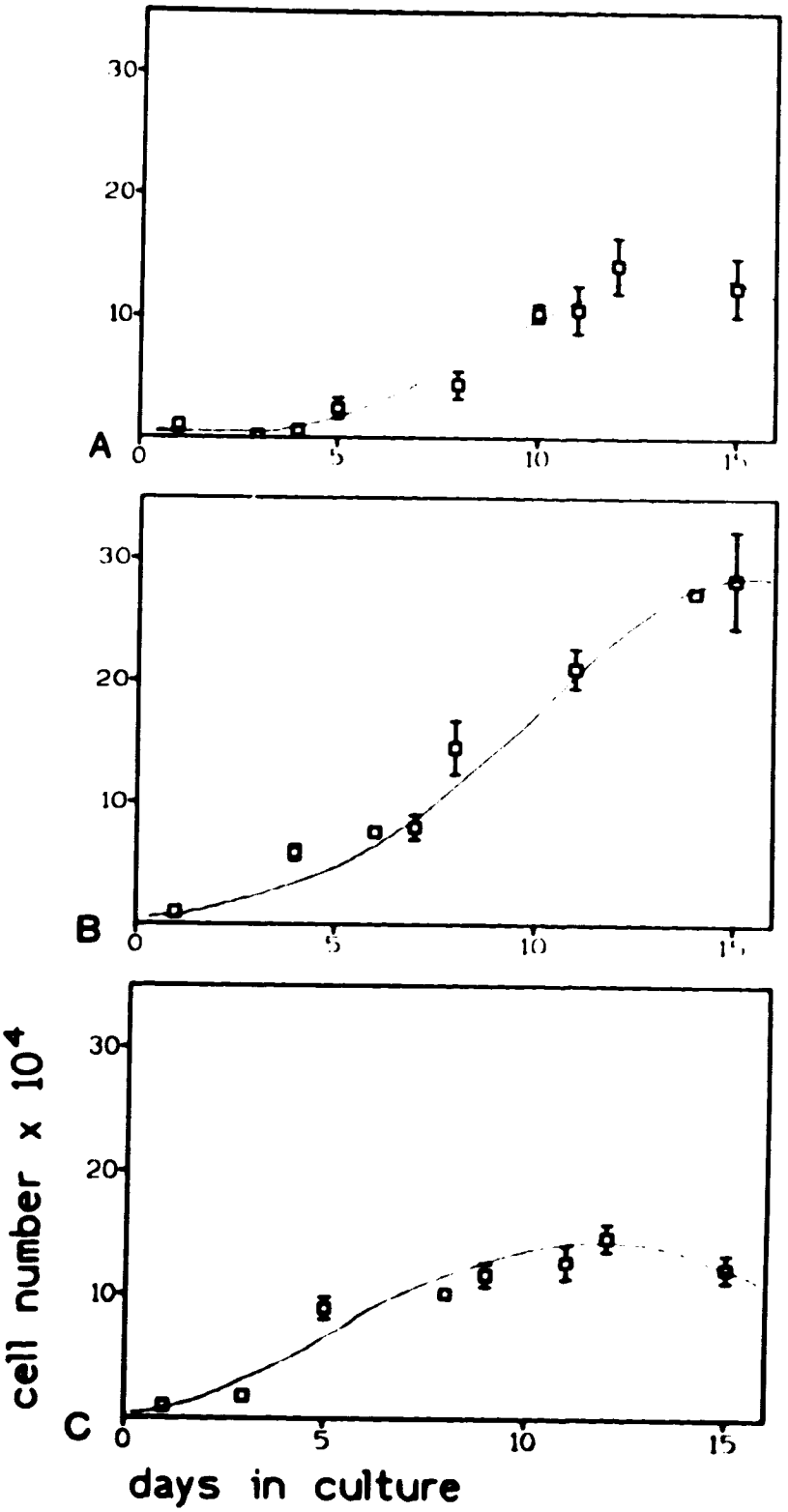


Fig. 4.12. Growth curves generated for cell strain 3 of bovine gingival fibroblasts in various passages. A) passage 4, t_d= 3.0 days B) passage 6, t_d= 3.9 days C) passage 9, t_d= 3.0 days.

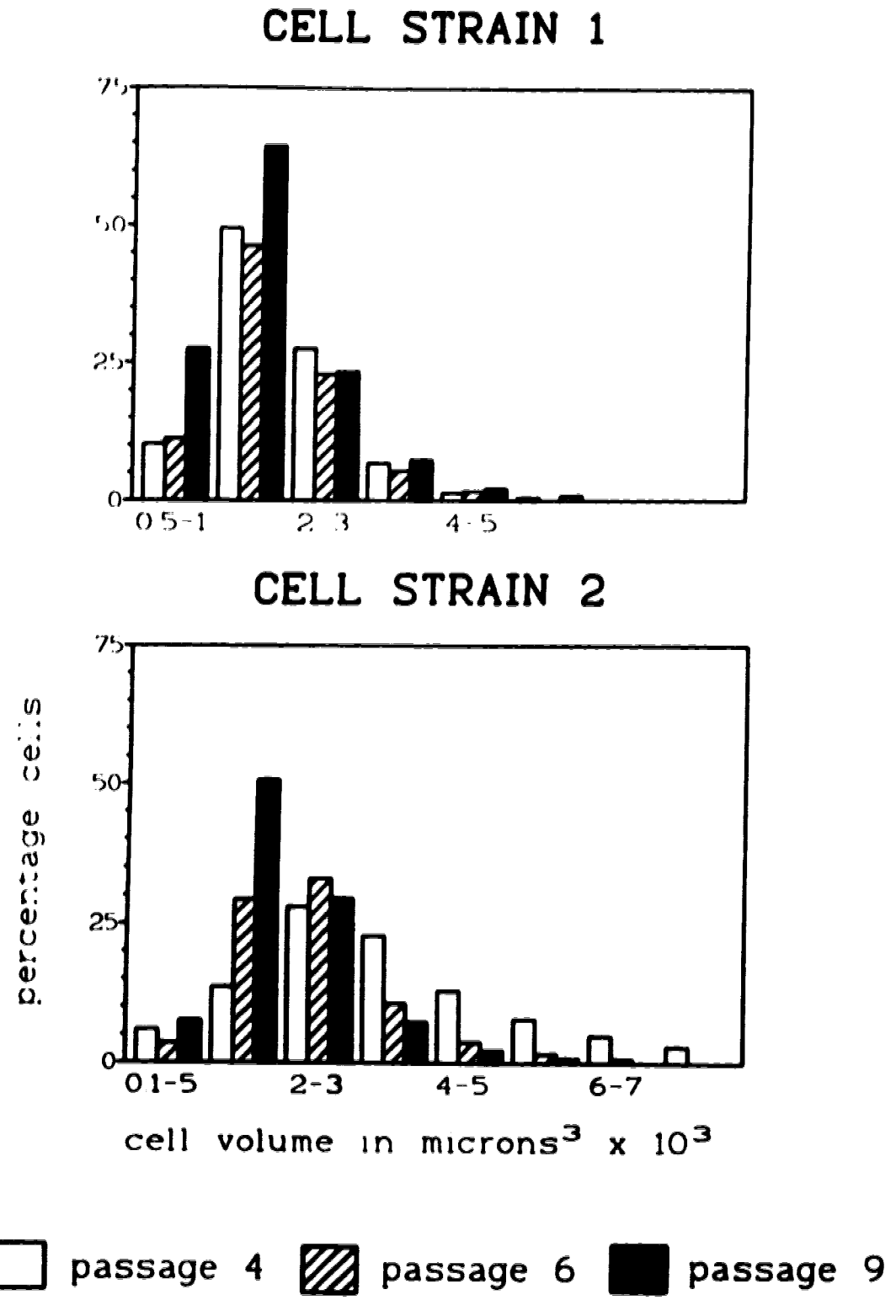


Fig. 4.13. Cell volumes as determined for cell strains 1 and 2 of bovine tendon fibroblasts in various passages.

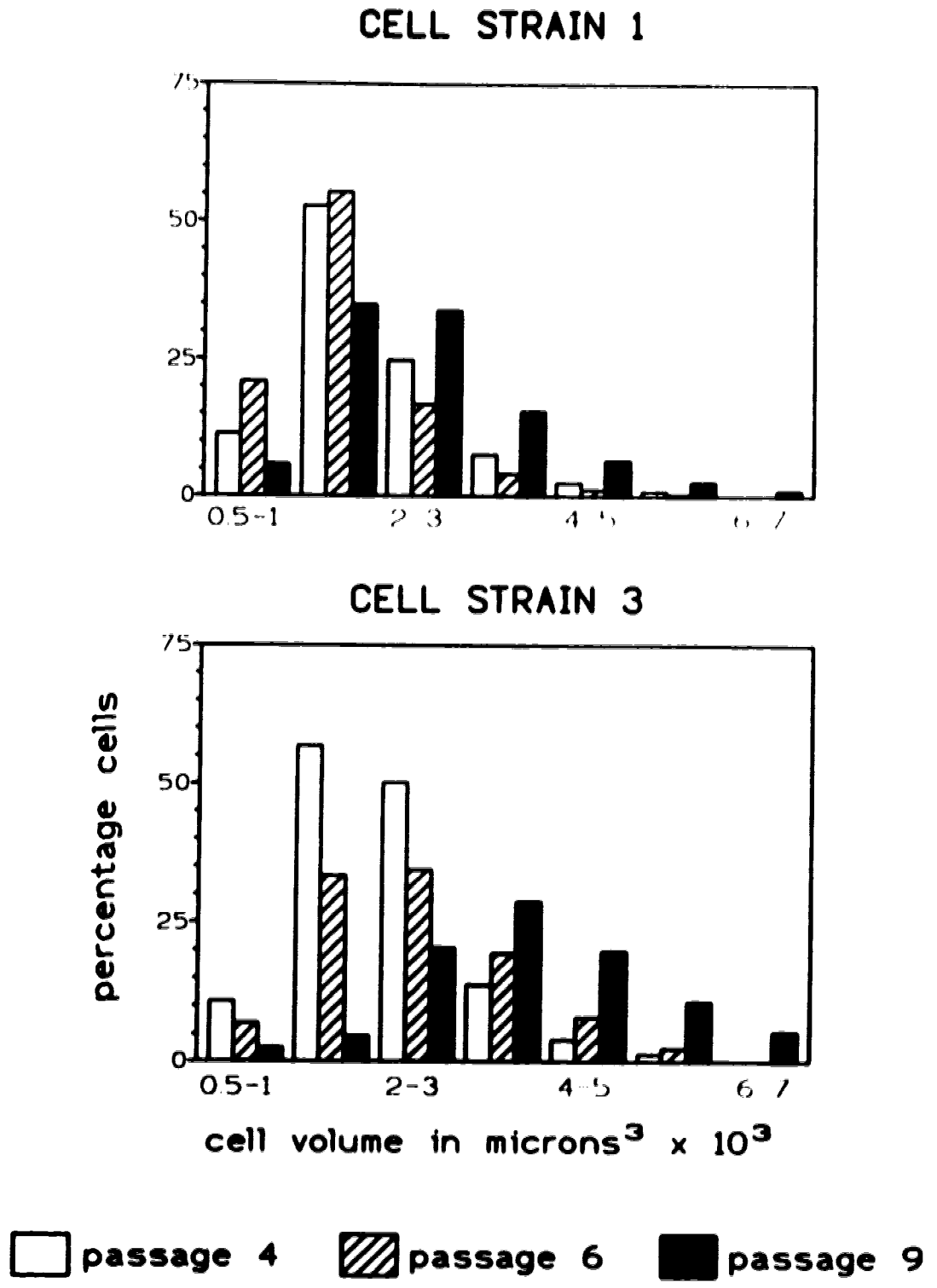
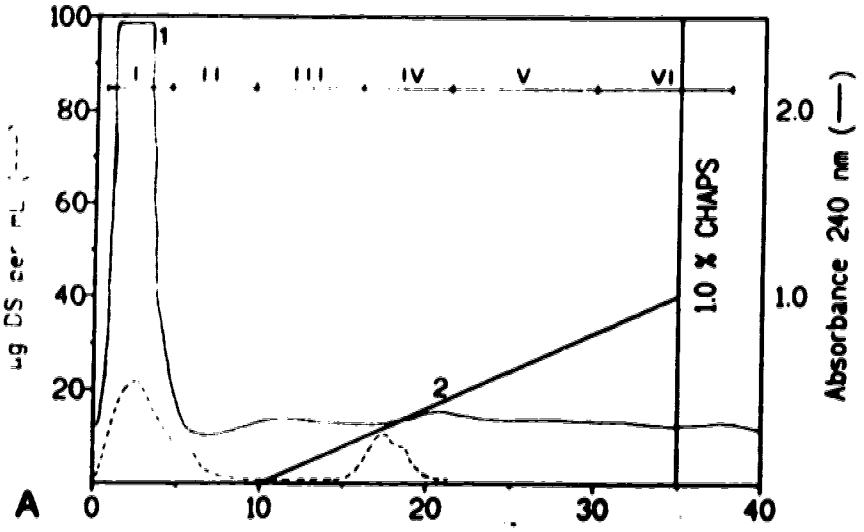
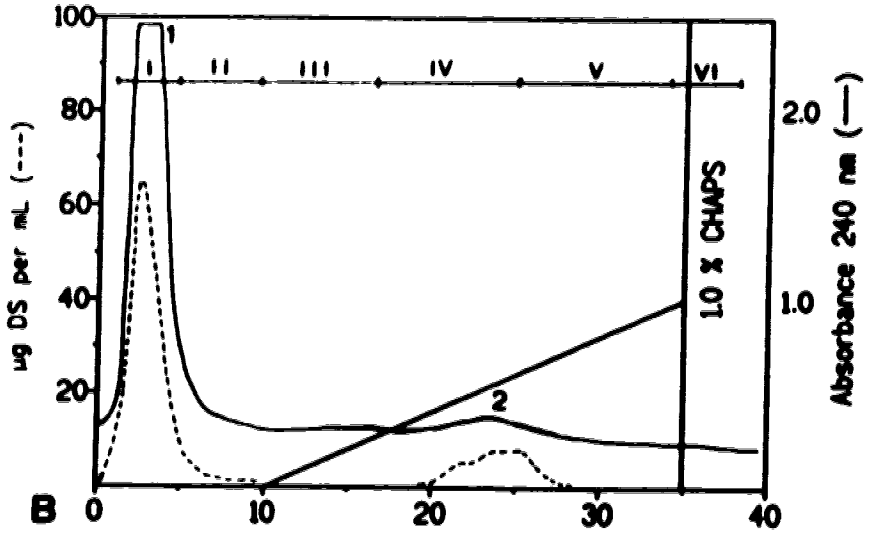


Fig. 4.14. Cell volumes as determined for cell strains 1 and 3 of bovine gingival fibroblasts in various passages.



FRACTION



FRACTION

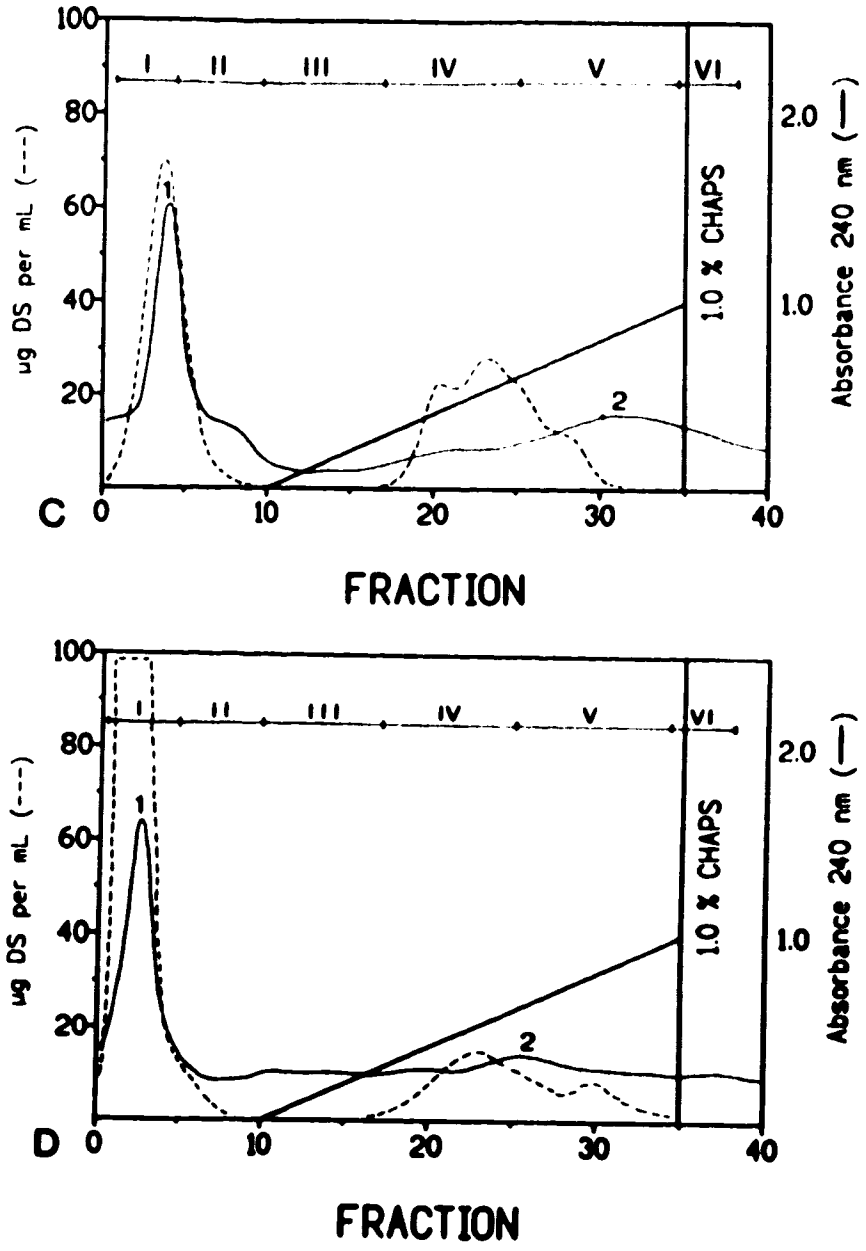


Fig 4.15. Purification of sulphated material extracted from various bovine tissues on Octyl-Sepharose. Samples (≈ 4.5 mg) of GAG positive material pooled from ion-exchange chromatography were applied to a 1.6 x 4.5 cm column equilibrated in 4M guanidine/0.15M sodium acetate and eluted with a 1% CHAPS gradient. 2mL fractions were collected and pooled as shown by bars. (---) is result of a dye binding assay. A) gingival extract B) scleral extract C) TMJ disc extract D) tendon extract.

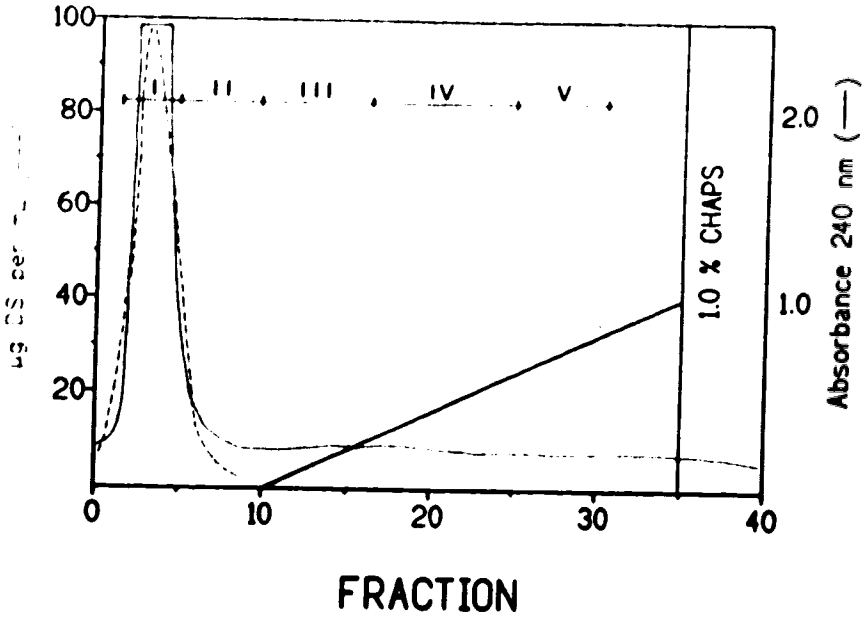
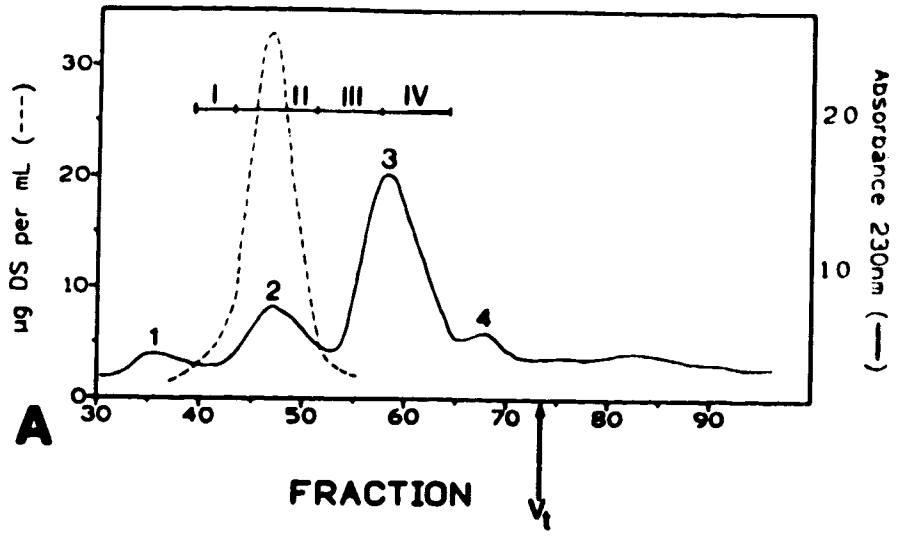
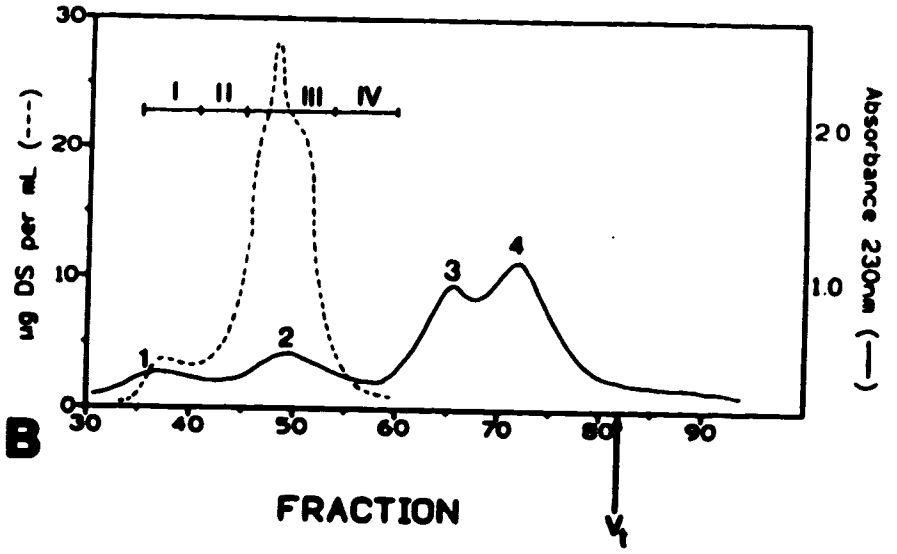


Fig 4.16. Purification of sulphated material extracted from bovine skin on Octyl-Sepharose. A sample (4.7 mg) of GAG positive material pooled from ion-exchange chromatography was applied to a 1.6 x 4.5 cm column equilibrated in 4M guanidine/0.15M sodium acetate and eluted with a 1% CHAPS gradient. 2mL fractions were collected and pooled as shown by bars. (---) is result of a dye binding assay.


µg DS per mL (---)
Absorbance 230nm (—)
I II III IV
1
2
3
4
A
FRACTION
V_t


µg DS per mL (---)
Absorbance 230nm (—)
I II III IV
1
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3
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B
FRACTION
V_t

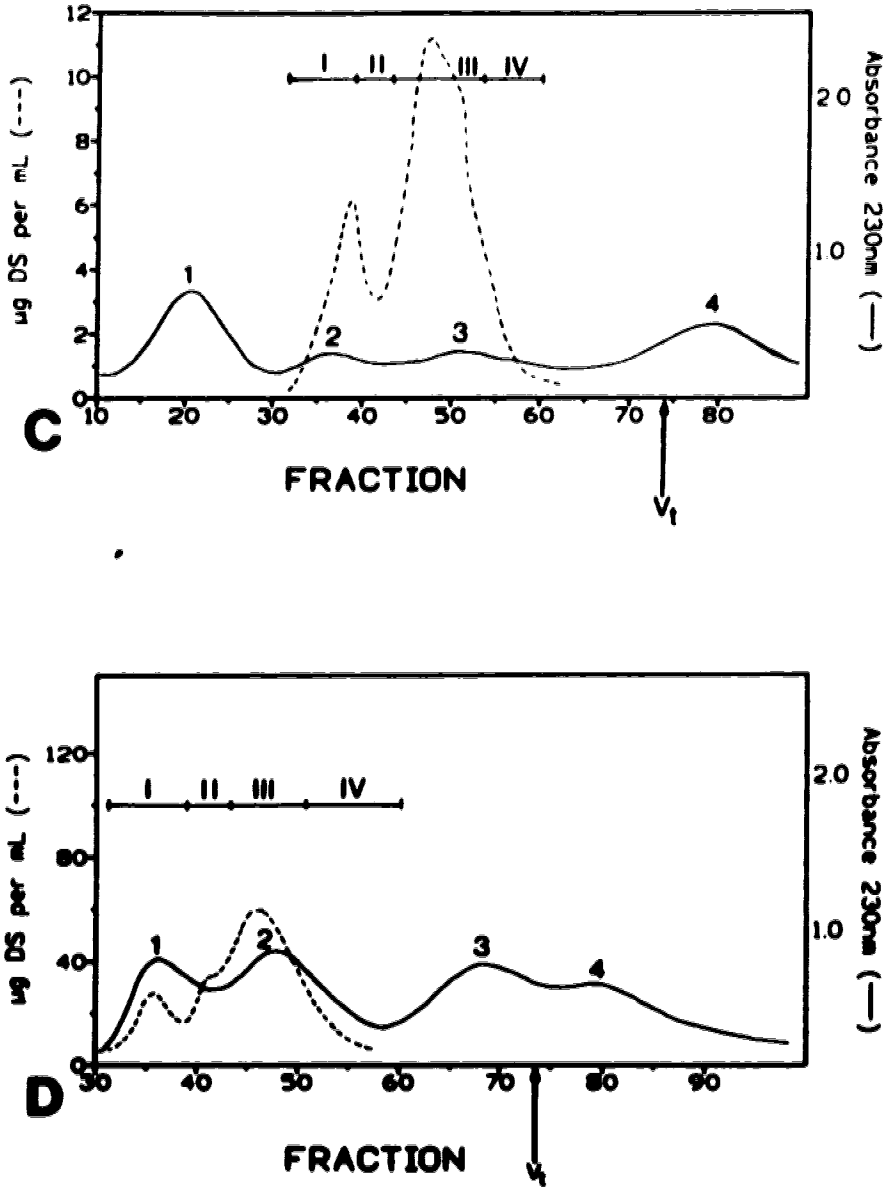


Fig. 4.17. Purification of sulphated material extracted from various bovine tissues on Sephacryl 400SF. Samples (≈ 3.0 mg) from fraction I of Octyl-Sepharose chromatography (fig. 4.15 and 4.16) were applied to a 1.6 x 135 cm column equilibrated and eluted with 25mM phosphate/0.5M NaCl. 3mL fractions were collected and pooled as shown by bars. (---) is result of a dye binding assay for GAGs. A) skin extract B) sclera extract C) TMJ disc extract D) tendon extract.

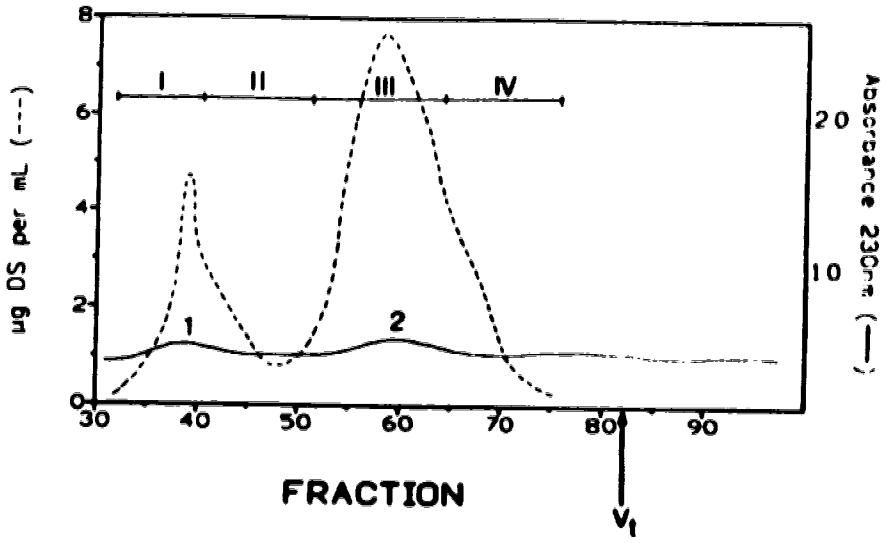


Fig. 4.18. Purification of sulphated material extracted from bovine gingiva on Sephacryl 400SF. A sample (≈ 3.0 mg) from fraction I of Octyl-Sepharose chromatography (fig. 4.15) was applied to a 1.6 x 135 cm column equilibrated and eluted with 25mM phosphate/0.5M NaCl with proteinase inhibitors. 3mL fractions were collected and pooled as shown by bars. (---) is result of a dye binding assay.

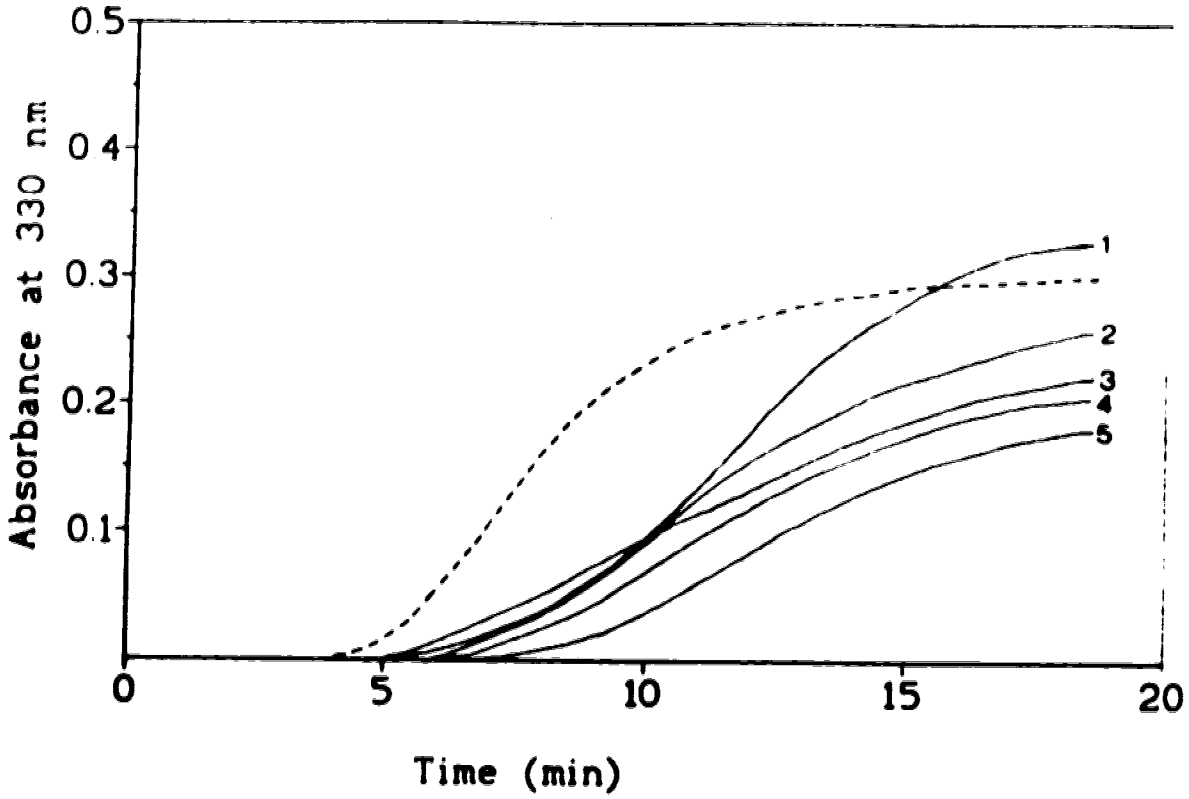


Fig. 4.19. The effect of DS-PGIIs extracted from various bovine tissues (or culture media for gingival sample) on the kinetics of collagen precipitation. (---) control (no added DS-PGII) 1) gingival DS-PGII 2) skin DS-PGII 3) TMJ disc DS-PGII 4) tendon DS-PGII 5) scleral DS-PGII. 30 μL of sample were used in each assay.

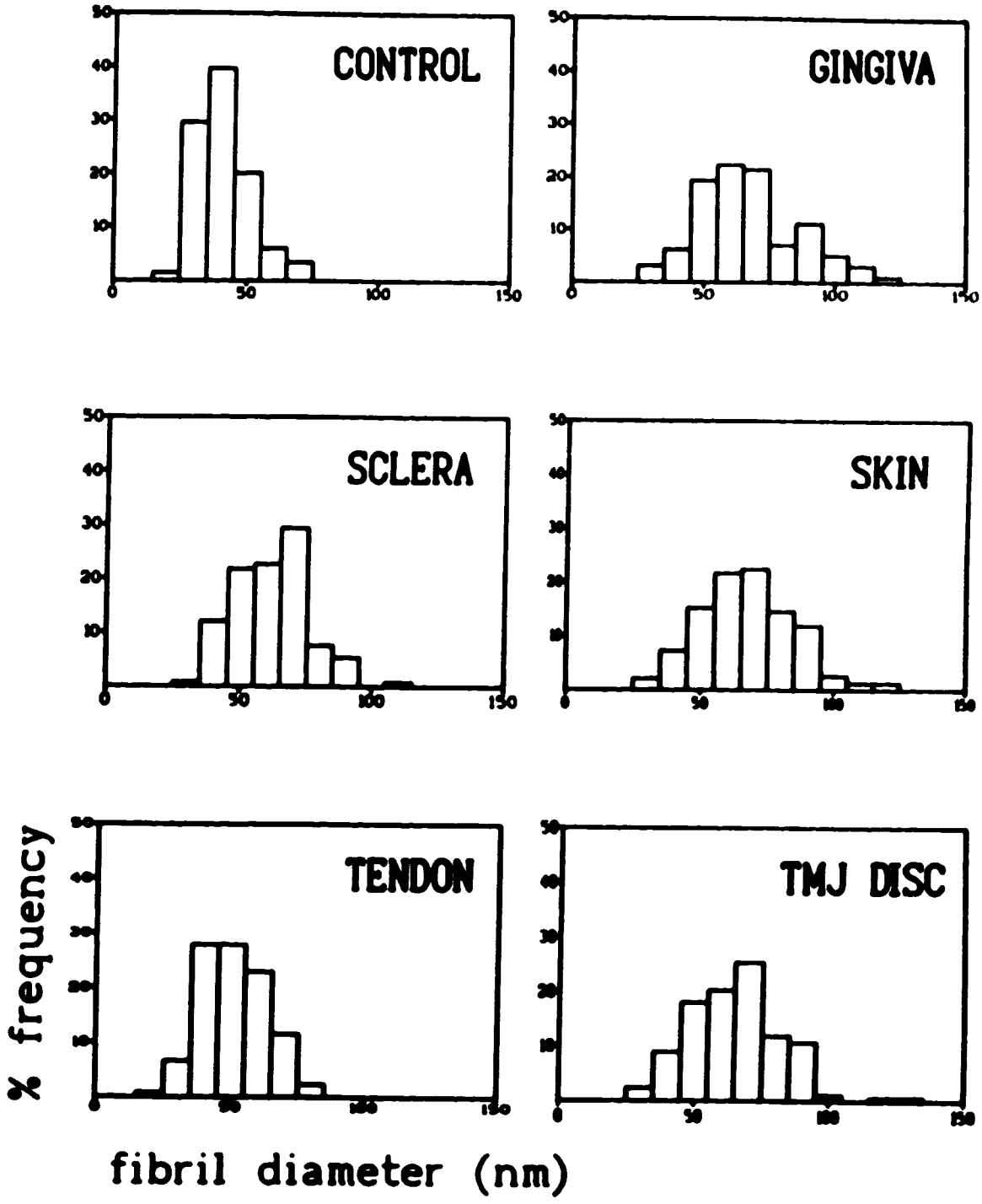


Fig. 4.20. Histograms illustrating the distributions of collagen fibril diameters formed *in vitro* in the presence of various tissue-specific SS-PGXIs.

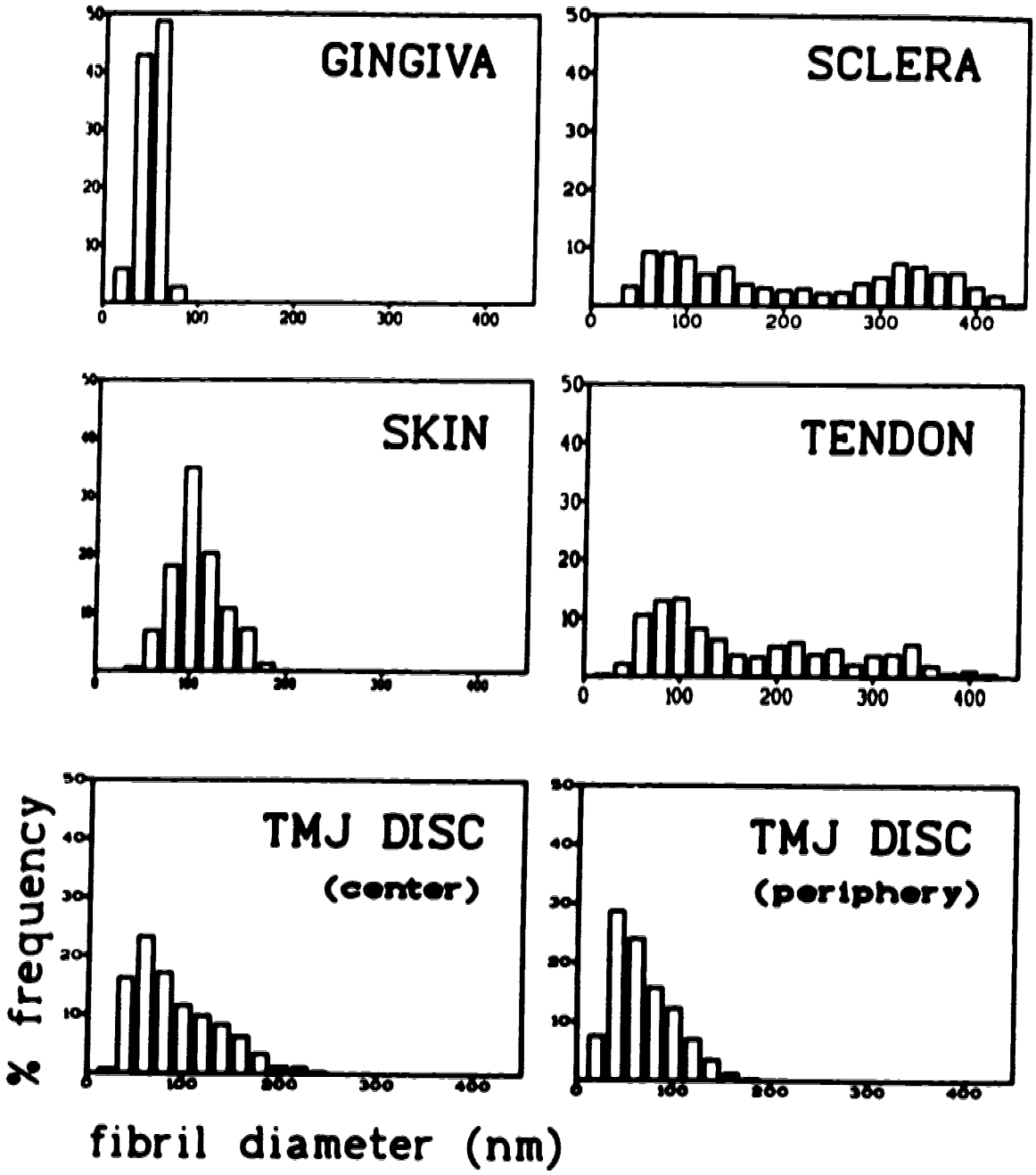


Fig. 4.21. Histograms illustrating the distributions of collagen fibril diameters in various bovine tissues.

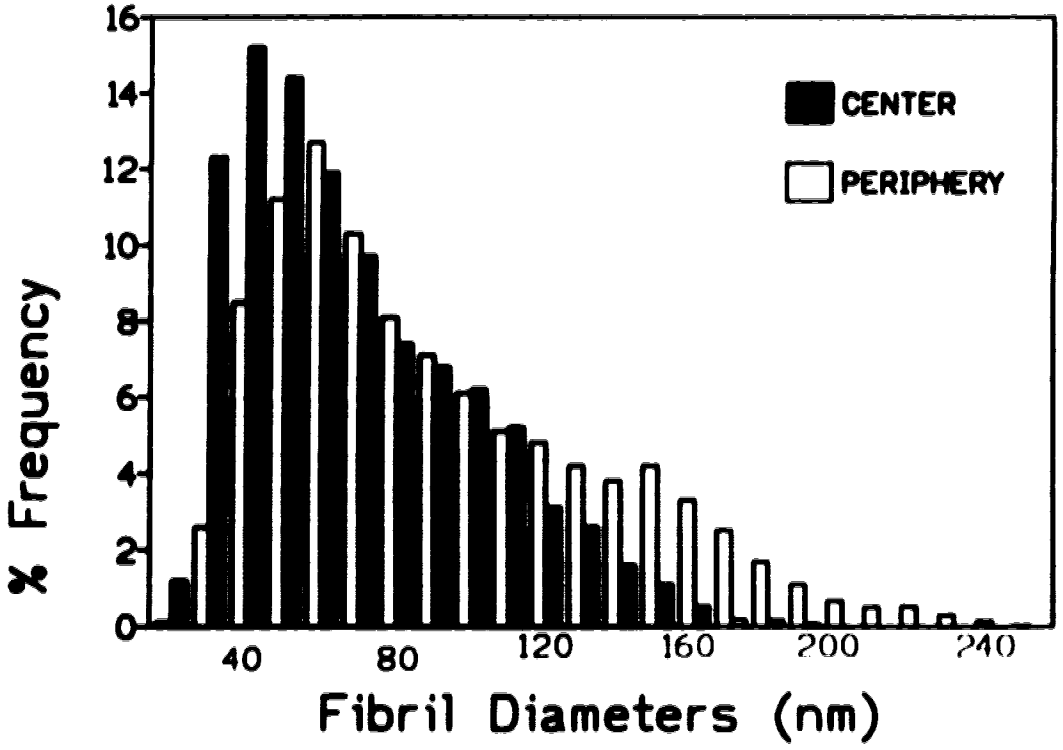


Fig. 4.22. Histogram illustrating the distributions of collagen fibril diameters in the central and peripheral zones of the TMJ disc.

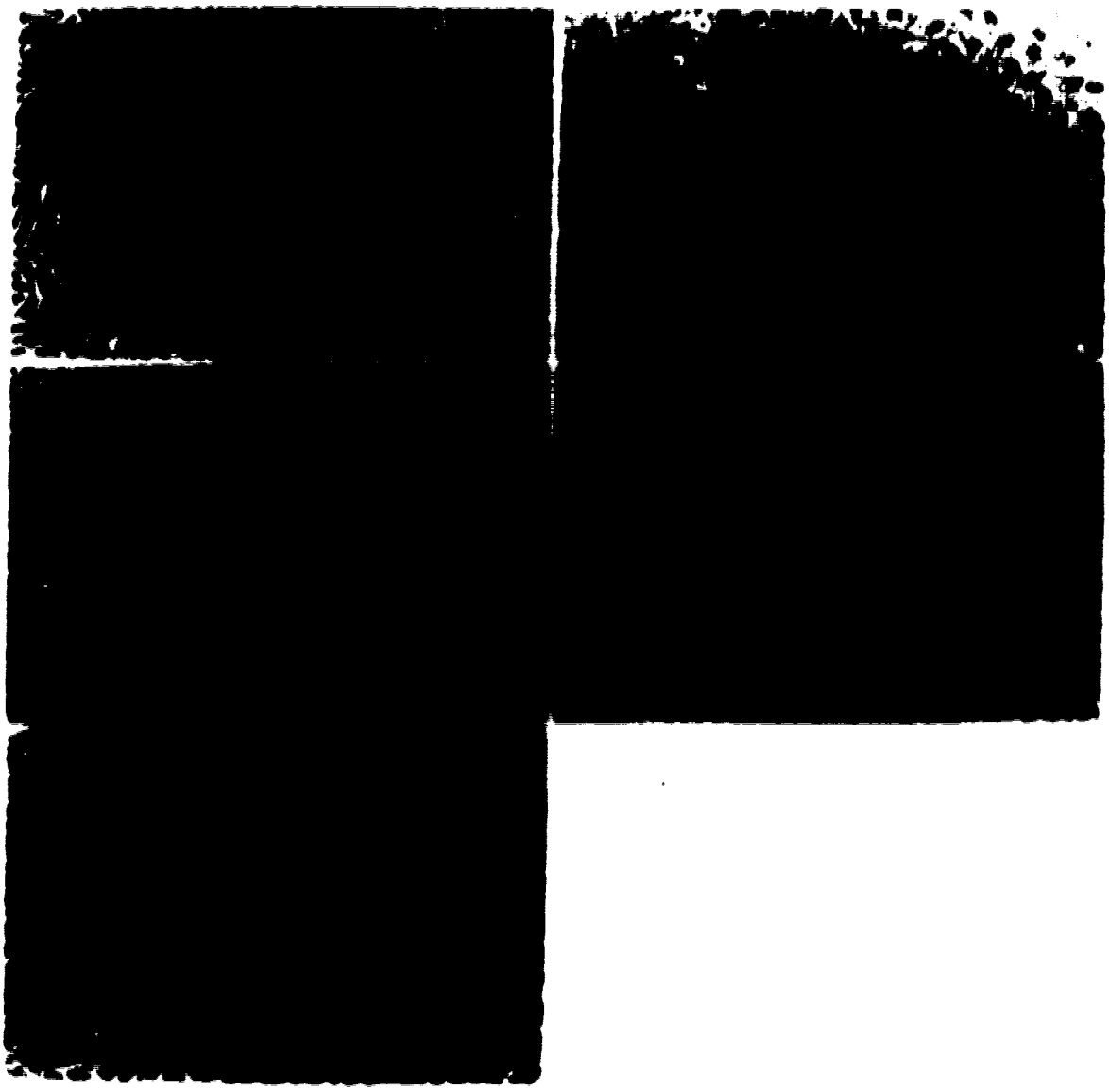


PLATE 4.1. Phase-contrast micrographs of fibroblasts cultured from various bovine fibrous tissues. Confluent cultures of fibroblasts were isolated from A) gingiva B) sclera C) skin D) proximal flexor tendon E) peripheral TMJ disc. X 550.

PLATE 4.2. Immunohistochemical localisation of DS-PGII in fibroblasts cultured from the periphery of the bovine TMJ disc. Arrows indicate perinuclear position of DS-PGII. X 2000.

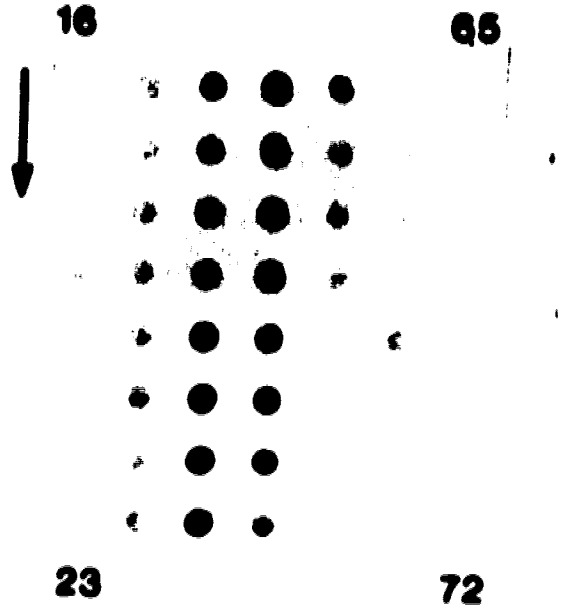


PLATE 4.3.
Dot-blot of DMMB positive fractions following ion-exchange chromatography of culture media from bovine skin fibroblasts. Blot is stained with mAbs to DS-PGII (6D6), and fraction numbers are shown.

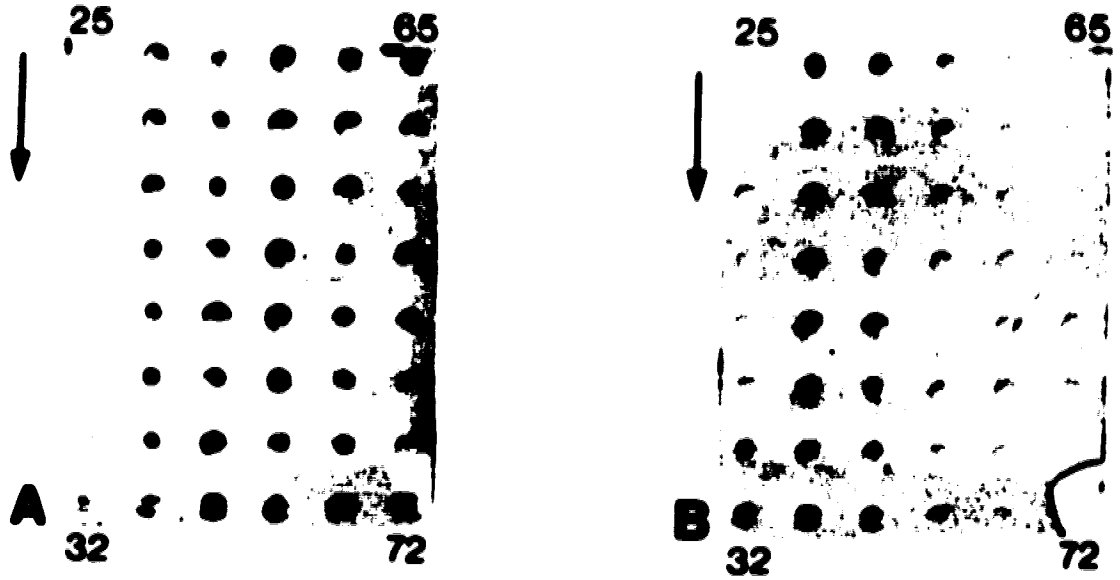
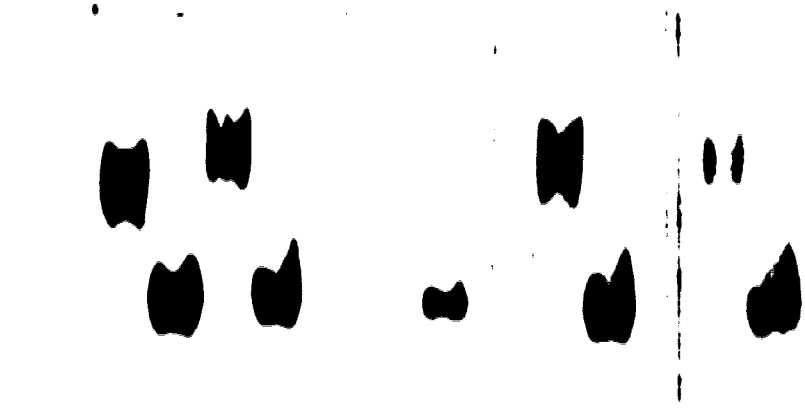


PLATE 4.4. Dot-blots of DMMB positive fractions following Sephacryl 400 SF chromatography (fig. 4.2B) of samples from cultured bovine TMJ disc fibroblasts. A) blot stained with mAbs to DS-PGII (6D6) B) blot stained with Abs to CS (CS-56). Fraction numbers are shown.

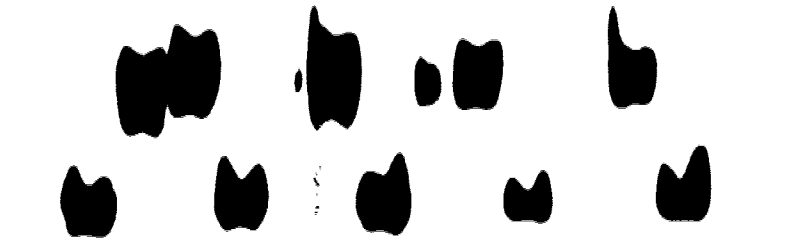
PLATE 4.5. Gel electrophoresis (7% polyacrylamide) of bovine skin DS-PGII and fractions I (II for TMJ disc material) from Sephacryl 400SF chromatography (figs. 4.3 to 4.6). Lanes 1 and 7- molecular weight standards; lanes 2 and 8- skin DS-PGII, lane 3- fraction I, from skin fibroblasts; lane 4- fraction I, from gingival fibroblasts; lane 5- fraction II, from TMJ disc fibroblasts; lane 6- fraction I, from tendon fibroblasts; lane 9, from scleral fibroblasts. All lanes are stained with Coomasie Blue.

PLATE 4.6. Gel electrophoresis (7% polyacrylamide) of bovine skin DS-PGII and fraction II of TMJ disc material from Sephacryl 400SF chromatography (fig. 4.3A). Lane 1- skin DS-PGII; lane 2- from TMJ disc fibroblasts. Gel is stained with Stains-All.

A 1 2 3 4 5 6 7 8 9 10



B 1 2 3 4 5 6 7 8 9 10



C 2 1 3 4 5 6 7 8 9 10

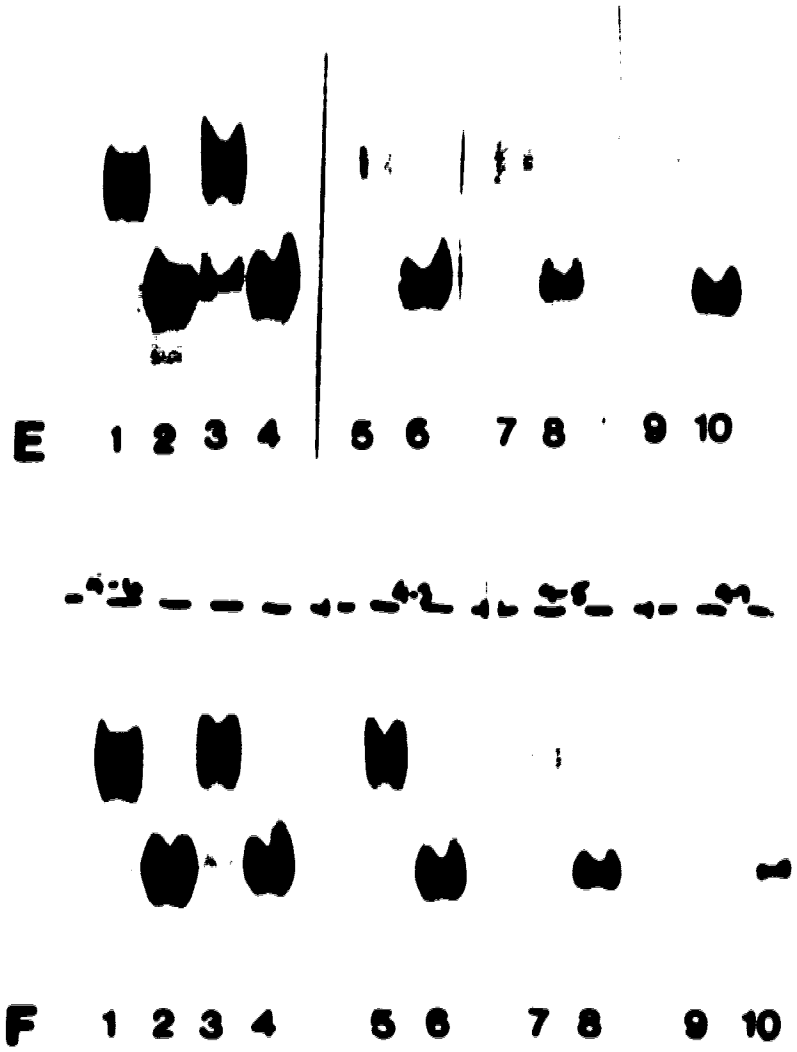


PLATE 4.7. Immunoblots (following gel electrophoresis on 7% polyacrylamide) of bovine skin DS-PGII and DS-PGII, fractions I (fraction II from TMJ disc material) from Sephacryl 400SF chromatography (figs. 4.3 to 4.6) stained with various mAbs. Gel A- from skin fibroblasts; gel B- from scleral fibroblasts; gel C- from gingival fibroblasts; gel E- from TMJ disc fibroblasts; gel F- from tendon fibroblasts. Odd-numbered lanes are intact DS-PGIIs, even-numbered lanes are protein cores of DS-PGIIs. Lanes 1 and 2 are bovine skin DS-PGII. Lanes 1 to 4 are stained with 6D6, lanes 5 and 6 are stained with 3B3, lanes 7 and 8 are stained with 5D1, lanes 9 and 10 are stained with 7B1.

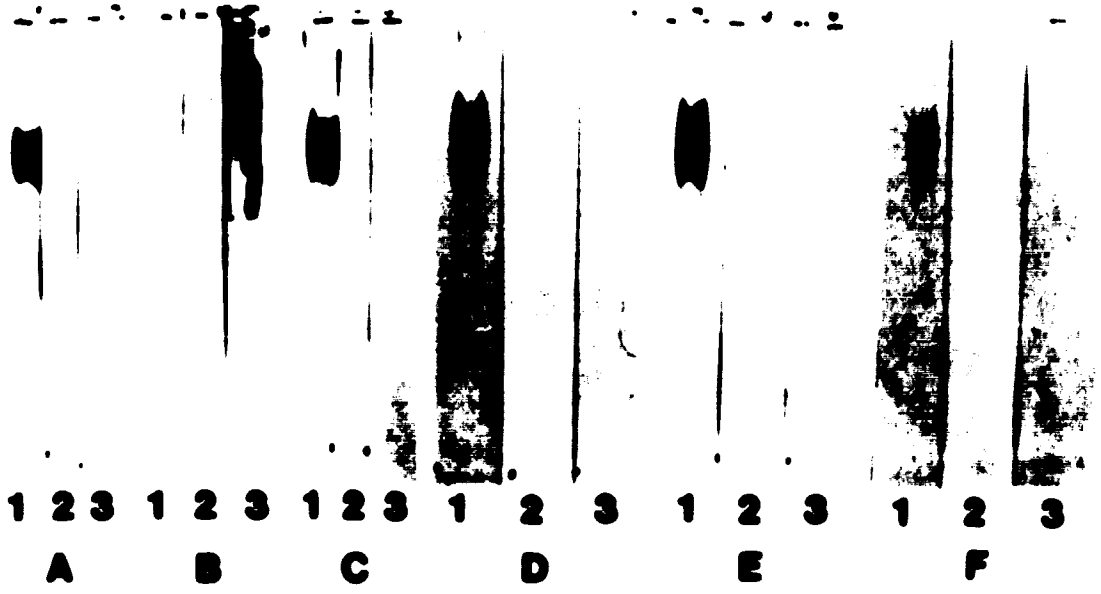


PLATE 4.8. Immunoblots (following gel electrophoresis on 7% polyacrylamide) of bovine skin DS-PGII, bovine nasal cartilage PGs and DS-PGII from fractions I (fraction II from TMJ disc material) from Sephacryl 400SF (figs. 4.3 to 4.6) stained with Abs to DS-PGII, CS and KS. Gel A- bovine skin DS-PGII; gel B- bovine nasal cartilge PGs; gel C- from skin fibroblasts; gel D- from gingival fibroblasts; gel E- from TMJ disc fibroblasts; gel F- from tendon fibroblasts. Lanes 1 stained with 6D6, lanes 2 stained with Abs to CS, lanes 3 stained with Abs to KS.

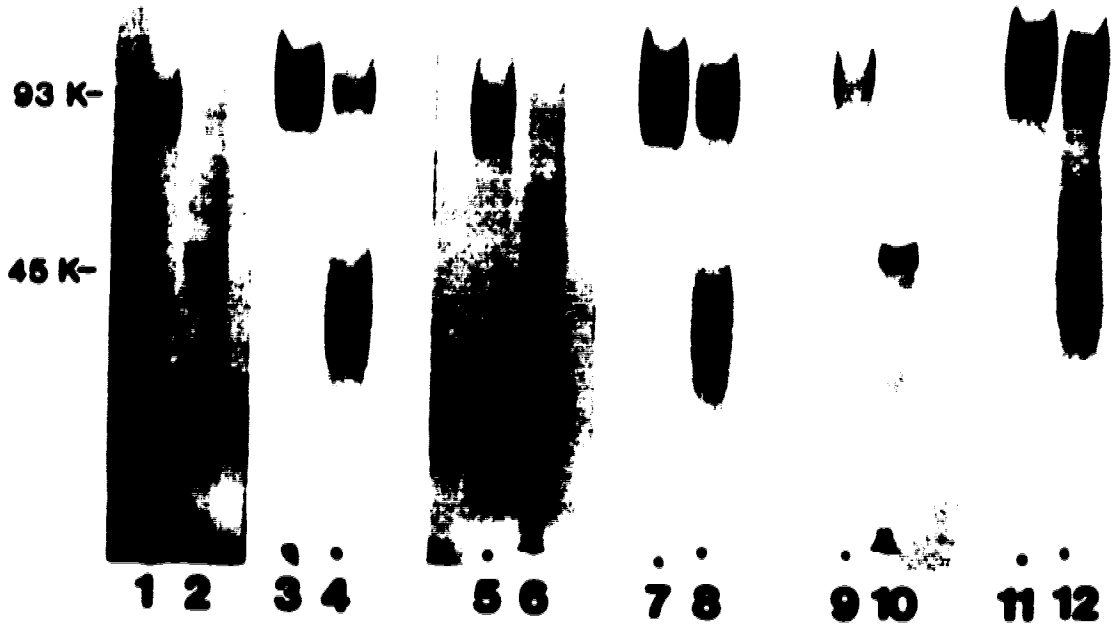


PLATE 4.9. Gel electrophoresis (7% polyacrylamide) of bovine skin DS-PGII and DS-PGII from fractions I from Sephacryl 400SF (figs. 4.5 and 4.6) chromatography digested with cathepsin C. Lanes 1 and 3- intact skin DS-PGII; lanes 5 and 7- intact fraction I from skin fibroblasts; lanes 9 and 11- intact fraction I from gingival fibroblasts; lanes 2 and 4- cathepsin C digested skin DS-PGII; lanes 6 and 8- cathepsin C digested fraction I from skin fibroblasts; lanes 10 and 12- cathepsin C digested fraction I from gingival fibroblasts. Lanes 1,2,5,6,9 and 10 are stained with Coomasie Blue. Lanes 3,4,7,8,11 and 12 are stained with Stains-All.

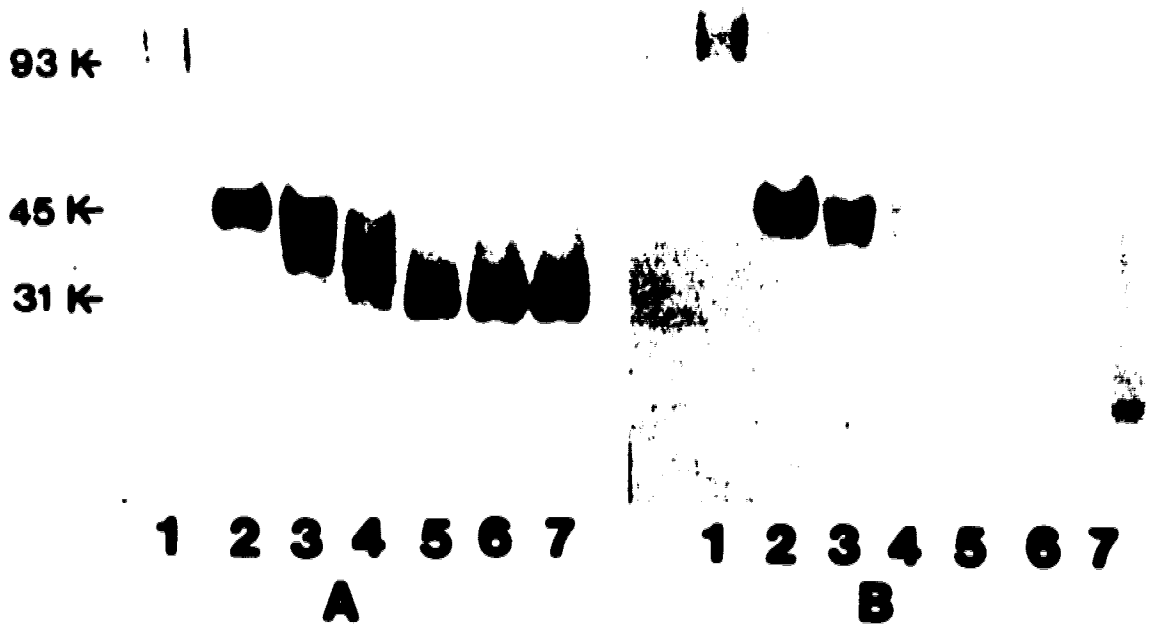


PLATE 4.10. Immunoblots (following gel electrophoresis on 7% polyacrylamide) of bovine tendon DS-PGII from cultured fibroblasts digested with varying concentrations of glycopeptidase F. Lanes 1- intact DS-PGII; lanes 2- protein core DS-PGII; lanes 3- core digested with 0.005 units; lanes 4- core digested with 0.025 units; lanes 5- core digested with 0.05 units; lanes 6- core digested with 0.25 units; lanes 7- core digested with 0.5 units. Blot A stained with 6D6, blot B stained with concanavalin A.

PLATE 4.11. Gel electrophoresis (7% polyacrylamide) of bovine skin DS-PGII and DS-PGII, fractions I (II for TMJ disc material), from Sephacryl 400SF chromatography (figs. 4.3 to 4.6) digested with papain. Lane 1- skin DS-PGII; lane 2- from skin fibroblasts; lane 3- from scleral fibroblasts; lane 4- from gingival fibroblasts; lane 5- from TMJ disc fibroblasts; lane 6- from tendon fibroblasts. All lanes are stained with Stains-All.

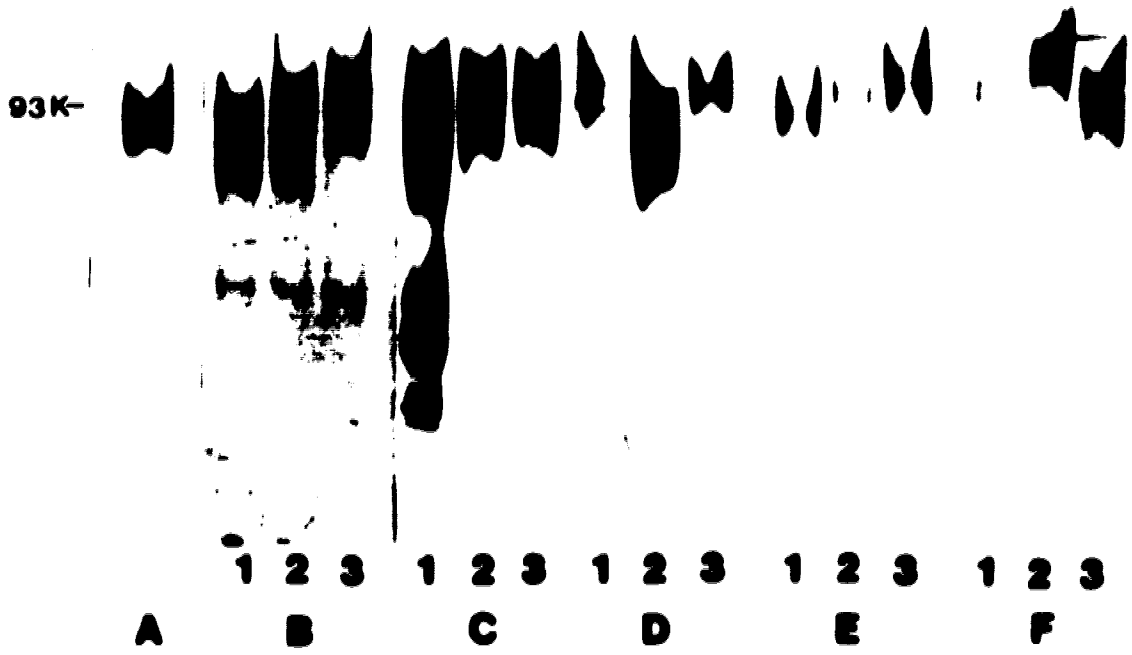


PLATE 4.12. Immunoblots (following gel electrophoresis on 7% polyacrylamide) of bovine skin DS-PGII and DS-PGII from various cultured cell strains. Gel A- intact skin DS-PGII; gel B- from skin fibroblasts; gel C- from scleral fibroblasts; gel D- from TMJ disc fibroblasts; gel E- from gingival fibroblasts; gel F- from tendon fibroblasts. Lanes 1 represent DS-PGIIs purified from fractions I (II for TMJ disc material) from Sephacryl 400SF chromatography. Lanes 2 and 3 are from additional cell strains. All lanes are stained with 6D6.

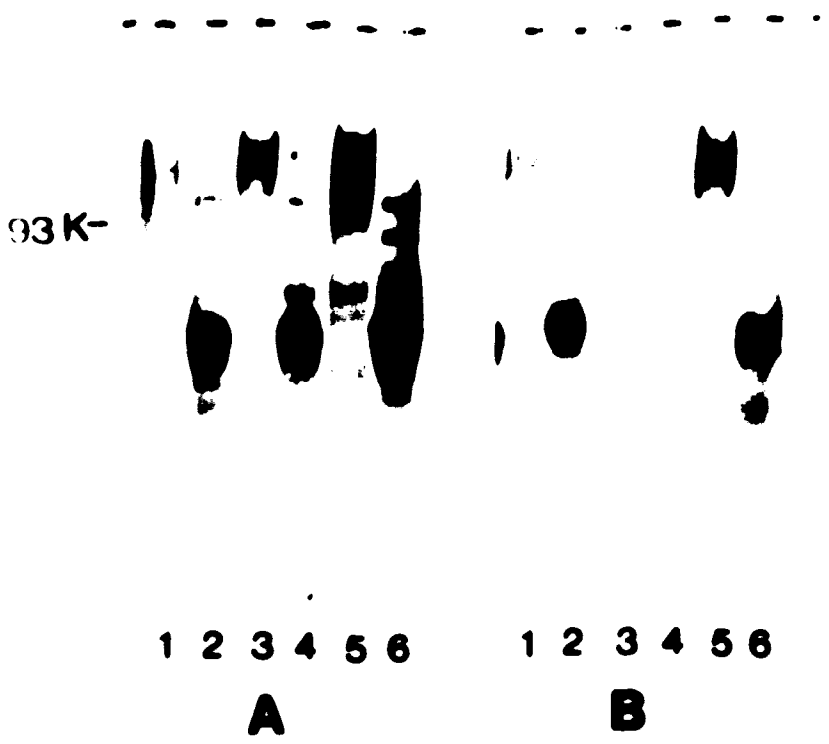


PLATE 4.13. Immunoblots (following gel electrophoresis on 7% polyacrylamide) of bovine tendon DS-PGII isolated from cell strains 1 and 2 at passage (p) 4,6 and 9. Gel A- cell strain 1; lane 1- intact DS-PGII, p4; lane 2- core DS-PGII, p4; lane 3- intact DS-PGII, p6; lane 4- core DS-PGII, p6; lane 5- intact DS-PGII, p9; lane 6- core DS-PGII, p9. Gel B- cell strain 2 with lanes 1 to 6 as described for gel A. All lanes are stained with 6D6.

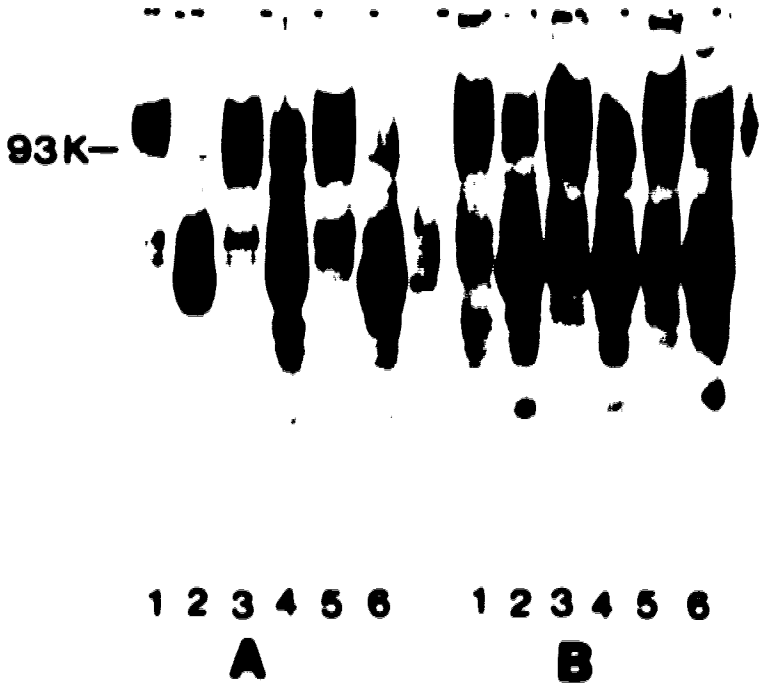
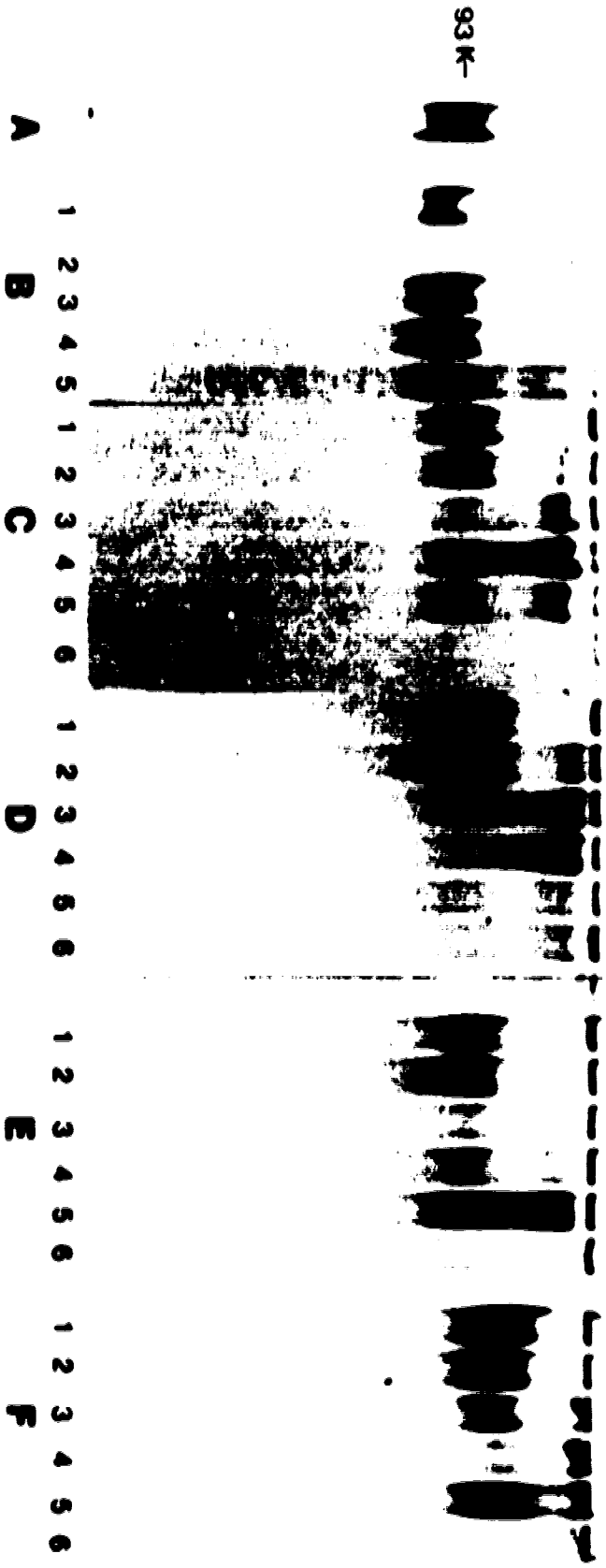


PLATE 4.14. Immunoblots (following gel electrophoresis on 7% polyacrylamide) of bovine gingiva DS-PGII isolated from cell strains 1 and 3 at passage (p) 4,6 and 9. Gel A- cell strain 1; lane 1- intact DS-PGII, p4; lane 2- core DS-PGII, p4; lane 3- intact DS-PGII, p6; lane 4- core DS-PGII, p6; lane 5- intact DS-PGII, p9; lane 6- core DS-PGII, p9. Gel B- cell strain 3 with lanes 1 to 6 as described for gel A. All lanes are stained with 6D6.

LATE 4.15. Gel electrophoresis (7% polyacrylamide) of bovine skin DS-PGII and fractions I to VI (V for skin extracts) from Octyl-Sepharose chromatography (figs. 4.15 and 4.16). Gel A- bovine skin DS-PGII; gel B- skin extract; gel C- scleral extract; gel D- TMJ disc extract; gel E- gingival extract; gel F- tendon extract. Lanes 1 through 6 represent fractions I through VI. All lanes are stained with Stains-All.


93K
A
B
C
D
E
F
1 2 3 4 5 6
1 2 3 4 5 6
1 2 3 4 5 6
1 2 3 4 5 6
1 2 3 4 5 6
1 2 3 4 5 6

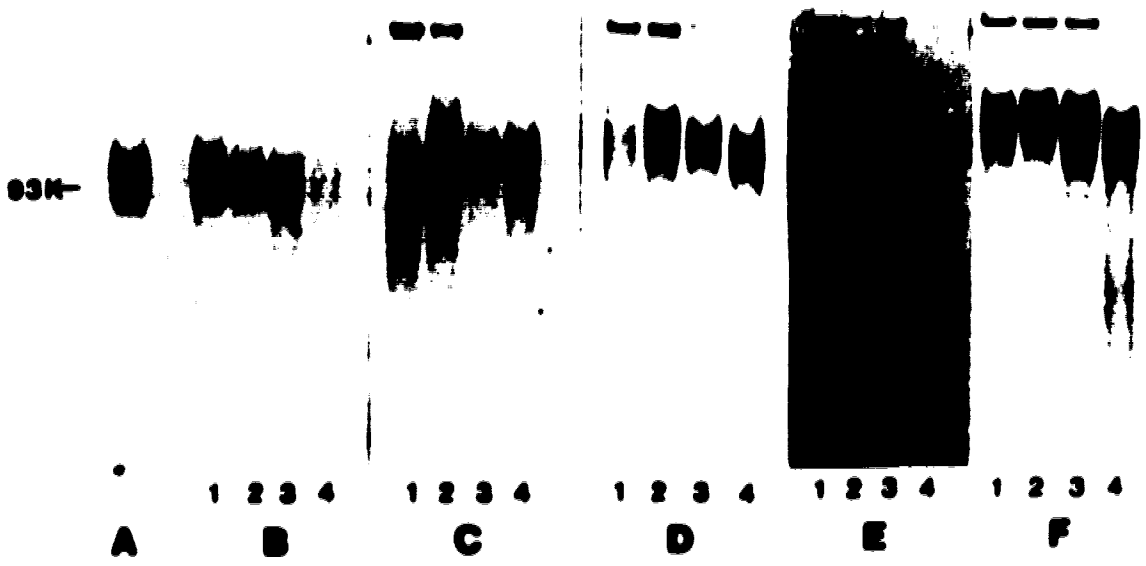


PLATE 4.16. Gel electrophoresis (7% polyacrylamide) of bovine skin DS-PGII and fractions I to IV from Sephacryl 400SF chromatography (figs. 4.17 and 4.18). Gel A- bovine skin DS-PGII; gel B- skin extract; gel C- scleral extract; gel D- TMJ disc extract; gel E- gingival extract; gel F- tendon extract. Lanes 1 through 4 represent fractions I through IV respectively. All lanes are stained with Stains-All.

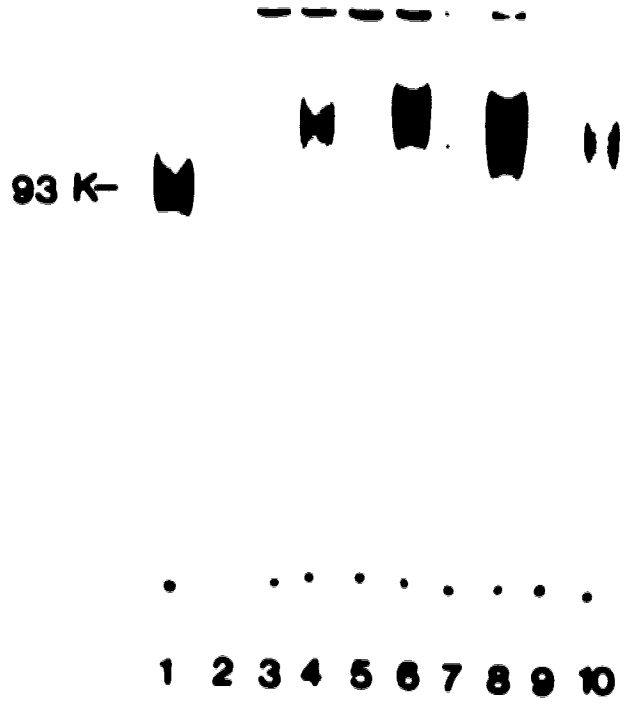
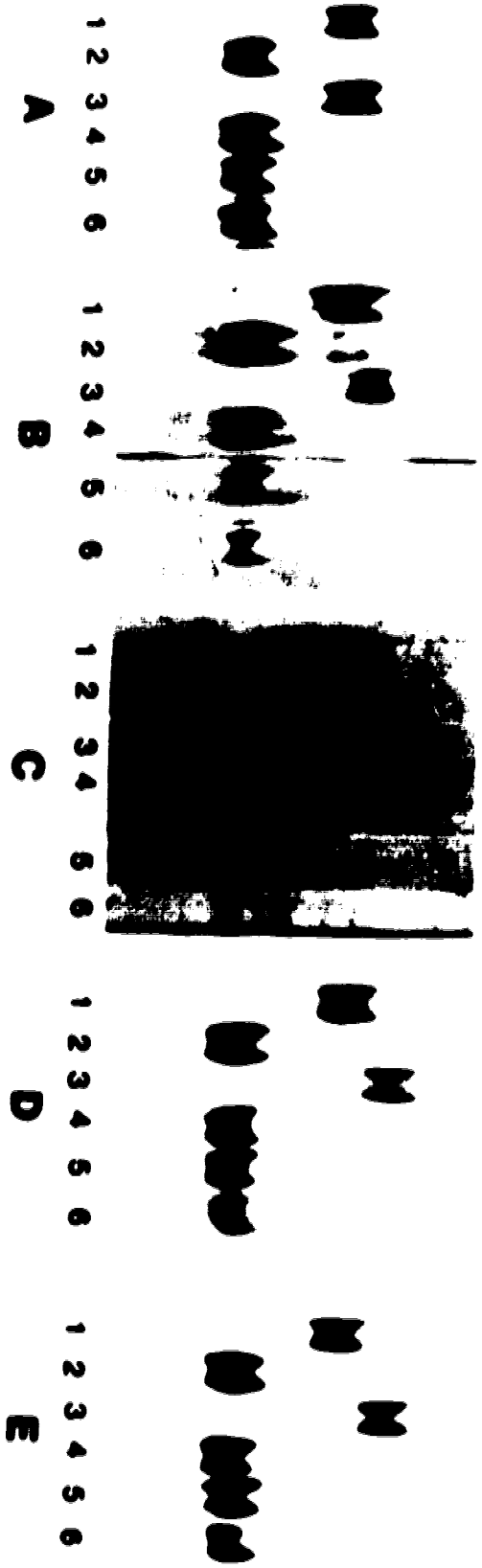


PLATE 4.17. Gel electrophoresis (7% polyacrylamide) of bovine skin DS-PGII and fractions I to IV from Sephacryl 400SF chromatography (fig. 4.17) of bovine tendon extract digested with chondroitinase B. Lane 1- intact skin DS-PGII; lane 2- digested skin DS-PGII; lanes 4,6,8 and 10- intact fractions I through IV respectively; lanes 3,5,7 and 9- digested fractions I through IV respectively. All lanes are stained with Stains-All.

PLATE 4.18. Immunoblots (following gel electrophoresis on 7% polyacrylamide) of skin DS-PGII and DS-PGII isolated from fractions III from Sephacryl 400SF chromatography (fig 4.17 and 4.18) stained with various mAbs. Gel A- skin extract; gel B- scleral extract; gel C- gingival extract; gel D- TMJ disc extract; gel E- tendon extract. Lanes 1- intact skin DS-PGII; lanes 2- protein core skin DS-PGII; lanes 3- intact DS-PGII from the various tissue extracts; lanes 4, 5 and 6- protein cores of DS-PGII from the various tissue extracts. Lanes 1 to 4 are stained with 6D6, lanes 4 are stained with 3B3 and lanes 5 are stained with 7B1.


A
1 2 3 4 5 6
B
1 2 3 4 5 6
C
1 2 3 4 5 6
D
1 2 3 4 5 6
E
1 2 3 4 5 6

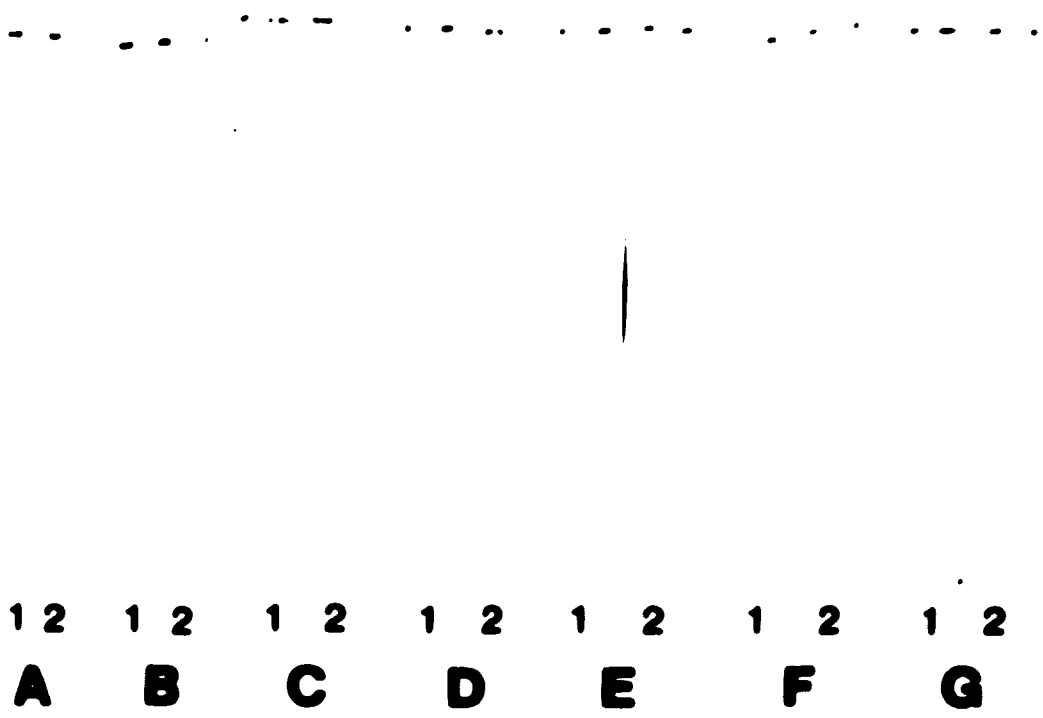


PLATE 4.19. Immunoblots (following gel electrophoresis on 7% polyacrylamide) of skin DS-PGII, bovine nasal cartilage PGs and DS-PGII, fractions III from Sephacryl 400SF chromatography (fig 4.17 and 4.18) stained with Abs to CS and KS. Gel A- bovine skin DS-PGII; gel B- bovine nasal cartilage PGs; gel C- skin extract; gel D- scleral extract; gel E- gingival extract; gel F- TMJ disc extract; gel G- tendon extract. Lanes 1 are stained CS-56, lanes 2 are stained with Abs to KS.

PLATE 4.20. Gel electrophoresis (7% polyacrylamide) of bovine skin DS-PGII and DS-PGII, fractions III from Sephacryl 400SF chromatography (figs 4.17 and 4.18) digested with papain. Lane 1- skin DS-PGII; lane 2- skin extract; lane 3- scleral extract; lane 4- TMJ disc extract; lane 5- gingival extract; lane 6- tendon extract. All lanes are stained with Stains-All.

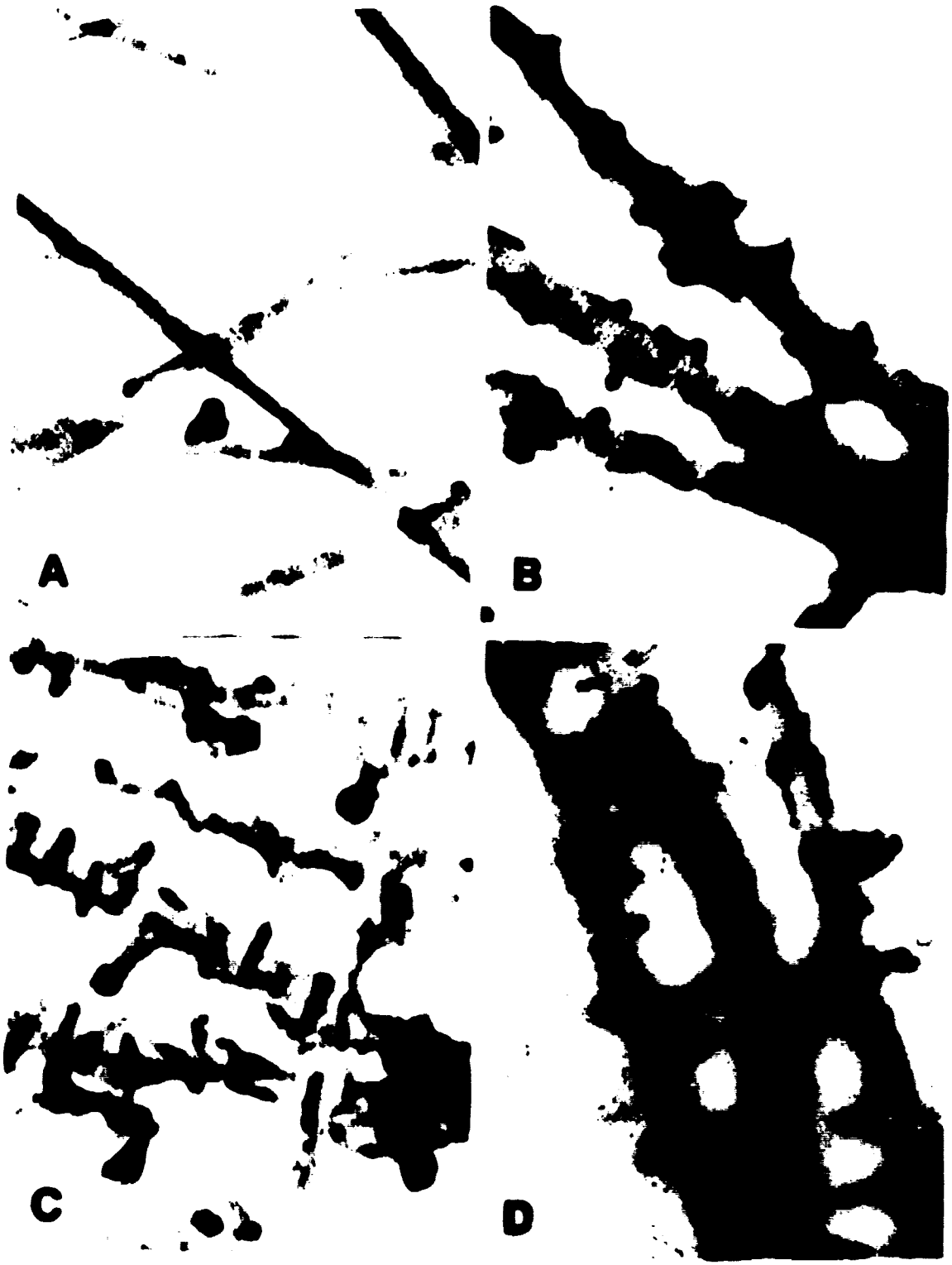
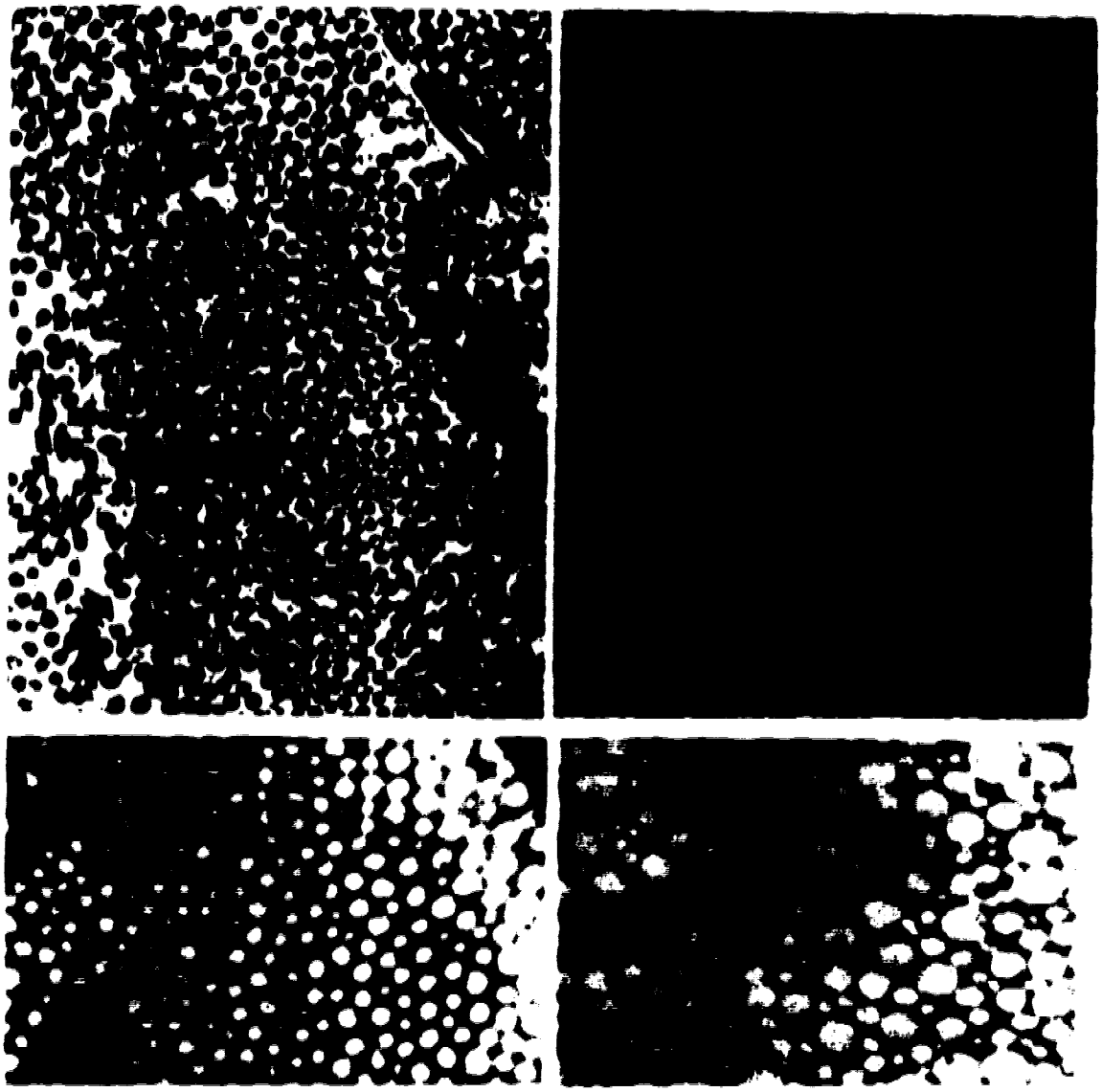

A
B
C
D

PLATE 4.21. Electron micrographs of collagen fibrils precipitated in the presence of various tissue-specific DS-PGIIs. A) collagen fibrils precipitated in the absence of DS-PGII B) precipitated with gingival DS-PGII C) with scleral DS-PGII D) with skin DS-PGII E) with TMJ disc DS-PGII F) with tendon DS-PGII. All preparations were fixed in 2.5% glutaraldehyde containing 0.05% Cuprolinic Blue. x 54,000.



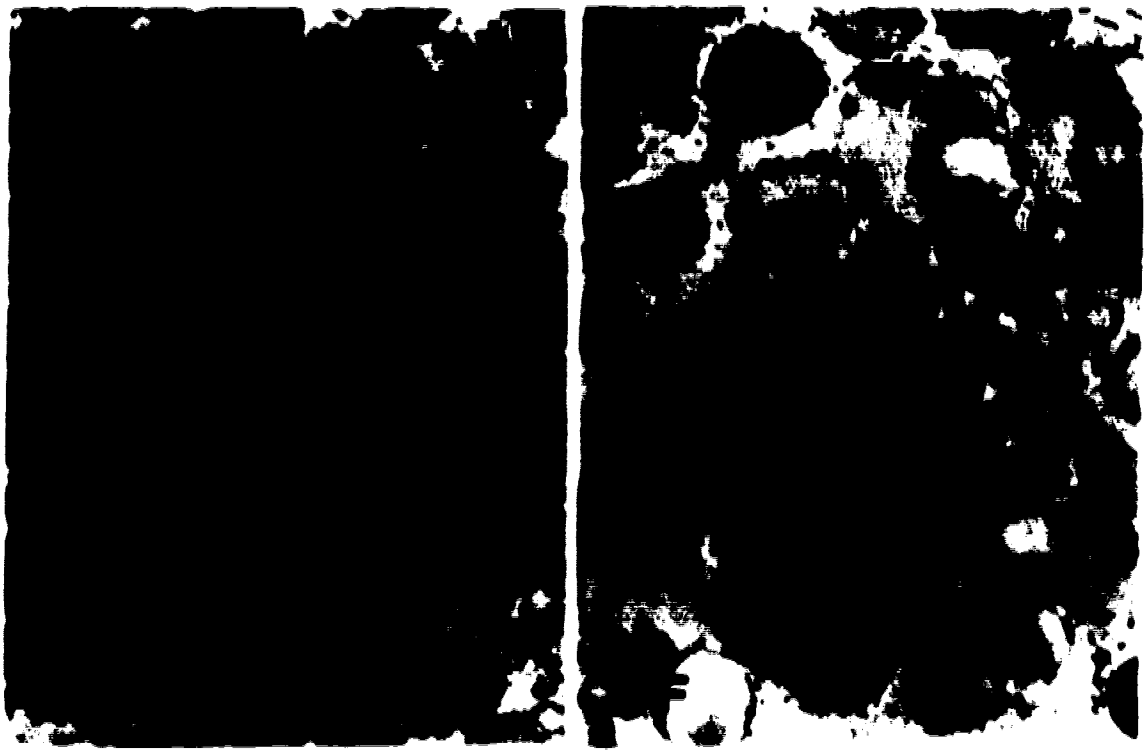
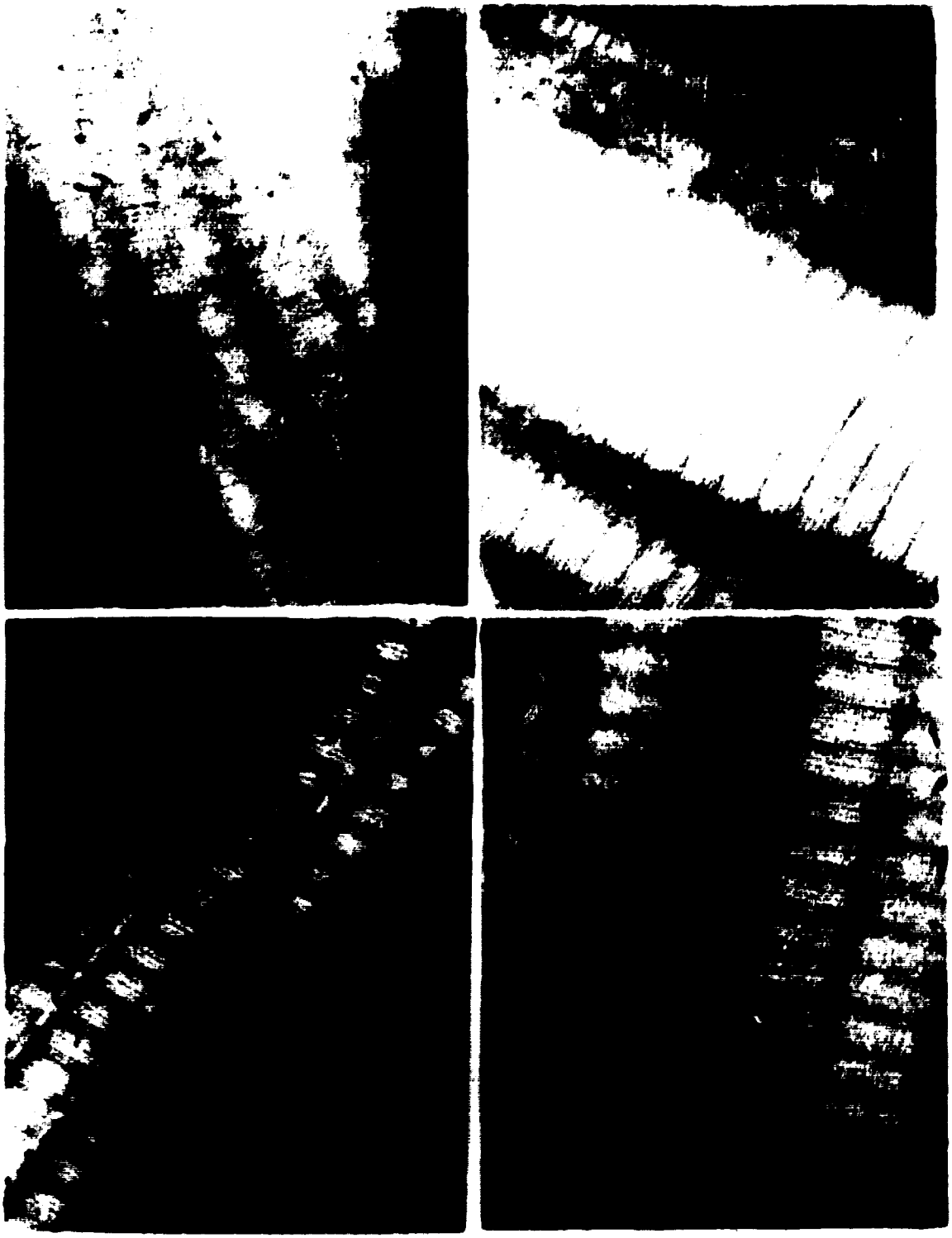


PLATE 4.22. Electron micrographs of collagen fibrils (transverse sections) from various bovine fibrous connective tissues. A) gingiva B) skin C) central zone of the TMJ disc D) peripheral zone of the TMJ disc E) tendon F) sclera. All sections were stained with uranyl acetate/lead citrate. x 30,000.



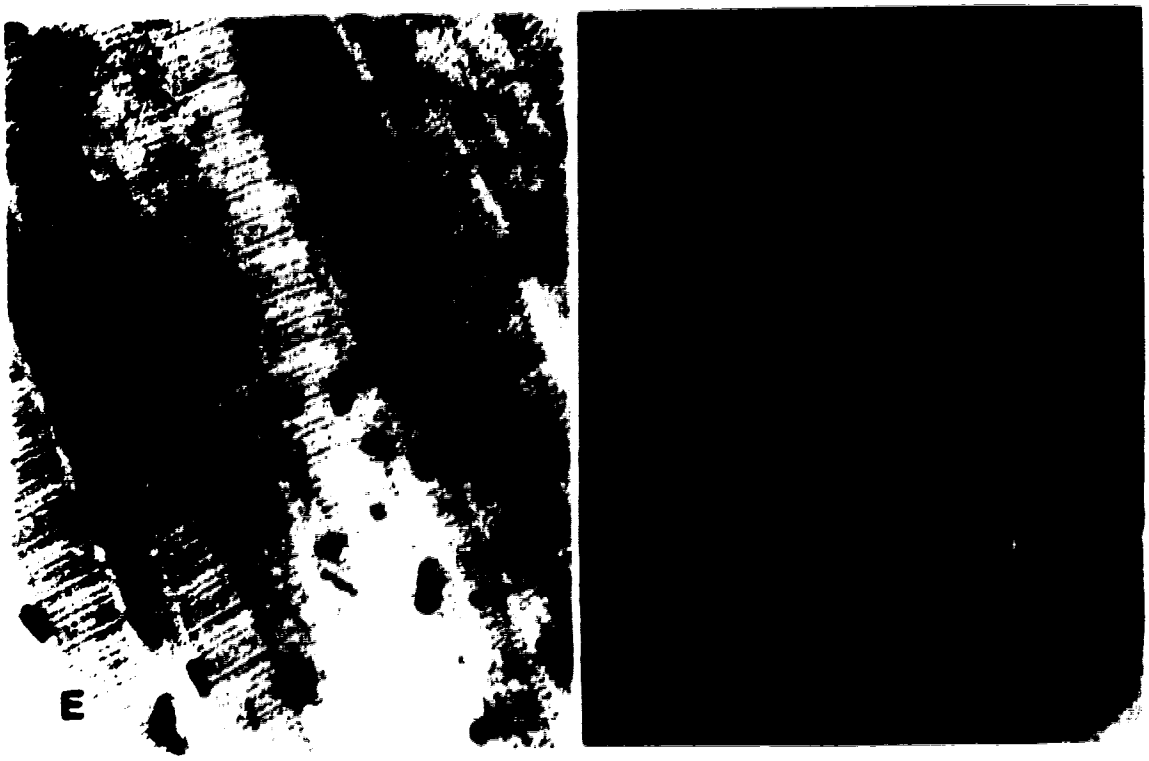


PLATE 4.23. Electron micrographs of collagen fibrils (longitudinal sections) from various bovine fibrous connective tissues. A) gingiva B) sclera C) skin D) peripheral zone of the TMJ disc E) central zone of the TMJ disc F) tendon. All sections were stained with 0.05% Cuprolinc Blue and counterstained with 2% uranyl acetate. Thin arrows indicate Cuprolinic Blue positive material aligned along the surface of fibrils in a periodic array. Thick arrows indicate Cuprolinic Blue positive material lying in a interfibrillar position. x 126,000.

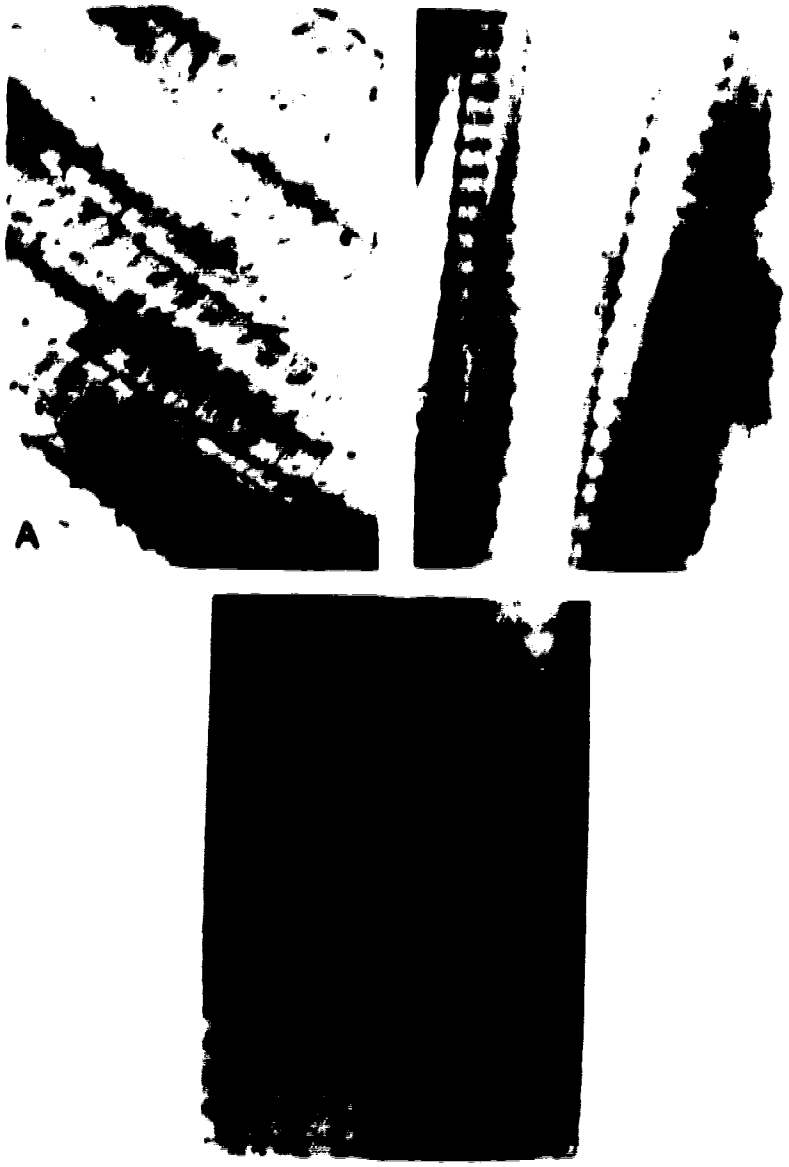


PLATE 4.24 Electron micrographs of collagen fibrils (longitudinal sections) from bovine skin digested with chondroitinase ABC and AC. A) no digestion B) digested with chondroitinase AC C) digested with chondroitinase ABC. All sections were stained with 0.05% Cuprolinic Blue and counterstained with 2% uranyl acetate followind digestion. x 71,400.

DISCUSSION

5.1. Fibroblast Morphology in Tissue Culture

Morphology is considered one of the simplest ways by which to identify cells. While it is generally accepted that cells with a "fibroblastic" origin are usually bipolar or multipolar, with a length more than twice their width (Freshney, 1983), variations in cell density, substrate and constitution of the medium can affect cellular morphology. In the present study, each of the cell strains established from gingiva, sclera, skin and tendon were considered to be fibroblastic in origin in that their general appearance conformed to the accepted criteria. The slight differences evident between cultures of scleral and tendon cells compared to those established from gingiva and skin (see Fig 4.1) can possibly be attributed to a seemingly slower growth rate and hence reduced cell density in the former cultures. The phase contrast micrographs shown in Plate 4.1 were all taken one week following subculture at the same seeding density.

Although it has been suggested that fibroblasts which exhibit more extensive cell spreading, and hence lower cell densities are reflective of senescent cells (Bayreuther et al, 1988), cells with a similar morphology to that shown for our cultured scleral and tendon fibroblasts have also been described in primary reticular skin fibroblast cultures (Schafer et al, 1985; Schönherr et al, 1993). In addition, the reticular cells were found to exhibit a lower rate of

biosynthesis of decorin when compared to cells cultured from the papillary dermis, a difference not related to cell aging (Schönherr et al, 1993). Our results are in agreement and further support the suggestion that cell morphology is not indicative of cell aging, but may be more reflective of synthetic activity. Fibroblasts from scleral, tendon and TMJ disc all secreted significantly less PGs (as determined by DMMB reactivity in fractions following ion-exchange chromatography) when compared to skin and gingival fibroblasts.

The morphological features of cell cultures established from the peripheral zone of the TMJ disc are unlike those described for the other cell strains, in that the cells are more polygonal. This may be a reflection of cell type. The joint disc of the sheep, which like the cow is a herbivorous animal, has a masticatory apparatus which has been described as "ungulate grinding" or "mill" type (Turnbull, 1970). Histologically, the central and posterior regions of the disc have been characterized as fibrocartilage, a cartilage subtype composed mainly of densely collagenous fibrous tissue, interspersed by layers of hyaline cartilage matrix with chondrocytes lined up in rows (Gartner and Hiatt, 1990; Wheater et al, 1987). Anteriorly, this merges into fibrous tissue containing the vascular sinusoids (Gillbe, 1973). Fibrocartilage was not identified in the peripheral zones of the disc. The rabbit disc (Mills et al, 1988) and the human disc (Provenza, 1964) have also been described as

fibrocartilage, although Mills et al (1988) found the most posterior attachments of the rabbit disc tended to be more like fibrous connective tissues. Histologically, the bovine disc also is composed of fibrocartilage (Peters, E., personal communication).

When Mills et al (1988) established explant cultures from the anterior and posterior regions of the rabbit disc, they found that their cultured cells were cartilage-like in shape (that is polygonal) and very similar in appearance to chondrocytes in culture (Kuettner et al, 1982). Cells from our bovine discs were also found to be very similar in appearance to those described for the rabbit disc, suggesting that they may be chondrocytic in origin.

Mills et al (1988) and others (Benya et al, 1978; Hunter et al 1984) have found that the chondrocyte-like phenotype is not stable in culture and that there is a gradual change to a more fibroblast-like phenotype with passage. The cells used in the present study were in passage three or four, however it was found that by the completion of a number of cycles of media collection, the cells were beginning to appear somewhat fibroblastic, although not to the same degree as seen for the other cell strains. This may be explained by the continual cell division that occurs in the cultures with time even though the cultures were not passaged. A number of cells were lost during each wash with PBS, however the flasks and cell factories were always allowed to become confluent prior to the next cycle of media collection. Nonetheless, positive

immunohistochemical staining, with mAbs to DS-PGII, in a perinuclear position (possibly in the endoplasmic reticulum) of cells cultured from the TMJ disc confirms the ability of these cells to manufacture DS-PGII, a product of mesenchymal cells.

The morphologic differences seen between the various cultures might be considered a reflection of phenotypic differences between the tissues of origin, since all the cell strains were cultured under identical conditions. As will be discussed, other features of these cells suggest that they come from phenotypically distinct connective tissues.

5.2. Secretion of DS-PGII by Fibroblasts in Culture

The presence of DS-PII in various bovine connective tissues, including gingiva (Pearson and Pringle, 1986), sclera (Cöster *et al* 1981), skin (Damle *et al*; Fujii and Nagai, 1981), tendon (Vogel and Heinegard, 1985) and the TMJ disc (Scott *et al*, 1989) has been previously demonstrated biochemically. We have shown the continued expression of DS-PGII in culture by cell strains derived from these various tissues and in addition have found tissue-related heterogeneity in the size of the DS-PGIIs secreted.

It has been shown that cells in culture are able to secrete a variety of proteoglycans and other extracellular matrix components. The secretion of small dermatan sulphate rich PGs by cultured fibroblasts from a variety of tissue types has been described by a number of authors (Carlstedt *et*

al, 1981; Gallagher et al, 1983, Glossl et al, 1984; Larjava et al, 1988; Schmidtchen and Fransson, 1992; Vogel et al, 1986; Warburton et al, 1992). In addition, other cell types in culture have been shown to secrete PGs that have been identified as decorin either by the size of their core proteins or their immunological reactivity, including vascular smooth muscle cells (Jarvelainen et al, 1991, Rauch et al. 1986, Asundi et al, 1990, Schönherr et al, 1990), myoepithelial cells (Warburton et al, 1992), bovine endothelial cells (Kinsella and Wight, 1988), chondrocytes (Sommarin and Heinegård, 1986) and human bone cells (Beresford et al, 1987).

5.2.1. Fibroblasts in culture secrete other proteoglycans

Fibroblasts in culture are not only capable of secreting DS-PGII but also a variety of other PGs. Cultures established from human embryonic skin have been shown to secrete large CS/DS-PGs (with versican-like 400-kDa and 600-kDa cores), small PGs (biglycan and decorin), membrane bound DS/CS-PGs with a 90-kDa core as well as a number of HS-PGs (Carlstedt et al, 1981; Fransson et al, 1991; Schmidtchen et al, 1990).

In the present study, we were interested only in purifying DS-PGII from the various cultures. As a result, any other PGs synthesized by our cultured fibroblasts were not identified. However, during the purification steps it became evident that some cell types secreted other PGs. In particular, cultures established from sclera, tendon and TMJ disc always showed the presence of a larger DSPG positive

molecule on initial Sephacryl-400 SF runs. It was suspected to be a large CS-PG; the presence of CS in this material was subsequently confirmed immunologically on dot blots. Others have shown the secrtion of large CS-PGs in cultures of bovine tendon (Vogel et al, 1986), rabbit TMJ disc (Mills et al, 1988), and bovine sclera (Rauch et al, 1986). No such higher molecular weight material was found in cultures established from skin and gingiva, although this may be due more to the sensitivity of our procedures rather than to a complete lack of secretion of larger PGs by these cultures. Secretion of larger molecular weight material has previously been shown for cultures of human skin fibroblasts (Schmidtchen and Fransson, 1992) and human gingival fibroblasts (Bartold et al, 1986). A smaller absorbance peak was evident on Sephacryl runs for material from gingiva and skin, but showed minimal reactivity in the DMMB assay and was not further examined. Cultured fibroblasts from the disc and tendon also appeared to secrete an additional molecule whose electrophoretic pattern suggests it may be DS-PGI or possibly an aggregate of DS-PGII. Although DS-PGI has been shown to aggregate under conditions of high ionic strength (Choi et al, 1989), it has long been observed in our laboratory that purified samples of DS-PGII will occasionally aggregate in our buffer conditions and appear as higher molecular weight species on SDS-PAGE.

5.2.2. Characterization of DS-PGII

In all instances, the molecular mass of the core proteins of purified DS-PGIIs, either following partial deglycosylation

with chondroitinase ABC or complete deglycosylation with chondroitinase ABC followed by glycopeptidase-F, were similar between fibroblasts cultured from different connective tissues. Usually one, but occasionally two core proteins, ranging between 45 kDa and 48 kDa, were evident on Western blots following digestion with chondroitinase ABC. The appearance of the doublet is thought to be due to differential glycosylation (Glössl et al, 1984), representing molecules with either 2 or 3 N-linked oligosaccharides. The doublet was more distinct when smaller samples were applied to the gels. Following the removal of N-linked oligosaccharides with glycopeptidase F from chondroitinase ABC digested core proteins, each of the cores no longer appeared as a doublet on Western blots, but rather as a single band with a molecular mass of about 38 kDa. This value corresponds to that determined by sequencing studies (Day et al, 1987; Krusius and Ruoslahti, 1986). The molecular mass also corresponds to that determined for the nonglycosylated core protein obtained from tunicamycin-treated fibroblasts (Glössl et al, 1984).

In addition, the amino acid compositions of the preparations showed them to be high in aspartic acid, glutamic acid and leucine, values which also correspond to sequencing studies of the core protein of DS-PGII (Day et al, 1987; Krusius and Ruoslahti, 1986). Although DS-PGI, DS-PGII and fibromodulin are all considered to be part of a family of leucine-rich proteoglycans (Heinegård and Oldberg, 1989), the amino acid composition of DS-PGI has shown it to contain

higher amounts of leucine than DS-PGII (Choi et al, 1989). The amino acid composition of our preparations suggest this not to be the case, and as such our preparations do not appear to be contaminated with any appreciable amounts of DS-PGI. The protein core of material isolated from tendon fibroblasts was additionally high in glycine resides, the significance of this however remains unknown. Nonetheless, significant amounts of DS-PGII were still present in the tendon preparation so as not to alter the final reactivity of DS-PGII to our panel of mAbs. Material isolated from tendon, as well as the other preparations, was not reactive to antibodies to CS or KS. Lack of reactivity to these antibodies, together with the molecular mass and amino acid composition of the preparations, confirms that they were not contaminated with the CS-substituted aggrecan (Doege et al, 1987) or versican (Zimmermann and Ruoslahti, 1989) or the KS-substituted fibromodulin (Hedbom and Heinegård, 1989).

Although the protein sequences of DS-PGI and fibromodulin are closely related to DS-PGII, further evidence to suggest that the material isolated from the various fibroblast cultures was in fact DS-PGII was obtained from the western blots. Recent results (Scott et al, 1993) have shown that none of the epitopes to 7B1, 3B3, 5D1 or 6D6 are present in their entirety in either bovine or human DS-PGI (Neame et al, 1989, Fisher et al, 1989) or in bovine fibromodulin (Oldberg et al, 1989). Results in our laboratory have also shown that none of the mAbs will stain DS-PGI or fibromodulin on immunoblots

(Scott P.G. and Dodd C.M., unpublished data).

Enzymatic digestion of the PG with cathepsin C further confirms that the preparations are PGII rather than PGI or fibromodulin, as they contain only a single GAG chain located within the first 6 amino acids of the core protein (Chopra et al, 1985). There are no attachment sites for GAG chains in the amino-terminal end of fibromodulin (Oldberg et al, 1989), while the slower migration of PGI on SDS-PAGE compared to that of PGII has led to the suggestion that PGI carries two GAG chains (Fisher et al, 1987). This hypothesis has been supported by sequencing studies which have shown Ser-Gly sites at position 5 and 11 or 10 (depending on the species) available for GAG substitution when compared to PGII, where only position 4 is substituted (Choi et al, 1989; Fisher et al, 1987; Neame et al, 1989). It appears however, that glycosylation of the additional sites in cartilage PGI may not always be complete (Neame et al, 1989).

Previous reports, from both cultured material and tissue-extracted DS-PGII, have shown the presence of 2-3 N-linked oligosaccharides attached to the core protein of the molecule (Glössl et al, 1984, Rauch et al, 1986; Larjava et al, 1988; Scott and Dodd, 1990). No O-linked oligosaccharides were detected. Nakamura et al (1983) however inferred the presence of a single N-linked oligosaccharide on DS-PG from fetal calf skin, and three O-linked oligosaccharides. Midura and Mascall (1989) have also suggested the presence of a single O-linked oligosaccharide, in addition to 3 N-linked oligosaccharides on

a DS-PG synthesized by corneal explants. The presence of galactosaminitol in their samples after alkaline borohydride reduction indicated the presence of O-glycosidically linked oligosaccharides.

In the present study, glycopeptidase F, which specifically hydrolyzes the glycosylamine linkage of asparagine-linked oligosaccharides (Tarentino et al, 1985), was used to confirm the presence of three N-glycosylamine linkages on the core proteins of the various secreted tissue-specific DS-PGIIs. In all of the samples, deglycosylation of DS-PGII with glycopeptidase F resulted in the formation of two glycosylated intermediates on acrylamide gels as well as a fully glycosylated molecule, thus confirming the presence of 3 N-linked oligosaccharides. Three sites for possible N-glycosidic substitution have previously been suggested by sequencing studies (Krusius and Ruohslahti, 1986). Although the isolated DS-PGII molecules were not subjected to any O-glycanase treatment such as endo-α-N-acetyl-D-galactosaminidase (Larjava et al, 1988), the removal of the asparagine-linked oligosaccharides in addition to the removal of any GAG chains resulted in an increase of electrophoretic mobility to a value consistent with the Mr predicted from cDNA and protein sequencing studies. As such it seems unlikely that any O-linked oligosaccharides are present. Scott and Dodd (1990) have found that the removal of the oligosaccharides did not prevent the binding of the DS-PGII molecule to collagen. However, the tendency for self-

association was enhanced and therefore may indirectly affect its interaction with collagen.

Cellulose acetate electrophoresis established the identity of the GAG chains on each of the purified molecules as DS. Although we did not study the copolymeric structure of the chains, the results of the electrophoresis suggests that each of the GAGs contained sufficient amounts of IduA so as to comigrate with the DS standard. Larjava et al (1988) have shown that DS-PGII purified from skin, synovial and gingiva cultures all reacted similarly to chondroitinase ACII digestion, suggesting that there were only marginal differences in IduA content between the different cell lines. They did not quantitate the amount of IduA in each DS-PGII. As our cultures conditions were similar to those of Larjava et al (1988), it is possible that our skin and gingival cell strains also had similar IduA contents, although species differences may exist.

The mechanisms involved in the substitution of proteoglycan core proteins with various GAGs are not fully understood. Not all mesenchymal cells secrete PGIIs substituted with DS chains. For example, small CS-PGs isolated from the mineralised matrix of bovine bone (Fisher et al, 1983; Day et al, 1987; Fransen and Heinegård, 1984) have been shown to contain the identical NH_2-terminal sequences as that of skin and cartilage DS-PGII (Chopra et al, 1985; Choi et al, 1989). Is the structure of the GAG regulated by the protein core or the cell type in which it is expressed?

Recently, Schmidtchen and Fransson (1992) characterized the CS, DS and HS chains found on various PGs secreted by human skin fibroblasts in culture. They were able to identify at least five types of chains, three of which were DS and CS chains as well as two populations of HS chains. The CS and DS chains were associated either with a large secreted PG, two smaller secreted PGs or a cell-associated PG. Although they displayed heterogeneity in terms of size and GlcuA content, the composition of the chains was dependent upon the core protein with which they were associated. The HS chains all had very similar compositions, and varied only in size. Since each of the various CS and DS chains were always localized to the same protein cores, their results suggest that the core proteins may be important in regulating the synthesis of GAG chains. However, their data do not address why GAG chains of PGs from different tissues possess the same core proteins but differ in IduA content. The biological consequences of one cell type secreting a PGII molecule substituted with a CS chain instead of DS are unknown.

A number of studies have suggested that sulphation plays an important role in the formation of IduA, such that in the absence of sulphation mainly GlcuA residues are formed (Malmström et al, 1975; Malmström, 1984; Silbert et al, 1986). Sulphation has also been shown to play a role in determining the degree of polymerization of GAG chains (Nakanishi et al, 1981; Inoue et al, 1986), with sulphation of the nonreducing terminus of the GAG chain stopping chain elongation. Noppe et

al (1985) however, have shown that glycosyltransferase II and sulfotransferase occur in separate cell compartments, implying that the two processess are independent. Using monensin to dilate the Golgi cisternae and block the secretory route in the medial part of the Golgi, they were able to show that chain polymerization and 6-sulphation occurred mainly in the cis -Golgi, while epimerization and 4-sulphation were predominant in the trans-Golgi complex. The influence of chlorate (an inhibitor of sulphate adenylyltransferase) on PG biosynthesis by cultured fibroblasts was investigated by Greve et al (1988). They were able to show that a reduction in medium inorganic sulphate did not affect the degree of polymerization of the GAGs on small DS-PGs, nor result in the formation of completely unsulphated GAG chains. In this situation, sulphate probably come from endogenous methionine and cysteine. Undersulphation however, did lead to a decrease in epimerization. Each of our preparations was found to be fully sulphated and under these conditions the influence of sulphate on the degree of polymerization and epimerization of the GAG chains can be considered minimal As well, each of the cultures was maintained under identical conditions and hence one would expect similar controls on epimerization and polymerization between cell strains if sulphate played a critical role in these processes.

Further work is required to assess whether or not any significant differences in IduA content exists between the tissue-specific molecules and how any differences may

influence the biological properties of DS-PGII, for example, self-association. Striking differences in the amount of IduA in the GAG chains of DS-PGII between different tissues (cartilage versus skin) has previously been shown (Choi et al, 1989). As well, human bone cells in culture switch from making CS-PGII to DS-PGII (Beresford et al, 1987). These results suggest that there may be some yet undefined tissue-specific mechanism which regulates the degree of epimerization of GlcuA to IduA.

5.2.3. GAG chain length heterogeneity occurs both within and between tissue types

The relative mobilities of the intact DS-PGIIs and the GAG chains of papain-digested DS-PGIIs clearly showed that differences in the molecular weight of the various tissue specific molecules were due to the size of the GAG chain. Similar tissue-related heterogeneity has been previously demonstrated. Larjava et al (1988) showed that cell lines established from human gingiva, skin and synovium secreted DS-PGs of varying sizes, with gingival and synovial fibroblasts producing larger DS-PGs than skin fibroblasts. They also attribute the differences in size to the GAG chain. Our cell strains, established from gingiva and skin, displayed similar differences in that gingival fibroblasts secreted larger DS-PGIIs than skin fibroblasts, although the apparent molecular weights of our intact molecules were smaller in size (89-103 kDa for bovine skin DS-PGII compared to 95-110 kDa for human skin DS-PG; 95-112 kDa for bovine gingival DS-PGII compared to

110-130 kDa for human gingival DS-PG). These differences may be the result of species variation or due to variations in the estimation of molecular mass by different techniques.

In order to accurately determine the size of a polysaccharide chain, additional experimental methods such as calibrated gel filtration columns or light scattering would be required. Because of the sparsity of our samples, further experimentation in this case was not feasible and only comparative estimates of the sizes of the GAG chains by SDS-PAGE were possible. Comparisons with other published data in this regard are unreliable. Zangrando et al (1989) found the DS chain of bovine skin DS-PGII to have a weight-average molecular weight of 24,000 when determined by light scattering. As all our tissue-specific DS chains displayed slower migration in acrylamide gels than the skin DS-PGII, it would appear that they all have a molecular weight greater than 24,000.

In addition, we have been able to demonstrate subtle differences in the size of DS-PGIIs secreted by subpopulations of fibroblasts from a single tissue. Functional heterogenity in fibroblast subpopulations has previously been shown by Hassell and Stanek (1983). They examined the proliferative rates, cell-size distributions, and synthesis of collagens and GAGs by six cultures of human fibroblasts derived from a single biopsy of normal gingival papilla. They found stable, significant differences in all aspects between the various cell lines. Others have shown functional heterogenity in

fibroblast subpopulations when examining such parameters as cell replication (Bordin et al, 1984; Smith and Hayflick, 1974), enzyme activity (Milunsky et al, 1972), hormone metabolism (Kaufman et al, 1975), synthesis of macromolecules (Korn et al, 1985; Hassell et al, 1986; Häkkinen and Larjava, 1992; Larjava et al, 1988) and phagocytic activity (Lockard et al, 1979). It has been hypothesised that functional heterogenity within fibroblast populations may allow for modulation of various cell subpopulations by serum mitogens or other factors. These subpopulations may then modulate the composition of the tissue and play a role in the pathogenesis of various connective tissue disorders such as keloid formation, burn scar or the formation of granulation tissue (Häkkinen and Larjava, 1992).

The variability in our results can thus be explained, however the biological significance of various fibroblast populations, both within and between tissue types, secreting identical DS-PGIIs which vary only in the length of their constituent GAG chains, remains unknown. In order to fully appreciate these differences, an understanding of the role of the GAG chain in these tissues is required. It is possible that the variety of GAG chain lengths may affect the packing of collagen fibrils in a tissue and thus influence such things as the mechanical properties of the tissues, or possibly affect the transport of macromolecules through the extracellular matrix by increasing or decreasing the interfibrillar space.

5.2.4. The relationship of culture age to the molecular mass of secreted DS-PGIIs and cell volume.

In general, the growth and synthetic activities of fibroblasts have been shown to be contolled by intrinsic genetic factors, in addition to various environmental factors. These can include components of tissue fluids such as growth factors, complement proteins, prostaglandins, and other inflammatory mediators (Bordin et al, 1984; Goldring et al, 1990; Ko et al, 1977; Korn et al, 1985, Narayanan et al, 1989). Differences in the proliferative rates of cultured fibroblasts have also been shown to influence the size and net charge of secreted GAGs (Cöster et al, 1986; Fedarko and Conrad, 1986), while various cytokines, including interferons, interleukin-1, lymphotoxin, transforming growth factor-β and tumor necrosis factor have all been shown to modulate the secretion of extracellular matrix molecules (Bassols and Massagüe, 1988; Bronson et al, 1987; Duncan and Berman, 1989a,b; Elias et al, 1988). It has also been shown that fibroblasts cultured from phenotypically distinct connective tissues display differences in the pattern of secretion of various PGs and GAGs reflecting the functional demands placed upon the tissues in vivo. Differences have been demonstrated in the pattern of secretion of PGs by tendon fibroblasts taken from distinct biomechanical regions; either compressional or tensional (Daniel and Mills, 1988; Vogel et al, 1986). Loss of stimulus in the compressive regions of the tendon, either by removal of pressure loads in vivo (Gillard et al, 1979), or

by failing to provide compressive loads *in vitro* (Koob and Vogel, 1987) results in a decrease in the synthesis of large PGs.

We have shown a variation in the growth and synthetic activities of four subpopulations of fibroblasts. An apparent correlation was observed between doubling times and size of DS-PGII secreted; larger mass-average DS-PGIIs were secreted by cultures that exhibited slower doubling times. We speculate that cells which maintain slower doubling times may exhibit an overall slower intracellular transport rate of PGs. It is known that in cultured human skin fibroblasts, pulse-labeled PG core protein is converted to mature DS-PGs with a half-life of about 12 minutes (Glössl *et al*, 1984). In order to address the present hypothesis, pulse labelling experiments would be needed to be done to determine if transport times of PGs varied between the cell strains which exhibited differences in doubling times.

The maintenance of cells in culture for a long time results in a general decrease in synthetic activities and proliferative rates (Hayflick, 1965; Hassell and Stanek, 1983), although there appears to be a wide range in the proliferative potential of isolated clones of cells throughout the life-span (Smith and Hayflick, 1974; Smith and Whitney, 1980). It is generally assumed that fibroblast senescence occurs after approximately 40 population doublings (Hayflick, 1965), although it is difficult to predict the exact age of a clone of cells in culture due to the influence of various

culture conditions such as cell density. When a culture becomes confluent, cell proliferation ceases due to a phenomenon known as "contact inhibition" (Freshney, 1983).

An inverse relationship has been shown between cell density and the synthesis of extracellular matrix components such as collagen and glycosaminoglycans (Chen, 1981; Hassell et al, 1986; Steinmann et al, 1982). The decrease in synthetic activity has been attributed to a suppression of membrane transport (Hassell et al, 1986) or an increase in intracellular adenosine 3',5'-cyclic monophosphate (cAMP) (Ahn et al, 1978), although it has been suggested that there may be a relative increase in specialized versus structural proteins with increasing cell density (Freshney, 1983).

In the present study, cultures were maintained over 9 passages. It can be assumed that as passage number increases, the number of cells approaching senescence also increase, although as mentioned, there is no accurate way to determine the "age" of the cultures other than using cloned cells. Only one cell strain (bovine flexor tendon, cell strain 2) demonstrated an increase in doubling time with passage number, indicating that perhaps the cultures were not maintained long enough for cell aging to manifest itself in an increase in doubling times.

We also do not know if the quantity of PGs secreted in culture are reduced once the cells reach senescence and begin to show an increase in doubling times. It is to be expected that a decrease in the synthetic activity of proteins occurs

with increasing age of cultures, and it could be suggested that the core protein would spend more time within each of the intracellular compartments prior to secretion. If this were so, then the size of DS-PGs should increase as the cultures aged. An additional time in the Golgi apparatus may allow for increased polymerization of the GAG chain. Although we were able to show an apparent correlation between doubling times and Mr of the DS-PGIIs, we were unable to demonstrate a correlation between increasing passage number, and hence cellular aging, and the size of the DS-PGs secreted. There may however, merely be an overall decrease in the amount of the PGs secreted.

There is also a concomitant increase in the cell size, due to an increased accumulation of slow or nonreplicating cells, as a cell population ages (Bayreuther et al, 1988; Häkkinen and Larjava, 1992; Mitsui and Scheider, 1976). When measuring cell volume as a function of cell passage, gingival fibroblasts showed an increase in cell volume with increasing passage number, although as mentioned this was not reflected in doubling times. However, tendon fibroblasts did not, and despite a significant increase in doubling time with passage (2.5 days in passage 4 to 4.3 days in passage 9), the cells became smaller. The significance of this difference between the cell strains is unknown.

5.3. Tissue Forms of DS-PGII

The results of our studies on extracted DS-PGIIs, in

which we purified DS-PGIIs directly from fibrous connective tissues, support our culture studies. Each of the tissues was found to contain a DS-PGII molecule whose relative size was comparable to that secreted by fibroblasts in culture. As with the *in vitro* material, variation in size between intact molecules was due to the length of the GAG chain.

Octyl Sepharose chromatography has been shown to be an efficient method for separating PGI from PGII (Beresford *et al*, 1987; Choi *et al*, 1989). By exploiting the differences in the hydrophobic properties of the core proteins of PGI and PGII, Choi *et al* (1989) were able to define the conditions for the separation of DS-PGI from DS-PGII. Their protocol was followed in the present study, and indirectly allowed us to further define tissues in which DS-PGI is present. DS-PGI, on the basis of its binding to Octyl Sepharose and relative mobility on SDS-PAGE was found in gingiva, sclera, tendon and TMJ disc tissue. Previous studies have reported the presence of PGI in bovine fetal tendon (Vogel and Evanko, 1987), bovine fetal skin (Choi *et al*, 1989), and bovine sclera (Cöster and Fransson, 1981). It has been reported that the proximal/tensional zone of adult bovine tendon contains predominantely DS-PGII, while both DS-PGI and DS-PGII have been identified in the distal/compressive zone of the tendon (Vogel *et al*, 1993). While we were able to demonstrate the presence of DS-PGI in the proximal zone of the tendon, the amounts may be quite small and it appears that DS-PGII is in fact the predominant PG in this area. Although positive

immunostaining for DS-PGI has been show in the upper layer of the adult human epidermis and a small layer of the dermis adjacent to the epidermis (Schönherr et al, 1993), no reports, including the present study have demonstrated the extraction of DS-PGI directly from adult skin. The detection of extremely small amounts of DS-PGI are probably beyond the sensitivity of the present technique.

Positive staining on Western blots with mAbs to DS-PGII demonstrated that the core proteins of each of our tissue specific molecules, following partial deglycosylation with chondroitinase ABC, appeared as either a single broad band or a doublet, ranging between 45 kDa and 48 kDa. As well, the amino acid compositions of the core proteins further confirm that the molecules are DS-PGII. Although our gingival samples were reactive to the DS-PGII specific mAbs and not to the CS or KS antibodies, the amino acid composition showed that the samples were high in serine and glycine, but low in leucine when compared to our DS-PGII standard. This suggests, along with the staining on Western blots, that the contamination was probably not with the other leucine-rich small PGs, namely fibromodulin or DS-PGI. However, the large CS-PGs contain a chondroitin sulphate rich region made up of a number Ser-Gly residues substituted with CS chains (Doege et al, 1987). It is possible that our samples were contaminated with small amounts of CS-PG core protein and as the CS antibody (CS-56, Avnur and Geiger, 1984) is reactive to the GAG chain rather than the core protein, staining would not be evident on the

blots. As well, fragments of the core protein may be present in the preparation, in light of the problems encountered with proteolysis during purification of DS-PGII from gingiva.

Tissue-related heterogeneity, with regards to the size of the constituent GAG chain(s), was maintained between the various molecules. As with the cultured material, the GAG chain liberated from skin DS-PGII had the lowest molecular weight, while that from tendon DS-PGII appeared to be the largest, although the GAG chain of secreted DS-PGII was not as large as that found for extracted DS-PGII. The difference may be a reflection of the loss of environmental controls. As mentioned, the weight average molecular weight of GAG chains of skin DS-PII has been found to be around 24,000 (Zangrado et al, 1989), while the that for bovine tendon DS-PGII is 37,000 (Vogel and Heinegård, 1985). These values define the upper and lower limits of the size of the GAG chains from the various molecules studied here. Poole (1986) reported that molecular weights for DS chains from the small PGs varyed from 15,000 to 55,000.

8.4. Collagen Fibrillogenesis

8.4.1. DS-PGIIs reduce the rate of fibrillogenesis in vitro

Using DS-PGIIs isolated from various bovine fibrous connective tissues, and type I collagen from bovine skin, collagen fibril formation was studied turbidimetrically. A number of studies have examined the role of PGs in the assembly of collagen molecules into fibrils (Birk and Lande,

1981; Chandrasekhar et al, 1984; Garg et al, 1989; Oegema et al, 1975; Scott et al, 1986; Toole and Lowther, 1968; Uldbjerg and Danielsen, 1988; Vogel et al, 1984). The results of these studies suggest that the type of PG, the source of collagen as well as pH, temperature and ionic strength of the system all affect the kinetics of collagen fibril formation. Results of studies are therefore difficult to compare. The advantage of the current study is that DS-PGIIs purified from a number of tissues were used in the same system under identical conditions. This rules out the chance that possible differences in effect were due to differences in the system as opposed to tissue-specific variations in the PG molecules.

The rate of fibrillogenesis was delayed in all cases regardless of the source of DS-PGIIs. As we have shown, each of the various tissue specific molecules had identical core proteins, varying only in the length of the consitituent GAG chain. The contribution of the GAG chain in fibrillogenesis is considered to be minimal. While earlier studies demonstrated in vitro effects of various GAG preparations on collagen fibrillogenesis (Obrink, 1973; Oegema et al, 1975; Snowden and Swann, 1980; Wood, 1960), more recently, Scott et al (1986) and Vogel et al (1984) have clearly shown that the influence of DS-PGII on fibrillogenesis is mediated by the core protein, as the specificity of binding to collagen lies in the core (Brown and Vogel, 1989). Both of these studies, using bovine skin DS-PGs (Scott et al, 1986) and bovine tendon DS-PGs (Vogel et al, 1984), found a decrease in the rate of

fibrillogenesis similar to that demonstrated here for the various tissue specific DS-PGIIs.

Uldbjerg and Danielsen (1988) examined the interaction of a small DS-PG isolated from human uterine cervix with type I collagen and found that the proteoglycan did not affect the rate of fibrillogenesis, but increased the final turbidity. DS chains or CS-PG did not have a similar effect on absorbance. Electron micrographs of collagen fibrils reconstituted with uterine DS-PGs demonstrated a slight increase in diameter, but a marked increase in the lateral alignment of adjacent fibrils. The authors suggest that intact DS-PGs are essential for lateral alignment; binding of the PGs to collagen being facilitated by the core proteins but the association of adjacent DS chains influencing the interaction between the fibrils. The speculation is interesting and gains support from the earlier work of Cöster et al (1981), in which they demonstrated self-association of DS-PGs from bovine sclera.

Although the DS-PG isolated from uterine cervix (Uldbjerg et al, 1983) had biochemical characteristics similar to other small DS-PG molecules described in a number of tissues, the differences with our results and others (Scott et al, 1986; Vogel et al, 1984) may be due to differences in the core proteins of the molecules. It is possible that the small DS-PG from uterine cervix may be contaminated with, or in fact is, DS-PGI. Contamination is more probable since Uldbjerg and Danielsen (1988) were still able to demonstrate the binding of their molecule to collagen in an ELISA. It is known that DS-

PGI is unable to bind to type I collagen (Hedbom and Heinegård, 1989). In addition, they suggest that uterine cervix DS-PG may contain 2 or 3 GAG side chains; DS-PGI is thought to contain two GAG chains attached to serine residues at position 5 and 11 or 10 (Choi et al, 1989; Fisher et al, 1987). However, it is difficult to determine the exact number of GAG chains on a protein core without examining the protein sequence of the core or, in the case of DS-PGs, enzymatically removing amino acids from the NH_2-terminal of the core and simultaneously removing any attached GAGs.

Results from our laboratory have shown that when DS-PGI (obtained from bovine TMJ disc) is used in the fibrillogenesis assay, there was no reduction in the rate of fibrillogenesis, but the final turbidity of the solution was increased as described for uterine cervix DS-PG. Vogel et al (1984) have also shown that small PGs isolated from bovine cartilage and aorta, both of the PGI type (Heinegård et al, 1985), did not affect the rate of fibrillogenesis, but did increase the final absorbance of the collagen preparation. The data strongly argues that fibrillogensis is affected by a small population of distinct PGs, namely PGII. The present study further supports this assumption, as we have shown that immunologically identical DS-PGII molecules, regardless of their tissue of origin, are able to reduce the rate of collagen fibrillogensis.

Limitations of sample availiability prevented us from conducting the assay with core protein only and carrying out

additional control studies with each of the GAGs. Such studies may have proved useful since the effect on the rate of fibrillogenesis between the various molecules was not consistent. We found no significant differences in the final absorbance of our solutions when intact DS-PGIIs were used and it is unlikely that we would have seen differences in absorbance when either DS chains or core proteins were used. The addition of a commercially available preparation of DS in the assay did not affect fibrillogenesis. It is possible however, that variations in the size of the tissue-specific GAG chains or differences in their disaccharide composition, when part of the intact molecule, may account for the differences in the rate of fibrillogenesis. Although we have shown variations in size between the DS chains, the copolymeric structure of the GAGs of these various molecules has not been addressed. It is known that DS is a copolymer of GlcuA and IduA. There may be 2 negative charges on IduA, as a result of a possible sulphate at position 2 (Rodén, 1980). The proportion of IduA in the GAG chain can vary from a few percent to almost 100% (Poole, 1986). Öbrink (1973) suggested that weak electrostatic interactions between GAGs and collagen may affect the rate of fibrillogenesis. We performed the turbidimetric assay under physiologic conditions, in which the electrostatic interaction of GAGs with charged groups along the collagen molecule are probably minimal. Evidence supporting this is derived from experiments in which GAGs, from bovine skin DS-PGII, applied to collagen-Sepharose

columns under physiologic conditions, do not bind (Scott and Dodd, unpublished work). It should be noted however, that various PG or GAG preparations may influence collagen fibril formation merely by the physical-chemical property of excluded volume, whereby large molecules in solution affect the equilibrium of different conformational states favouring more compact conformations (Ruggeri and Benazzo, 1984).

Nantö-Salonen et al (1987) characterized the DS chains of small PGs secreted in culture by skin fibroblasts taken from a patient with aspartylglycosaminuria (a lysosomal storage disorder of glycoprotein degradation). They were able to show an increased sensitivity of the GAGs to chondroitinase AC digestion over controls, suggesting a decrease in epimerization with a higher frequency of GlcuA residues. They had previously described an increase in thinner collagen fibril diameters in aspartylglycosaminuria patients over controls (Nantö-Salonen et al, 1984). These results suggest that there may be a relationship between epimerization and collagen fibril diameters.

When comparing the rate of fibrillogenesis in vitro to in vivo collagen fibril diameters, a correlation was found between increasing rates of fibrillogenesis and decreasing fibril diameters. In particular, gingival DS-PGII reduced the rate of fibrillogenesis by a factor of 2; gingival tissue in vivo was found to have a mean collagen fibril diameter of 51.1 nm (± 11.8). Compare these results to those found for scleral DS-PGII, where the rate of fibrillogenesis was reduced by a

factor of 4 while scleral tissues were found to have fibrils with a mean diameter of 214.7 nm (± 121.2). These results would suggest that as the rate of collagen formation is reduced, the opportunity for lateral aggregation of fibrils increases, thus resulting in increased collagen fibril diameters. Whether or not lateral aggregation is mediated by the self-association of the GAG, which in turn is thought to be affected by the copolymeric structure of the DS chain (Cöster et al, 1981), is not known.

5.4.2. DS-PGIIs alter the morphology of collagen fibrils formed in vitro.

It has been suggested that small PGs inhibit lateral aggregation, since collagen fibrils formed in the presence of DS-PGII purified from tendon were significantly smaller than fibrils formed in the absence of PGs (Vogel and Trotter, 1987). Our results are in disagreement with these. Morphometric analysis of fibrils reconstituted in vitro with the various DS-PGIIs show an increase in width beyond control solutions regardless of source of the DS-PGIIs. Uldbjerg and Danielsen (1988) also found a slight increase in the diameter of individual fibrils formed in the presence of DS-PGs; PGs isolated from cartilage have been found to form thicker fibrils (Chandrasekhar et al, 1984; Oegema et al, 1975; Toole and Lowther, 1968) while a mixture of PGs isolated from sclera and cornea (Birk and Lande, 1981) had no effect on collagen fibril diameters. As with the results examining the effects of PGs on the rate of collagen fibrillogenesis, these results

must be interpreted with caution. Various mixtures of PGs have been used, or the PGs have not been fully characterized, particularly with respect to their core proteins.

Tissues rich in DS-PGs have increased fibril widths. A number of authors have described a strong correlation between increasing collagen fibril diameters and increasing proportions of DS of the total GAGs (Flint et al, 1984; Merrilees et al, 1987; Parry et al, 1982). Conversely, tissues in which thin collagen fibrils predominate have increased proportions of hyaluronic acid and CS of the total GAGs (Scott et al, 1981). As well, tissues with increasing collagen fibril diameters are able more effectively withstand tensile forces (Hukins and Aspden, 1985). Whether or not the thickening of collagen fibrils, which allows for an increase in interfibrillar cross-link density and hence tensile strength, occurs prior to an increase in the proportion of DS-PGs in tissues is not known. It would appear more probable that as tensile stresses are placed upon a tissue, resident cells are stimulated to secrete proportionally more DS-PGs, eventually resulting in an environment which would favour an increase in the lateral aggregation of collagen fibrils. Our results support this hypothesis, although the mechanism by which increasing proportions of DS results in an increase in fibril diameters is unknown.

A correlation was also seen between the amount of PGs extracted from the various tissues by ion-exchange chromatography and the diameters of collagen fibrils found in

these tissues. Interestingly, tissues from which a greater amount of PGs were extracted (tendon and sclera) had the largest mean collagen fibril diameter. Caution needs to be exercised when interpreting these results however, as the percentage of DS-PGII in the total population of PGs was not determined.

No correlation could be established between diameters of collagen fibrils formed *in vitro* in the presence of DS-PGII and those found *in vivo*. The differences probably reflect the absence of other matrix molecules which may play a role in determining final collagen diameters (see section 5.5.1). It has previously been shown that collagen fibril diameters formed *in vitro* are not similar to those seen *in vivo* (Cooper, 1970).

5.5. Electron Microscopy Studies

5.5.1. Collagen fibril diameters

The diameter of collagen fibrils, as seen in electron micrographs, has been shown to vary markedly between different connective tissues, ranging from an average of 51.1 (± 11.8) nm in the gingiva to 214.7 (± 121.1) nm in the sclera. It is assumed that the fibrils being measured are predominantly type I collagen. The assumption is based on the fact that type I collagen accounts for 90% of collagen in mammals and is predominant in skin, organ capsules, fibrous cartilages, ligament and tendons (Montes *et al*, 1984). As well, type I collagen and other interstitial collagens have typical

ultrastructural features, namely a characteristic periodic transverse striation, resulting in a 64-67 nm banded staining pattern. It was previously thought that type I collagen could be distinguished from other interstitial collagens, in particular type III, on the basis of ultrastructural features. Fleischmajer et al (1981) reported that type I collagen was the main constituent in fibrils whose diameters were in the range of 100-500 nm, while type III collagen fibril diameters were no greater than 60 nm. However, more recent studies using immunoelectron microscopy were able to show that type III collagen is present in all fibrils with a 67 nm periodicity regardless of their diameter (Keene et al, 1987) and that type I and III collagen can actually form hybrid fibrils (Fleischmajer et al, 1990). The results of these studies ask whether or not heterotypic fibrils of type I and III collagen may play a role in the regulation of collagen fibril diameters. As well, are the variations in the fibril diameters seen between the various fibrous connective tissues due to the influence of other collagen types?

A role for type V collagen in modulating fibril diameters has been suggested. Martinez-Hernandez et al (1982) initially demonstrated the association of type V collagen with other collagen fibrils, including type I and IV collagen in rat kidney. Adachi and Hayashi (1986) were able to show that type V collagen interacts with type I collagen in vitro to limit the growth of type I fibrils. They found that when the proportion of type V collagen in their reconstituted collagen

fibrils was over 50%, the average diameter of the hybrid fibrils formed was under 50 nm. More recently Birk et al (1990), found a correlation in vitro between increasing molar ratios of type V to type I collagen and a decrease in collagen fibril diameters. Whether or not the small diameter fibrils seen in gingival tissues are hybrid fibrils containing type V collagen is not known. Type V collagen has been identified immunohistochemically in human skin, lung, and cornea both interstitially and as part of/or in close proximity to basement membranes (Konomi et al, 1984), and more recently as part of the small fibrils of chick cornea (Birk et al, 1988). Although it has been demonstrated that cornea has uniformly narrow fibrils in several species (approximately 25 nm) and that corneal stroma contains significantly more type V collagen than other type I collagen rich tissues (Birk et al, 1988; Tseng et al, 1982; Welch et al, 1980), no studies have examined the in vivo distribution of type V collagen and its relationship to fibril diameters.

It has also been suggested that collagen fibril diameters are reflective of the functional demands of the tissue. In their analysis of collagen fibril diameter distributions from various connective tissues, Parry et al (1978) found a correlation between fibril diameters and the mechanical properties of a tissue. They, and others (Merrilees and Flint, 1980; Parry et al, 1980), have speculated that smaller diameter fibrils allow for a greater interface between fibrils and the matrix and therefore enhance the creep resistance of

a tissue, allowing the tissues to withstand compressive forces. Conversely, thicker collagen fibrils are characteristic of tissues which function under tension (Hukins and Aspden, 1985). The greater number of covalent intermolecular cross-links present in these tissues is considered to enhance tensile strength. It has also been suggested that tissues which are subjected to long-term, high stress levels have a bimodal distribution of collagen fibrils diameters (Parry et al. 1978) and display an increase in packing density (i.e. collagen concentration) that may further contribute to the overall tensile strength of a tissue (Merrilees and Flint, 1980). The thicker, bimodally distributed collagen fibrils which occupy over half of the extracellular matrix, as found in sclera and tendon, may be an indication of the functional characteristics of these tissues.

Distinct ultrastructural differences were found between the central and peripheral zones of the TMJ disc, which may suggest that different areas of the disc function in different mechanical capacities. The thin central zone of the disc contained narrower collagen fibrils which occupied less of the extracellular matrix than those in the peripheral zone. This organization is consistent with that expected for a tissue adapted to withstand compressive forces (Berkovitz et al, 1992; Merilees and Flint, 1980), as is the unimodal distribution of fibril diameters (Parry, 1988). Recently Berkovitz and Robertshaw (1993) described the distribution of collagen fibril diameters in two zones of the rabbit TMJ disc.

They found that collagen fibril diameters were narrower in the thicker, peripheral zone of the disc compared to the thin central zone in which the fibrils were thicker. They also suggest that these differences may reflect the mechanical loads to which the tissues are subjected. The differences observed in the pattern of distribution to the bovine disc may be the result of differences in chewing patterns which are reflected in the loading of the disc.

5.5.2. Interfibrillar distances and their relationship to GAG chain length

One of our initial hypotheses was that DS-PGII, by virtue of binding onto the surface of type 1 collagen and the extension of an electrostatic "arm" of sugars into the surrounding extracellular space, may influence the packing of collagen fibrils in the extracellular matrix. Interfibrillar distances would then be reflective of the length of this highly charged "arm", which would function in keeping adjacent collagen fibrils apart. Conversely, if these electrostatic arms were able to interact, as shown for DS from sclera (Cöster et al, 1981), then in addition to keeping fibrils physically apart, they may also assist in keeping all the collagen fibrils in a fiber together as a unit. Either way, the distance between the fibrils would be a function of the length of the "arm". A number of problems were encountered when trying to find evidence to support this hypothesis. First, an accurate measurement of interfibrillar distances is difficult to obtain unless one is dealing with a strictly

unimodal distribution of fibril diameters. Bimodal distributions reflect variations in the packing of fibrils, with smaller fibrils filling in the spaces between the larger fibrils. Is it then reasonable to assume that the distance between a large fibril and a small fibril is the same as that between two small or two large diameter fibrils. At best, one can only hope to obtain an average of this distance. This was the approach used in the current analysis of interfibrillar distances.

Secondly, the influence of other extracellular matrix components on the packing of collagen fibrils is currently not known. The large aggregating CS-PGs, by virtue of their size, occupy very large hydrodynamic volumes and therefore act as a type of "waterbed" around collagen fibrils (Heinegård and Paulsson, 1984). The interfibrillar location of these PGs may therefore help in keeping collagen fibrils apart. For example, CS-PGs together with the collagen binding PGs, are thought to give sclera its typical appearance of irregularly organized collagen fibrils of varying sizes (Cöster, 1991). It is noteworthy that in hyaline and articular cartilages, whose main PG consituent is aggrecan (Hascall, 1988), collagen fibrils appear to be randomly arranged and widely spaced (Montes et al, 1984; Ruggeri and Benazzo, 1984).

No correlation between the relative size and length of the GAG chains of the various tissue specific DS-PGII molecules (purified directly from tissues) to interfibrillar distances was evident (Table 5.1). The large standard

deviations (Table 4.19) encountered when assessing interfibrillar distances in scleral and tendon tissues are probably a reflection of their bimodal distributions.

Table 5.1: The relative size of GAGs compared to interfibrillar distances in matched tissues.

Relative size of GAGs of various tissue-specific DS-PGIIs	tendon > gingiva > disc (periphery) > sclera > skin
Interfibrillar distances	sclera > tendon > disc (periphery) > skin > gingiva

However, when comparing the peripheral and central zones of the TMJ disc, a correlation could be established between the length of DS chains of area-specific DS-PGII and interfibrillar distances. Nakano T., Dodd C.M. and Scott P.G. (unpublished data), compared the relative sizes of DS chains found on DS-PGIIs specific to the peripheral and central zones of the disc (which were identical to the zones used in the present E.M. study). They found the DS chain from the central zone to be longer. It was also found that the collagen fibrils from the central zone of the disc were more widely spaced (43.8 nm ± 5.8) than in the peripheral zone (38.0 nm ± 7.8), although the difference between the two was not statistically significant. These results suggest that the length of the single GAG chain found on DS-PGII may play a role in the packing of collagen fibrils in a tissue. The increase in interfibrillar distances may however, reflect the presence of other matrix molecules in the central zone of the disc, namely CS-PGs. In order to further test the hypothesis, additional

comparisons between different zones in the same tissues need to be done. For example, differences have been observed in collagen fibril diameters between the papillary and reticular layer of dermis, with those from the papillary layer being narrower (Parry and Craig, 1984). As well, it has recently been suggested that fibroblasts from the reticular layer of the dermis secrete a DS-PGII with a slightly longer GAG chain compared to that of papillary fibroblasts (Schönherr *et al*, 1993). Whether or not these differences can be used to support the current hypothesis merits consideration. Merilees *et al* (1987) have also shown differences in collagen fibril diameters between the intima and the adventitia of the artery wall, offering yet another system for investigation.

Recently, Hahn and Birk (1992) lent support to our hypothesis. They disrupted the synthesis of DS-PGs during the development of avian corneal stroma by the addition of β-D-xyloside *in ovo*, thus forming under- or non-glycosylated core proteins. Corneal stroma also contains KS-PGs which, like DS-PGs, have been shown to associate with specific bands of corneal collagen fibrils (Scott and Haigh, 1985, 1988). However unlike the DS chains on DS-PGs, the corneal KS chains are N-glycosidically linked to the protein core. Their biosynthesis would not be disturbed by the addition of β-D-xyloside, which disrupts the synthesis of xylose-mediated O-linked PGs (Schwartz *et al*, 1974). They found that a reduction in the synthesis of normally glycosylated DS-PG correlated with focal alterations in collagen fibril packing

and a disruption of lamellar organization. There were no observed alterations in collagen fibril diameters. Their results suggest that while DS-PGs are not involved in the regulation of corneal collagen fibril diameters, they may be important in fibril spacing.

5.5.1. The visualization of DS-PGIIs with Cuprolinic Blue

When the polyvalent cationic dye Cuprolinic Blue is used in combination with an electrolyte such as Mg^{++} at a critical electrolyte concentration, the selective staining of polyanions, in particular sulphated GAGs, is possible (Scott and Hughes, 1983). Since the stain is electron dense, it can be used to examine proteoglycans in the extracellular matrix of various connective tissues at the ultrastructural level, as these are heavily sulphated and hence anionic. Electron dense filamentous structures have been visualized radiating in a periodic array from the surfaces of collagen fibrils in stained electron micrographs of bovine skin (Scott *et al*, 1986), canine articular cartilage (Orford and Gardner, 1984), in human and rabbit sclera (Young 1985), in rabbit skin and cornea (Scott and Haigh, 1985), in bovine lung (vanKuppevelt *et al*, 1987) and bovine tendon (Evanko and Vogel, 1990). We exploited the properties of Cuprolinic Blue to confirm the *in vivo* presence of PGs in the extracellular matrix of the tissues used in the present study, as well as to confirm the binding of purified DS-PGII onto the surfaces of collagen fibrils formed *in vitro*.

It has been suggested that the filaments seen after

staining with the Cuprolinic Blue are the result of the collapse of the GAG chain(s) onto the proteoglycan core following binding of the dye (Scott, 1980; van Kuppevelt *et al*, 1984b). If the filaments are indeed collapsing onto what is now known to be a globular protein core (Scott *et al*, 1986), the appearance of filamentous structures would be contrary to this hypothesis. A more reasonable suggestion may be that the extended filaments are representive of the GAG chains radiating outwards from the core protein and highlighted by bound Cuprolinic Blue (Scott *et al*, 1986). Cuprolinic Blue staining of our *in vitro* collagen fibril preparations, formed in the presence of the various tissue-specific DS-PGIIs, supports this suggestion. We were able to demonstrate electron dense filamentous structures, representing the DS chain of DS-PGII, radiating outwards from the surface of collagen fibrils. In numerous instances the staining pattern was periodic.

Young (1985) examined the distribution of proteoglycans and their association with collagen fibrils in rabbit and human sclera following staining with Cuprolinic Blue. He was able to visualise filaments in three orientations, around, along and radiating from the d band of collagen fibrils. We were also able to demonstrate, in bovine sclera, a periodic array of electron dense filaments on the surface of both large and small diameter collagen fibrils (Plate 4.23). Although the micrographs may suggest that the filaments are overlapped onto the large fibrils from adjacent small diameter fibrils,

cross-sectional views clearly show that they are associated with both types of fibrils (micrograph not shown). This was not shown by Young (1985) in his micrographs, although this may be a reflection of species differences in collagen fibril diameters. Our result confirm those of van Kuppevelt et al (1987), in which they showed a similar pattern of staining in micrographs of Cuprolinic Blue stained bovine sclera, although they did not place any significance on the staining of various diameter fibrils in a single tissue. This observation is significant in that it argues against the idea that DS-PGII plays a role in determining collagen fibril diameters by limiting lateral growth (Vogel and Trotter, 1987).

Cuprolinic Blue stained sections taken from the peripheral and central zones of the TMJ disc display differences in staining patterns which may reflect PG composition. Sections from the periphery of the disc display a pattern of staining that is reminiscent of other fibrous connective tissues containing DS-PGs. These data are consistent with immunohistochemical results that show DS-PGII to be concentrated in the peripheral zones of the disc (Nakano and Scott, 1989). However, the large leaf-like structures seen in the central zone are suggestive of a different PG species, most probably the large CS-PGs found in cartilaginous tissues. Similar structures have been described in hyaline cartilage stained with Alcian Blue (Ruggeri and Benazzo, 1984). Biochemically, CS-PGs have been described in the bovine disc (Nakano et al, 1989), but presently it is not

known if they are aggrecan or versican.

In a comparison between the distal and proximal zones of bovine tendon, which have been shown to represent distinct functional areas subjected to tension (proximal) or compression (distal), Evanko and Vogel (1990) found similar patterns of Cuprolinic Blue staining as described here for the TMJ disc. Sections taken from the proximal zone of the tendon revealed small "rodlike" precipitates associated with collagen fibrils, while the large, electron dense, globular precipitates confined to the interfibrillar spaces were predominant in the distal, compressed regions. Their results also correspond to biochemical differences between these zones, namely the presence of small DS-PGs throughout both the tensional and compressed regions with a preponderance of large CS-PGs in the pressure-bearing regions of mature bovine tendon (Evanko and Vogel, 1990).

The pattern of Cuprolinic Blue staining in samples of tendon and skin are similar to those described by others (Evanko and Vogel, 1990; Scott et al, 1981; van Kuppevelt et al, 1987). Occasionally, larger globular precipitates were also seen in sections of tendon, and in all likelihood these too are large CS-PGs. Cuprolinic Blue staining of the gingival matrix appears to be the first report, although the staining is once again similar to the staining pattern seen in the other DS-PGII containing tissues.

Chondroitinase ABC and ACII digestions of sections of skin and both the peripheral and central zones of the disc

confirm the identity of the electron dense filaments as GAG, but do not distinguish between CS or DS. Previous studies on rat-tail tendon have found a linear correlation between collagen fibril diameters and the ratio of hydroxyproline to DS, confirming that the electron dense filaments seen on the surface of collagen fibrils following staining with Cuprolinic Blue are DS (Scott and Orford, 1981).

In tissues in which a variety of PGs are to be expected, a panel of enzymes including chondroitinase ABC and AC, hyaluronidase, heparinase, neuraminidase in addition to nitrous acid have been used prior to staining with Cuprolinic Blue as an adjunct to identication (Chan and Wong, 1989; van Kuppevelt et al, 1984b). In many instances however, the specificity of the enzymes is such that a distinction may be made between hyaluronic acid and CS or DS, but not convincingly between CS and DS. Also, identification of the PGs is based on GAG chains only and does not take into account protein cores. In order to facilitate the identity of the large leaf-like structures in the central zone of the disc, selective enzymatic digestions could also be done. For example, chondroitinase B has been shown to specifically digest DS (Michelacci and Dietrich, 1975), and when used in this situation could theoretically distinguish between CS and DS. Immunological identification using antibodies to various core proteins would produce a definitive answer.

8.6. Conclusions

The purpose of the present study was to further define the biological roles of DS-PGII. To that end we have shown that;

1) various tissue-specific fibroblasts are capable of secreting DS-PGII in culture. Each of the molecules have identical core proteins, confirming that they are the product of a single gene (Krusius and Ruoslahti, 1986), are substituted with 3 N-linked oligosaccharides and a single DS chain. We have also shown that fibroblasts taken from different fibrous connective tissues represent fundamentally dissimilar phenotypic cell types. The evidence for this is provided by two observations. First, fibroblasts derived from the different tissues and cultured under identical conditions secrete different combinations of PGs reflecting their tissues of origin. Secondly, differences were found in the length of the single DS chain both between and within fibroblast cell strains. These differences were maintained when comparing secreted DS-PGII with DS-PGII purified directly from tissue extracts. The functional significance of this remains unknown, however fibroblast heterogeneity may play an important role in the selection of cells expressed during various pathogenic processess. The function of the GAG chain in DS-PGII also remains unknown, however by establishing differences in the length of the GAG chain on an otherwise identical core protein, we can begin to explore the significance of this finding.

2) DS-PGII, regardless of its tissue of origin, is able

to bind to fibrillar collagen and inhibit fibrillogenesis *in vitro*. As such, this molecule may play an important role as a modulator of fibril assembly. Collagen fibrils formed in the presence of the DS-PGII molecules were found to be thicker than those formed without, thus disputing the notion that DS-PGII may inhibit the lateral growth of collagen fibrils by interfering with the addition of collagen monomers onto growing fibrils (Scott, 1984; Scott *et al*, 1981; Vogel and Trotter, 1987). The mechanisms by which forming collagen fibrils are able to increase in diameter under the influence of DS-PGII is not known.

3) DS-PGII may play a role in the packing of collagen fibrils *in vivo*. By correlating ultrastructural and biochemical data for the bovine TMJ disc, we have been able to show that variations in interfibrillar distances may be related to the length of the GAG chain on DS-PGII.

8.7. Future studies

Although a number of functions have been ascribed to DS-PGII, including those suggested above, the precise role of this PG in the extracellular matrix has yet to be defined. As highlighted in the Discussion, the influence of other matrix molecules is difficult to assess. However, a number of other avenues can be explored in the hopes of further defining the functions of DS-PGII. These include;

1) characterising the copolymeric structure of the DS chain of our various preparations to further investigate if

any possible role exists for the GAG chain in fibrillogenesis.

2) further exploring the relationship between the age and/or synthetic activity of the various tissue-specific fibroblasts and the size and amount of PGs secreted. Pulse-labeling studies could be used to define transport times of PGs through various cell compartments, while a radioimmunoassay could be used to quantitate the PGs. Differences in the rate of secretion of the cells may help to explain variations in the length of the DS chain between various fibroblast populations.

3) determining whether the fibril diameters seen in our tissues are due to type I collagen or if cohybridization of various collagen types, such as type I and type V, plays a role in determining the final diameter of a fibril. This would be an asset in understanding the importance of DS-PGII in influencing collagen fibril diameters

4) determining whether the differences demonstrated by Cuprolinic Blue staining in the two zones of the TMJ disc reflect different PG populations. Selective enzymatic digestions could be performed, or more specifically, the immunolocalization of different PGs in the tissues using antibodies to core proteins.

5) conduct additional comparative ultrastructural and biochemical studies between different zones of the same tissue (for example, the papillary and reticular layer of dermis or the adventitia and intima of arteries) to establish the role of the GAG chain in influencing interfibrillar distances.

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