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Development and Application of Non-Rejectable Skin substitute as a Wound Coverage: Role of Indoleamine 2,3-Dioxygenase (IDO) as a Local Immunosuppressive Factor

by

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ABSTRACT

A major advancement in optimizing the care of severe burn victims become possible by improving the clinical procedure of early excision and skin grafting. In past decades, the shortage in availability of normal skin to be used as wound coverage for massive burn patients led researchers and clinicians to develop skin substitutes with biological comparability to normal skin. Although the significant achievements were obtained recently both in vitro and in different animal models, the feasibility and mass production of skin substitutes for human use remain a major challenge for researchers and clinicians. Problems related to immuno-rejection due to slow re-vascularization and immune response of host cells to foreign antigens is a common obstacle in engraftment of either allo- or xeno-geneic skin substitute. To address these issues, several approaches have been adopted including the use of immunosuppressive genes, endothelial cells, and vascularization stimulatory factor, to improve the outcome of skin substitutes and other tissue-engineering devices. In this study, I propose and have tested the use of indoleamine 2,3 dioxygenase (IDO) as a local immunosuppressive factor to protect xeno-genic skin substitute as a wound coverage.

I have used a co-culture system and demonstrated that IDO expressed by fibroblasts induces bystander CD4+ Jurkat cells, THP-1 monocytes, and peripheral blood mononuclear cells to undergo apoptosis through tryptophan depletion, and did not significantly affect the proliferation and viability of normal primary skin cells and endothelial cells. IDO expressed in skin keratinocytes significantly down-regulated cell membrane-associated major histocompatibility complex (MHC) class I antigen via the

degradation of tryptophan. Engraftment of IDO expressing human fibroblasts embedded within bovine collagen gels accelerated wound healing in a rat model. IDO expressing human fibroblasts were not rejected immunologically in this xenografting model. In addition, IDO initiated neovascularization in an in *vivo* model. This result was further confirmed by an *in vitro* angiogenesis assay.

Based on these results, I propose that both the local immune cell damage and down-regulation of MHC class I antigen in target cells may be the molecular mechanism by which IDO functions as a local immunosuppressive factor.

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ABBREVIATIONS

Ad	adenovirus
AIDS	acquired immunodeficiency syndrome
AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	analysis of variance
APC	antigen-presenting cell
ATP	adenosine triphosphate
BCA	bicinochoninic acid
bp	base pair
CD	cluster of differentiation
CD4	T helper cell subpopulation
CD8	cytotoxic T cell subpopulation
CIHI	Canadian Institute for Health Information
CMV	cytomegalovirus
CSF	cerebrospinal fluid
CTL	cytotoxic T lymphocyte
CTLA	cytotoxic T lymphocyte associated antigen
DAB	3,3'-diaminobenzidine
DC	dendritic cell
DiI	dioctadecyl-3,3,3',3'-tetramethylindocarbocyanin
DMEM	Dulbecco's modified Eagle's medium
DNA	denxyribonucleic acid

ECGS	endothelial cell growth supplement
ECL	enhanced chemiluminescence
E. coli,	*eEscherichia coli*
EDTA	ethylenediamin tetraacetic acid
EGTA	ethyleneglycol-bis (β-aminoethylether)-N-N'-tetraacetic acid
eIF2α	α subunit of eukaryotic initiation factor 2
FACS	fluorescence-activated cell sorter
FasL	Fas ligand
FBS	fetal bovine serum
FDA	Food and Drug Administration
FIFC	fluorescein isothiocyanate
GAG	glycosaminoglycan
GFP	green fluorescence protein
H&E	hematoxylin and eosin
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HPR	horseradish peroxidase
HUEVC	human umbilical vein endothelial cell
IDO	indoleamine 2,3-dioxygenase
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IRES	internal ribosome entry site

IRF	interferon-regulatory factor
ISR	integrated stress response
kDa	kilodalton(s)
KSFM	serum-free keratinocyte medium
LAP	latency-associated peptide
LC-MS	Liquid Chromatography / Mass Spectrometry
LPS	lipopolysaccharide
MCSF	macrophage colony-stimulating factor
MHC	major histocompatibility complex
min	minute(s)
MOI	multiplicity of infection
mTOR	mammalian target of rapamycin
NAD	nicotinamide adenine dinucleotide
NaOH	sodium hydroxide
NK	natural killer
NMDA	N-methyl-D-aspartate
NOD	nonobese diabetic
NP-40	Nonidet P-40
p	probability
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin

PECAM	platelet-endothelial cell adhesion molecule
PI	propidium iodide
PVDF	polyvinylidene difluoride
RNA	ribonucleic acid
rpm	revolution(s) per minute
RPMI	Roswell Park Memorial Institute
RT	reverse transcriptase
RT	room temperature
SD	standard deviation
STAT	signal transducer and activator of transcription
TAA	tumor-associated antigen
TBSA	total body surface area
TCA	trichloroacetic acid
TCR	T-cell receptor
TDO	tryptophan 2,3-dioxygenase
TdT	terminal deoxynucleotidyltransferase
TGF-β	transforming growth factor-beta
Th	T helper
Tris	tris(hydroxymethyl)-aminomethane
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP nick end- labeling
UTP	uridine triphophate
VEGF	vascular endothelial growth factor

Chapter 1

Skin Substitutes, Immunosuppressive Factors, and Indoleamine 2,3-dioxygenase (IDO) (A Literature Review)

I. Introduction

Skin, the largest and most highly complex organ in the human body, is the most easily affected organ by different types of insults (1). Massive injury resulting in extensive damage to the skin necessitates immediate coverage to aid repair and regeneration, so that normal skin functions can be restored. Meshing and expansion of split-thickness skin grafts permits coverage of large areas of excised wounds with a given amount of skin. However, patients with extensive burns cannot be grafted by this conventional method due to the lack of available donor sites. As a consequence, the final coverage is delayed until donor site wounds are allowed to heal so that skin can be re-harvested. In addition, because widely meshed skin grafts cover only a small area, the remainder of the wound area must heal by re-epithelialization. As a consequence, cosmetic and functional results are severely compromised by scarring, pigmentation change, and contraction. These defects in wound healing are the major driving force in search to develop and apply a non-rejectable skin substitute. In the last decade, academic and industrial scientists have established a cultured skin substitute that can be reproduced and used as a wound coverage(2). However, techniques to establish a satisfactory shelf-ready wound coverage within which skin cells such as fibroblasts and epidermal cells, as a source wound healing promoting factors, are

embedded is lacking. Re-vascularization of grafted skin substitutes and immune responses to either allogeneic or xenogeneic skin cells are currently considered to be two main obstacles for a successful engraftment. To address these problems, this thesis has tested the immuno-protective role of a specific local immunosuppressive protein known as indoleamine 2, 3 –dioxygenase (IDO) in engraftment of a allogenic skin substitute in animal model. As a background information related to this project, the development and clinical applications of skin substitutes, mechanisms of immune rejection and current study interests for the improvement of immune rejection by immunosuppressive genes are presented.

II. Basic Structure and Functions of Skins

Skin is anatomically divided into two primary layers: the epidermis and the dermis, and are separated by a basement membrane (3). Beneath the dermis is a layer of loose connective tissue called the hypodermis. The epidermal layer is composed of stratified squamous epithelial cells, or keratinocytes, and is divided into five layers (3). These layers, beginning from the outermost to the innermost include the stratum corneum, stratum lucidum, stratum granulosum, stratum spiosum, and stratum basal. The basement membrane zone is subdivided into two distinct zones: lamina lucida and lamina densa (3). The major proteins

found in the basement membrane zone are fibronectin, lamimin, type IV and type VII collagens, as well as heparan sulfate proteoglycan (4). The dermis is subdivided into two layers: the papillary dermis and the reticular dermis and is the thickest tissue layer of the skin. Dermis is populated primarily by fibroblasts and is vascularized and innervated (4). The two major proteins that are found within the dermis are collagen and elastin (4). Collagen which is the major structural protein found in the dermis, is secreted by dermal fibroblasts. Collagen type I and III are fibrotic proteins that are important in architectural integrity and tensile strength of skin (4). This is because normal human dermis is primarily composed of fiber-forming proteins, the type I and type III collagen. The major amino acids of collagen are proline, glycine, hydroxyproline and hydroxylysine. Elastin, like collagen, is a fibre forming protein, but provides elastic recoil and is not composed of hydroxyproline (4).

The major function of skin is to provide protection against aqueous, chemical and mechanical assaults; bacterial and viral pathogens; and ultraviolet radiation (5). Protection against mechanical assaults is mainly provided by collagen and elastin. Protection against aqueous, chemical, bacterial and viral pathogens is provided by physical barrier of the stratum corneum as well as secretion of anti-pathogenic factors by cells of the sebaceous glands as well as the immuno-protective role of Langerhans' cells present within epidermal layer (6-7). Protection against

ultraviolet radiation is provided by skin pigmentation, which results from synthesis of the pigment melanin (8).

In addition to the mechanical protection of skin, skin also participates in thermoregulation of the body by sweating, homeostasis by preventing fluid and electrolytes loss, skin sensation resulting in burning, tickling, and itching, synthesizing vitamin D to participate in calcium and phosphate metabolism. Skin also provides communication to other organs of the body in response to a variety of stimuli (9, 10).

III. Burn Wounds and Skin Transplantation

The annual incidence of burn injuries in the United States is approximately 1.25 million (11). Among these patients, 50,000 people require inpatient care, 5000 to 12,000 people die. The size of total body surface area (TBSA) burned in 75% of these patients is less than 10%. Analysis of data from Statistics Canada and the Canadian Institute for Health Information (CIHI) revealed that in 1998, 300 Canadians died and 3,493 more were hospitalized due to fire and burn-related injuries (12).

The epidermis is capable of regeneration. If the injuries were limited to the epidermis (first-degree burn), the skin would heal by the migration of keratinocytes from the periphery of the wound, from

remnants of the stratum basal at the base of the wound, and from keratinocyte proliferation out of the epidermal appendages (13). This process could completely regenerate the epidermis, without scar formation, pigmentation changes and contraction.

The dermis cannot be regenerated. When serious injuries such as deep second (deeper partial-thickness) or third to fourth (full-thickness) degree occur, the dermis and epidermal appendages are destroyed. In this case, the dermis is repaired as a sheet of scar tissue without significant polarity (13, 14). Since the dermis cannot be regenerated, near-full-to full-thickness injuries are true breaches in the body envelope, and the skin cannot be reconstructed without the skin transplantation.

A most important treatment for severe burn patients is removal of burned and devitalized skin by surgical excision, in order to eliminate the risk of infection (15). Devitalization has increased the survival of burn patients by reducing the risk of sepsis and local inflammation (16, 17). Another important surgical treatment is to cover the larger areas of excised wounds with split-thickness or full-thickness skin. The process can increase the wound closure area and shorten the time required for permanent wound closure. Immediate wound closure is important because it abrogates the intense inflammatory cascade and eliminates the ongoing loss of water, electrolytes, protein and heat that contribute to the hyper-

catabolism of the burned patients. This treatment could also significantly increase survival of burned patients (18, 19).

The practice of skin grafting originated in the Hindu tilemade caste, approximately 2500 to 3000 years ago. Early surgeons used free skin graft of the gluteus region to replace noses amputated as punishment for theft and adultery (20). Interestingly, despite the early invention of skin grafting, nothing of importance was done in regards to skin transplantation until the 19th century. When several skin grafting techniques were published in 19th century (21), skin grafts were used for the purpose of reconstruction.

Skin graft is a life-saving procedure for patients who suffer extensive skin loss. According to the structure of grafted skins, skin graft could be divided into four different categories: (a) full thickness skin graft, (b) split thickness skin graft, (c) composite graft and (d) free cartilage graft (21).

Full thickness skin grafts are composed of the epidermis and the entire thickness of dermis. It also includes adnexal structures such as hair follicles, sweat glands and nerves. Split thickness skin grafts are composed of the full thickness epidermis and the partial thickness of dermis. Composite grafts are composed of at least two different tissue

types. Most commonly, composite grafts consist of the skin and the cartilage. Free cartilage grafts consist of the cartilage with its overlying prechondrium.

In reconstructive surgery, full thickness skin grafts are the most commonly used for the repair of facial defects resulting from the removal of skin cancers (22). It can provide good color, texture, and thickness matching to the selected facial defects. Wound contraction is minimized and the dermal adnexal structures remain intact (22). Full thickness skin grafts are also useful for cosmetically and functionally acceptable repair of defects of the nasal tip, nasal dorsum, nasal ala, lateral nasal sidewall, lower eyelid, and ear.

Split-thickness skin grafts vary in thickness from approximately 0.005 to 0.030 inches, and are classified as thin (0.005 to 0.012 inches), medium (0.012 to 0.018 inches) or thick (0.018 to 0.030 inches), depending on the ratio of dermis included in the graft (21). Split-thickness skin grafts have several advantages over full thickness skin grafts. These advantages include less tissue requiring revascularization so that there is an increase chance of survival under conditions of vascular compromise. In addition, the application of split-thickness grafts is easier than that of full-thickness skin graft. They have the ability to act as a "window" for a recurrence of high-risk lesions, and they can be used to cover large defects

(23). The main disadvantages of split thickness skin grafts include a suboptimal cosmetic appearance, the presence of a granulating donor site wound requiring postoperative care, greater graft contraction, and the special equipment required to harvest larger grafts (21).

Composite grafts are modified full thickness skin grafts, composed of two or more tissue layers. Composite grafts are especially useful for the repair of full thickness nasal alar rim defects, and nasal tip defects resulting in cartilage loss (24).

Partial-thickness nasal alar defects extending into deep soft tissue or approaching the alar rim often lead to the collapse of the alar rim producing a functional as well as a cosmetic deficit. Free cartilagle grafts can be used to avert this potential problem (25). The grafted cartilage provides a rigid but flexible cartilaginous framework that braces the alar rim against collapse during inspiration and expiration.

According to the derivations of grafted skin, skin graft could be divided into autologous skin grafts, allogeneic skin grafts, and xenogeneic skin grafts.

The best material for wound coverage is the patient's own skin (autograft). Split-thickness skin grafts harvested from a patient's

uninjured skin is the most common type of graft utilized. However, this approach suffers from several disadvantages such as (26):

a. The donor site is a new wound, which is painful for the patients and adds to the area of open wound driving the patient's system inflammatory response.
b. The donor site is subject to scarring and pigmentation changes.
c. The dermis taken from the donor site is not replaced, leaving the donor with a permanently thinner dermis.
d. The limited supply of donor sites on a patient with an extensive burn makes it impossible to cover massive damage.

One approach to solve the problem of limited availability of the donor sites from patient's own skin is through allograft. Allograft skin, when practical, is used as a biological dressing in the management of severely burned patients. The meshed split auto skin is interleaved with allograft either by overlaying (27) or insertion of allograft into holes cut in autograft sheets (28). The lower rate of graft take is the major disadvantage of using allograft materials compared to that of autograft in the long term. Rejection commonly occurs before 3 months and often within the first 3 weeks (29).

Xenograft skin is another alternative that can be used to cover wounds. This is primarily used as an initial short-term biological dressing.

This temporary substitute has to eventually be replaced by a patient's own healed donor skin, which are available for repeated harvesting, to avoid an occurrence of severe immune rejection (30). Although this approach could save a patient's life, delays in grafting may increase scarring with a consequent loss of function and disfigurement.

IV. Development of Skin Substitute and Its Clinical Evaluation

Development of skin substitute for the treatments of deep burn skin consists of early excision of burned tissue followed by immediate coverage with an autograft. The using of this method resulted to a significant increase in the survival rate of patients with extensive full thickness burns. However, in the case of extensive burns, healthy skin is insufficient to cover massive wounds. Moreover, when donor sites have been multiply harvested, healing occurs slowly and the quality of donor skin is diminished. These problems emphasize the fact that there is an essential need for skin substitutes. Different approaches have been attempted for temporary or permanent coverage of the wounds. Synthetic and biological dressings have been developed for these purposes in the last decades.

A. Synthetic Dressings

Synthetic dressings are defined as some kind of skin substitutes

made of polymers with two or more layers. The outer layer is designed for durability, and the inner layer is designed for maximum adherence and elasticity. In the late 1960s, Burke and Yannas began their attempts to create an artificial skin (synthetic dressing) (31, 32). They used polysiloxane (silicone rubber) as an epidermal equivalent to provide appropriate evaporation and microbial protection, and used glutaraldehyde-cross-linked collagen as a dermis replacement in early experiments (33). Consequently, a hydrophilic glycosaminoglycan (GAG) was included to signal the dermis to begin synthesis of normal connective tissue matrix fibers. Synthetic dressings usually do not stimulating inflammation, foreign-body reaction, or immunologic reaction (34). This artificial skin was approved in 1997 by the FDA for use in burn patients as Integra artificial skin (Integra LifeSciences Corporation, Plainsboro, NJ).

Preliminary clinical results on the use of "artificial skin" as a wound coverage for excised burns were published by Burke et al in 1981(33). Clinical and histological experience in a relatively short follow-up period (2 to16 months) in 10 patients indicated that the artificial skin retains some of the anatomic characteristics and behavior of normal dermis, thus promising improvement in functional and cosmetic results as well as providing physiologic function as a skin substitute. Heimbach also published the results of his study from a multi-centre randomized clinical trial for artificial skin use in the major burns in 1988 (35). In this study, the artificial dermis was compared

with the investigator's usual skin grafting materials including autograft, allograft, xenograft, or a synthetic dressing epidermal grafts. Once the artificial dermis was vascularized, patients were grafted with a very thin meshed auto split skin. At 136 sites in 106 patients, the rate of graft take of artificial dermis graft was significantly lower than all controls (80 vs. 95%, $p<0.0001$). Meanwhile, when it was compared with either allograft or xenograft, there was no significant difference in the rate of graft take. In addition, a common complaint from patients and surgeons was the inconvenience of a second operation to cover the dermis.

Another major synthetic dressing is named Dermagraft (Advance Tissue Sciences Inc, La Jolla, CA), which is composed of a synthetic matrix and allogeneic fibroblasts. In this artificial skin, a silicone sheet is used as the epidermal layer, while bioabsorbable mesh within which neonatal foreskin fibroblasts are embedded is used as dermal layer of this composite. The skin substitutes are then sealed in a sterile bag with circulating nutrients (36). Dermagraft can be used as a temporary wound coverage for excised burn wounds. As with cadaver allogeneic fibroblasts and silicone material, Dermagraft skin has to be removed and replaced with autologous skin grafts. If biodegradable polyglactin acid mesh (without silicone layer) is used in this kind of graft, it will be absorbed after 3 to 4 weeks.

Several clinical trials have been performed on Dermagraft with various results. One pre-clinical experiment of using a combination of the Dermagraft overlaid by a human meshed split skin graft in athymic mice demonstrated that the Dermagraft could be vascularized in the wound bed and support attachment (37). Vascularization of a meshed split skin graft could result in neodermis formation beneath the skin graft. Another clinical trial was carried out by Hansbrough and colleagues on 17 patients with full thickness burns (38). Dermagraft plus an overlying split thickness meshed skin autograft was compared with a meshed autograft alone, on an adjacent site. The results showed a slightly lower rate of graft take of Dermagraft plus split skin autografts compared to the control (meshed autograft alone). For this kind of skin substitute, the use of thinner meshed autografts did not appear to improve the rate of graft take. The finding also shows no evidence of immunological or other adverse responses towards the nylon mesh.

Dermagraft has been approved by the FDA to apply for some cutaneous defects, in particular diabetic foot ulcers. The results of clinical trials demonstrated that faster and more complete healing of diabetic ulcers was obtained when a greater amount of Dermagraft was applied and when it was applied more frequently (39).

B. Biological Dressings

Biological dressings are reconstituting artificial skin from collagen film, gel and sponges from bovine and other sources of collagen combined with glycosaminoglycan (GAG) with or without cells (40). Although biological dressings could reduce healing time and improve the quality of healing amongst other properties, most of them usually serve only as a temporary wound coverage.

One type of biological dressing called Alloderm, produced by Life Cell Inc., is based on collageneic human dermis from which all cellular material is removed using techniques claimed to optimize the residual matrix for subsequent implantation (41). As it lacks the epithelial component and dermal cellular component of cadaveric allograft, Alloderm is well tolerated by patients' immune system.

Clinical trails using Alloderm in combination with a meshed split skin autograft on full thickness wound in 67 patients were reported (42). The success of the combination graft was shown to be technique-dependent, as it was more sensitive to shear stresses and prone to dressing. Overall graft take in the combination grafting was worse than the split skin graft alone, however, most of this was due to surgeons failing to comply with the optimal dressing technique. There was no immunological sensitization of the patients to the different components of

Alloderm up to 70 days post grafting. Other advantage that Alloderm has over Integra is that it can be grafted simultaneously with thin epithelial autografts, saving the patient a return trip to the operating room. For the most part, Alloderm is used to replace small skin defects.

Apligraft, another commercially available biological dressing, and formerly called Graftskin (Organogensis Inc.), has been approved by the FDA for treatment of partial thickness and full thickness skin loss due to venous stasis ulcers in 2000.

Apligraf is a bi-layered living skin with appearance and handing characteristics similar to normal skin (43). The dermis layer is comprised of a combination of bovine type I collagen and human fibroblasts. The collagen/fibroblast mixture undergoes fibrillogenesis to form a gel, followed by fibroblast-induced condensation of collagen fibres. Casting and anchoring the original collagen/fibroblast mixture onto the filter disc of a cell culture insert ensures that the volume reduction is in the dorso-lateral plane rather than exhibiting radial shrinkage. At late stages, keratinocytes are seeded onto the "dermal equivalent" in submerged culture and later raised to the air-liquid interface to allow keratinocyte differentiation and stratum corneum formation.

A multi-center study on Apligraft involving a total of 107

patients with surgical wounds (largely skin cancer excisions) was reported (44). The Apligraf was applied once, immediately after excisional surgery, and patients were followed up for 1 year. There was no evidence of an immunological response to either the allogeneic cellular components or the xenogeneic bovine components. Graft persistence was 73% at 1 week, falling to 53% at 1 month, and 31% at 1 year. Rate of infection was 10.5%.

Apligraf has also been applied to chronic cutaneous wounds. A multi-centre study involving 233 patients with venous ulcers was reported (45). At 6 months post grafting, the Apligraf treatment demonstrated a greater frequency of complete wound healing (61 *vs* 44% for the control $p=0.12$), and a more rapid median time to complete wound closure (57 days *vs* 181 days, $p= 0.007$).

Recently, another bilayer skin substitute called OrCel^{TM} , developed by Ortec International Inc (New York, USA), and containing living allogeneic human skin cells, has been approved by the FDA (46). Different with Apligraf, in OrCel^{TM}, human allogeneic epidermal keratinocytes and dermal fibroblasts are cultured into two separate layers on a type I bovine collagen sponge. Fibroblasts are cultured on the porous sponge side of the collagen matrix while keratinocytes are cultured on the coated, non-porous side of the collagen matrix. Application of OrCel^{TM} to

donor sites in burn patients showed a significant shorter of the healing time than that treated with Biobrane-L (47).

V. Mechanisms of Immune Rejection to Foreign Tissues and Cells

Due to limitation in autograft tissues, allograft and xenogeneic grafts are considered to be a valuable alternative to be used as a coverage for either damaged wounds or replacement of a damaged organ. However, rejection of transplantated foreign tissues often occurs because the immune system of the recipient recognizes and responds to foreign tissue histocompatibility antigens expressed in the graft

Many processes participate in host response to grafted foreign tissues. These processes include local inflammatory response to surgery, processes that initiate wound repair and vascular endothelialization, and the immune response to the recognition of foreign antigens. The host major histocompatibility complex (MHC) plays the defining role in the acceptance or rejection of a graft. The more closely the donor graft and the host are matched for MHC, the greater the likelihood of the graft acceptance by the host.

Host T cells recognize a foreign antigen and generate an immune response, only when foreign antigens are " presented" to its T-

cell receptor (TCR) by a major histocompatibility complex (MHC) class I or Class II molecule. Antigens are taken up by endocytosis, processed and presented to the host immune system by antigen presenting cells (APCs) (48). After presented graft antigens by APC via MHC class II, host lymphocytes (T helper cells) become sensitized, activated, and proliferate. The cytokines secreted by activated T helper cells continue to activate more T helper cells. In addition, T helper cells activate CD8 lymphocytes, leading to greater proliferation and differentiation into cytotoxic T lymphocytes (CTL). CTL are responsible for recognition of foreign tissues via the T cell receptor (TCR)-MHC class I complex which induces the release of cytotoxic granules.

The role of antibodies in graft rejection is controversial. T lymphocytes alone are sufficient to reject a foreign tissue. However, the humoral response to the graft could participate in this process either by synergizing with T cells or by antagonizing them so as to impede rejection.

Several mechanisms have been postulated to explain the reaction of a foreign tissue by the immune system. Grafts can be destroyed either directly by delivery of a "lethal hit" from cytotoxic T lymphocytes or indirectly by molecules such as cytokines. Both mechanisms involve a broad diversity of elements within the cellular events of the immune

response (49).

A. Cytotoxic T Lymphocytes (CTL)

Generation of antigen-specific CTL is a major immunological effector mechanism in allograft rejection. The CTL recognize antigen in the context of MHC class I molecules, and, therefore, are important in allograft rejection where the graft itself can present alloantigen to immune system. The role of CTL in the rejection has long been known. For example, in the 1970s, Strom and colleagues (50) showed that donor-specific CTL could be eluted from rejecting human renal allografts. Later on, Rosenberg and associates (51) showed that adoptive transfer of CD8+ effector T cells was sufficient to induce rejection of MHC Class I mismatched skin grafts in mice.

There are at least two major mechanisms by which CTL can deliver a lethal hit to the target cells through direct cell-cell contact. One is the interaction of Fas ligand on the activated CTL with Fas expressed on the target cells. The other is the delivery of cytotoxic molecules termed granzyme B and perforin.

B. Fas and Fas Ligand

Fas (CD95) and FasL (CD95L) are members of the tumor necrosis factor (TNF) family. The defects in this pathway have been

implicated in animals with a defect in apoptosis and autoimmunity (52). Fas is a widely expressed glycosylated cell surface molecule with molecular weight approximately 45 to 52 kDa. It is a type I transmembrane receptor, and also exists in several soluble forms (53, 54). Fas is expressed in a more restricted way than Fas ligand, and is a TNF-related type II transmembrane molecule (55). The Fas-FasL pathway has been implicated in clonal selection and control of lymphocyte activation (56-57), as well as in killing target tissues mediated by cytotoxic T cells (58). The expression of Fas, by commonly transplanted organs such as the heart (59), lung (60) and kidney (61), suggests that these tissues may be targeted by FasL-expressing allo-specific cytotoxic T lymphocytes. In addition to a deleterious role in the destruction of graft tissue, Fas/FasL has also been proposed as a local immunosuppressive factor to protect grafted tissues or cells as described in the following.

C. Perforin and Granzyme B

Perforin is a cytotoxic molecule released into target cells by CTL during conjugate formation within CTL and target cells. The role of perforin in cytotoxity may be directly related to the formation of holes in the target cell membrane to facilitate the toxic granzyme B entrance. The importance of perforin in transplantation has been highlighted by the observation that mice that are genetically deficient in perforin, are deficient in their ability to lyse allo-specific targets in vitro (62).

Granzymes are neutral serine proteases that are stored in the specialized lytic granules of cytotoxic T lymphocytes. Although the biological functions of most granzymes remain unknown, it is very clear that granzyme B promotes DNA fragmentation, and is directly involved in cell death (63). A mutation or deficiency of granzyme B leads to a severe defect in the ability of cytotoxic T lymphocytes to induce apoptosis in susceptible target cells (64), and reduces the severity of class I-dependent acute graft-versus-host disease (65). Like perforin, the expression of granzyme B has been used as predictive markers for the acute and chronic rejection of organ transplantation (66-68)

D. Natural Killer Cells

Natural killer cells (NK cells), like CTL, kill target cells via perforin and granzymes. However, NK cells disrupt target cells in a MHC-unrestricted manner via their receptors that are not antigen specific. The role of NK cells in allograft rejection is uncertain. Early research implicated that NK cells participate in the rejection of bone marrow allografts (69). Later, Ogura and colleagues (70) reported that transplantation of rat liver into CD8+ T-cell-depleted recipients resulted in the rejection and intra-graft expression of granzyme B and FasL similar to that of unmanipulated allograft recipient controls. It is also found that infiltrated NK cells might contribute to the rejection in those CD8+ T cell-

depleted recipients. Slightly more provocative is a murine cardiac allograft model in which removal of the CD28-costimulatory signal did not afford long-term graft acceptance unless accompanied by the depletion of recipient NK-receptor-bearing cells (71). However, the depletion of NK cells alone does not prevent allograft rejection. Taken together, these studies suggest that NK cells might play a role in the alloimmune response, but their importance in a host with normal T-cell function has not been clearly demonstrated.

E. Macrophages

Macrophages play multiple roles in initiating and propagating immune response to grafts. Early studies showed that macrophages are presented in sufficient numbers at relevant sites of rejected allografts, and their absence would be interfered with the rejection (72). Macrophages are required for T cell infiltration and the rejection of xenograft (73). It has been demonstrated that macrophages are the dominant infiltrating cells in pancreatic islet or islet-like cell cluster (ICC) xenografts undergoing rejection (74). Macrophage recruitment in xenograft rejection might result from several mechanisms (75): (1) binding to deposited xenoantibodies through macrophages expression of Fc receptors for IgG; (2) the action of chemokines; and (3) a novel lectin-dependent interaction. However, few studies detail the mechanism through which xenograft rejection occurs.

F. Natural Antibodies and Complement

Natural antibodies appear to have a role only in xenograft, not allograft rejection. The predominant natural antibodies are those directed against the α-galactosyl residues presenting on many nonprimate mammalian endothelial cells. They bind to xenogeneic endothelium and fix complement, leading to vascular leakage and thrombosis. The process can cause xenograft rejection in minutes to hours (Hyperacute rejection) (76). Xenoreactive natural antibodies in humans are of at least two types (1) the polyreactive natural antibodies, and (2) the monoreactive anti-carbohydrate antibodies. Polyreactive antibodies, which are thought to be the product of B1 B cells, bind to a variety of structures on foreign cell surfaces (77). The monoreactive antibodies specific for Galα1,3Gal are thought to be of preeminent importance in humans (78, 79).

Complement appears to have a role in both allo- and xenograft rejection (80, 81). It can perforate the target cell membrane and create a lethal electrolyte imbalance. Alternatively, it can form a complex with bound antibody and form a potent adhesion complex for the binding of macrophages and neutrophils, targeting these cells to the graft (82, 83). These functions of complement in immune rejection depend on the generation of an immune response to the graft that leads to antibody formation and antibody-complement complex formation.

G. Cytokines

Cytokines play both destructive and immunomodulatory role in graft rejection (84). Cytokines that participate in immune rejection include TNF-α, interferon-γ, and interleukin-1 (IL-1). They contribute to graft destruction either directly or via activating effector cells (85). Cytokines thought to be capable of impairing graft rejection include IL-4, IL-10, and transforming growth factor β (TGF-β). The roles of these cytokines in immunomodulation will be discussed in detail in the following section.

VI. Application of Immunosuppressive Drugs in Transplantation

In large part, the success of solid organ transplantation lies in the appropriate utilization of immunosuppressive medication (86). The concept of suppressing immune response at various sites by blocking precursor cell formation, immunocompetent cell stimulation, and proliferation or differentiation of lymphocytes, has led to the development of immunosuppressive drugs. The earliest immunosuppressive drugs, which were used for immunosuppression after transplantation, were composed of cytotoxic drugs and antimetabolites (87). These drugs include anti-proliferative drug 6-mercaptopurine (6-MP), alkylating agent cyclophosphamide and purine analogue azathioprine. Like radiation, in addition to attacking immune cells and preventing immune rejection, these

drugs also damage normal cells that are essential for survival, particularly hematopoietic cells. The second groups of immunosuppressive agents include corticosterioids, calcineurin inhibitors, mycophenolate mofetil, rapamycin, monoclonal antibodies against CD3 and CD25 (88).

Corticosteroids are natural hormones secreted by the adrenal cortex. They inhibit T cell proliferation and inhibit cytokine production. Corticosteroids are usually combined with other immunosuppressive drugs in order to improve efficacy and reduce toxicity. During the 1960s and 1970s, immunosuppressive therapy usually combined corticosteroids and azathioprine, with or without antilymphocyte globulin (89). Although this combination was moderately successful in prolonging allograft survival, it caused a variety of toxic side effects such as overwhelming and sometimes fatal infections, direct organ toxicities, impaired wound healing, anemia, diabetes, and malignancies (90).

The combination of corticosteroids and azathioprine was used as a standard therapy until the 1980s when cyclosporine A was developed. Cyclosporine was the first transplant-specific drug applied to specifically target effector T cells (91). Cyclosporine had changed the entire field of transplantation by significantly increasing the survival times of all grafted organs at the time. The adverse effects of cyclosporine, which related to the concentration of the drug, include nephrotoxicity, hypertension,

hyperlipidemia, gingival hyperplasia, hirsutism, and tremor (92).

Over the past decade, the development of several new drugs continued to improve transplanted organ survival times and reduce systemic drug toxicity. These drugs include: monoclonal antibodies such as muromonab-CD3 (93), inhibitors of co-stimulatory signal such as cytotoxic-T-lymphocyte-associated antigen 4 (CTLA-4) immune globulin (94), and inhibitors of T- cell differentiation such as sirolimus (rapamycin) (95).

Although immunosuppressive drug therapy has become a universal treatment to prevent rejection of allograft organs, there are still some major obstacles for using these drugs for prevention of skin allograft rejection as the skin possesses a high degree of immunogenicity. It was widely assumed that the dosage of immunosuppressive drugs required to prevent skin graft rejection were too high to be used safely in the clinical setting. For example, in 1989, Frame et al. (96) treated three patients with cyclosporin after allografting. The allografts survived during drug treatment but were rejected in two cases after cessation of therapy. In the third case, a meshed alloautografts sandwich technique was used to improve the graft take. Although there was no visible evidence of rejection, it was suggested that the allograft was replaced by a creeping substitution with autologous cells. It is likely that the same process

occurred in the first reported case in the long-term allograft survival after a 4-month course of cyclosporin administration was used.(97). In addition, the use of immunosuppressive drugs to prevent skin allograft immune rejection would encounter serious problems with sepsis as a result of immunodeficiency in massive burned patients.

VII. Experimental Studies of Immunosuppressive Genes in Prevention of Graft Rejection

Gene therapy was originally conceived as an intervention strategy to replace, or correct defective genes in patients with inherited diseases. Deriving mostly from knowledge of the mechanisms of cell transformation by tumor viruses, gene therapy strategies were developed through vector technology. The realization that it may have a much broader potential for the delivery of therapeutic protein agents such as cytokines, antibodies and recombinant ligands, has stimulated interest in many biological fields including transplantation.

Gene transfer offers the possibility of delivering molecules with immunomodulating activity to the donor organ, which may either reduce graft immunogenicity or increase the graft's protective mechanisms against the host's immune or inflammatory responses. Graft modulation resulting from the expression of several "protective" genes including IL-

10, TGF-β, FasL, CTLA4-Ig, and IL-12, has been considered for ex vivo gene transfer (98). Immunosuppressive cytokines are potential candidates for gene therapy (99).

Delivery of biologically active molecules to the graft can be achieved in two different strategies, either by co-transplantation of genetically modified cells along with the graft, or by genetic modification of the graft itself. The use of carrier cells that are transplanted along with the graft could be an efficient way of delivering immunosuppressive molecules. But, as carrier cells, they must be easy to transfer and express the gene, and must be able to survive when transplanted to the selected site in vivo. Most importantly, they must not have any tumorogenic potential. These requirements limit the number of potential applications using this approach.

Lau and colleagues had described this strategy by co-transplantation of gene modified myofibroblasts and islets. They transfected myofibroblasts of recipient origin ex vivo with an expression plasmid containing the cDNA for either CTLA4-Ig or Fas ligand (FasL) (100, 101). Myofibroblasts are relatively robust cells that could survive after transfection and selection in vitro. They are ideal vehicles for this type of application as they have the potential capacity of anti-apoptosis. According to their description, to prepare the composite islet-muscle cell

grafts, allogeneic islets and the transfected myofibroblasts were mixed before transplantation into the kidney capsule of recipient mice. In the case of co-transplantation of myofibroblasts expressing CTLA4-Ig, islet grafts were prolonged from a mean survival time of 11 days to 31 days. Expression of FasL by the myofibroblasts resulted in an even longer survival time of the co-transplanted allogeneic islets.

Direct modification of the transplanted organs or cells has also been explored as a strategy to suppress the immune response from allo- or xenografts. Several transfected approaches including retrovirus, adenovirus, adeno-associated virus, and nonviral plasmids (liposome and polymers) have been selected for this purpose. Their advantages and disadvantages were compared (98), and no obvious favorite has emerged in the literature published to date. Indeed, the preferred strategy may vary between organs depending on the susceptibility of the organ to transduction.

A. **IL-10.**

The Th2-type cytokine IL-10 has many effects that may modify the immune response. It has been shown to prevent APC activation and costimulation, permitting antigen presentation in the absence of co-stimulation signal, and inhibit adhesion molecule expression (102). It may also induce an anergic state in CD4+ cells (103), suppress production of

the proinflammatory cytokine IL2 by CD4+ T cells (103), and directly inhibit T cell proliferation (105). IL-10 has inconsistent effects which may delay (106), accelerate (107), or have no effect (108) on allograft rejection. For example, mouse islet allografts that express IL-10 by transfected beta cells, are rejected more rapidly (109, 110), or at the same rate as normal control islet graft (111), while transient viral IL-10 gene transfer to cardiac allografts could significantly delay their rejection (112, 113).

B. TGF-β

TGF-β belongs a member of a super-gene family in which consist of TGF-β, activins and bone morphogenic proteins. Three isoforms of TGF-β have been identified to date. TGF-β is released as an inactive precursor, in a complex bound with a latency-associated peptide (LAP) (114). The factors, which can release TGF-β from this complex, include alterations in pH, several proteolytic enzymes, and the protein thrombospondin (115).

TGF-β is a multi-functional cytokine that influences many physiological and pathological processes. TGF-β could prevent adhesion of neutrophils to endothelium (116) and their subsequent transmigration (117). It also inhibits the proliferation of monocytes and lymphocytes (118, 119), as well as induces lymphocyte apoptosis (120). TGF-β has also

been linked to inappropriate fibrosis in several pathological conditions (121). In vitro experiments showed that TGF-β could increase the production of matrix protein (122) and prevent the degradation of matrix protein (123).

Like IL-10, TGF-β has been attempted to improve allograft survival. In an animal model, Qin et al reported (124) that TGF-β could prolong syngeneic or allogeneic cardiac survival from 12.6 ± 1.1 days to 26.3 ± 2.5 days when purified plasmid DNA-encoding murine TGF-β1 was injected into the graft at surgery. The authors further demonstrated that TGF-β1 could reduce the precursor frequency of donor-specific cytotoxic T lymphocytes and total interleukin-2 producing helper T lymphocytes in graft-infiltrating cells, suggesting that cell-mediated immunity was inhibited by TGF-β1 (125). Recently, another research group reported that TGF-β could prolong cardiac allograft survival in approximately two-third of transplant recipients by DNA-liposome mediated transfection, while adenoviral delivery of TGF-β1 was not protective (126). These results suggest that TGF-β could be used as an immunosuppressive gene to prevention immune rejection.

C. FasL

Interaction of Fas with its ligand (FasL) is thought to play a

major role in the maintenance of immunological homeostasis and peripheral tolerance (127). Stimulation of Fas, a member of the tumor necrosis factor receptor super-family, either through interaction with FasL or by means of anti-Fas antibodies, initiates an intracellular signaling cascade, resulting in a programmed sequence of nuclear degradation and cytoplasmic alterations characteristic of apoptosis (128). Expression of FasL is tightly regulated, being expressed primarily by T cells after activation, where it serves as a self-regulatory mechanism for the immune response. The finding that FasL is expressed constitutively at sites of immune privilege such as the testes and the anterior chamber of the eye (129, 130), suggests that it may have immunosuppressive function. The ability of Sertoli cells to express high levels of FasL has promoted attempts to use them to provide immunological protection for other transplanted tissues in a kind of artifical immunologic privilege site. A mixture of Sertoli cells with islets before transplantation into the renal subcapsular space reportedly results in islet allograft survival in the absence of immunosuppression (131). Co-transplantation of FasL-transfected myofibroblasts in association with islets, was shown to prolong islet allograft survival (100). However, these results were conflicted with several studies. For instance, transplantation of islet allografts transfected with FasL recombinant adenovirus resulted in long-term allografts survival in only 1 of 30 recipients (132).

D. CTLA4-Ig

Recognition that co-stimulation signals are required to achieve T cell activation after TCR ligation with MHC containing antigen peptide, has led to several tolerance induction strategies, based on the blockade of this interaction. The fusion protein of CTLA4-immunoglobulin (CTLA4-Ig), which effectively blocks costimulation via CD28 by binding to B7-1/2 on antigen presenting cells, has been shown to prolong kidney (133, 134), heart (135, 136), and islet (101, 137) allograft survival. CTLA4-Ig could also prolong the survival of xenogeneic human islets in mice (138). Similarly, in several adenovirus mediated gene transfected experiments, CTLA4-Ig has been demonstrated to prolong the survival of allografted liver (13, 140), pancreas (142) and heart (143). Recently, CTLA4-Ig has been entered phase I clinical trials for the treatment of psoriasis, a T-cell mediated skin disease and treatment of graft-versus-host disease in allogeneic bone marrow transplantation. Its immunosuppressive effects coupled with features such as specificity of interaction and low toxicity, make CTLA4-Ig a promising new therapeutic agent for the induction of donor-specific immunological tolerance.

E. Major Histocompatibility Complex (MHC)

Pre-transplant organs exposured to the donor MHC antigens, are the highly successful strategy for tolerance induction in experimental models. It has long been known that blood transfusion prior to

transplantation has beneficial effects on kidney allograft survival (144). Fibroblasts of recipient origin being transfected with a donor-specific class I or II MHC gene and the transfected cells being infused back into the mice before cardiac allograft, would significantly prolong heart allograft function (145). In accordance with this data, recent experiments have demonstrated that injection of donor-type lymphopoietic cells, such as resting B cells or splenocytes, with donor origin MHC, allow indefinite prolongation of fully allogeneic cardiac allografts in mice (146).

A direct injection of MHC peptide could also induce graft tolerance. Following the study of Posselt and co-workers (147), who demonstrated MHC peptide induces tolerance in islet cells by injection intrathymically, numerous protocols have been produced to sustain unresponsiveness in allografts by injecting MHC-derived peptides (148).

The mechanisms of transfected allo-MHC molecules (peptide or gene) to recipient prior to transplantation inducing host tolerance were considered for the production of allo-MHC antibody and induction of anergic T cells. Allo-Class I MHC antibody alone could enhance graft survival by blocking the interaction of MHC with TCR. The expression of class I MHC in the absence of molecules such as class II MHC and costimulatory signal may lead to an incomplete activation of CD8+ T cells and their diversion to anergy.

VIII. Indoleamine 2,3-Dioxygenase as a Novel Local Immunosuppressive Factor

A. Metabolism of Tryptophan

Tryptophan is the least available essential amino acid in the body. Tryptophan is primarily synthesized from molecules such as phosphoenolpyruvate in bacteria, fungi and plants. It is then transferred into the body of mammals through the food chain. Dietary tryptophan is delivered to the liver through the hepatic portal system. Tryptophan is either distributed to the blood stream for protein synthesis and other functions, for example, as the only source of substrate for synthesis of serotonin, melatonin, and nicotinamide adenine dinucleotide (NAD+), or is degraded in the liver through a series of metabolic steps, namely the kynurenine pathway.

The kynurenine pathway represents the major catabolic route of tryptophan in mammals. The initial and rate-limiting reaction, in the breakdown of tryptophan to kynurenine, is the oxidative cleavage of the pyrrole ring of tryptophan by tryptophan 2,3-dioxygenase (TDO, EC 1.13.11.11) or ubiquitous and extrahepatic indoleamine 2,3-dioxygenase (IDO, EC 1.13.11.17). Kynurenine can be hydroxylated by the kynurenine 3-monooxygenases (EC 1.14.13.9) to form the aminophenol, 3-

hydroxykynurenine. The later can be converted to xanthurenic acid by kynurenine aminootransferase (EC 2.6.1.7). Kynurenine aminotransferase can also catalyze kynurenine into kynurenine acid. Alternatively, kynurenine or 3-hydroxykynurenine may be converted to anthranilic acid or 3-hydroxyanthranilic acid, respectively, via kynureninase (EC3.7.1.3). 3-hydroxyanthranilic acid can be cleaved by 3-hydroxyanthranilic acid 3,4-dioxygenase (EC 1.13.1.5) to 2-amino-3-carboxymuconi-semialdehyde. The unstable intermediate is then converted to stable quinolinic acid.

Quinolinic acid was shown to excite neurons via the activation of NMDA receptor (149), and consequently produced neuronal damage (150). When quinolinic acid is injected into the brain of rats, marked convulsant and neurodegenerative activity is noted (150). There has been much debate on whether the amounts of quinolinic acid encountered in the brain or the cerebrospinal fluid (CSF) would be sufficient to produce neuronal damage. The amount of quinolinate in the brain rarely exceeds 1 μM. Some studies showed that even the concentration of quinolinate in 1 μM, is sufficient to cause significant neuronal damage either by direct activation of NMDA receptors or via the release of endogenous glutamate (151, 152). Micromolar concentrations of quinolinic acid are toxic when cells are exposed to those concentrations for several hours (153, 154), and submicromolar concentrations can produce neurotoxicity in culture if

maintained for several weeks (155). Lower concentrations were also tested in different conditions, and showed to cause damage of neurons (156, 157). Substantial elevation of quinolinic acid concentrations in both cerebrospinal fluid and brain tissue have been reported in human patients and non-human primates subjected to a broad-spectrum of infectious and other inflammatory neurological diseases, including AIDS-related dementia (158-161). As these concentrations found in inflammatory diseases are comparable with those concentrations tested in experiments, the potential importance of this neurotoxicity to brain damage associated with this product of kynurenine pathway is clear.

In contrast to quinolinic acid, kynurenic acid was proved to be an antigonist of NMDA, kainite and AMPA receptors (162). Kynurenic acid can block glutamate receptors in rodents (162) and primates (163), as well distinguish subpopulations of kainite receptors (164). Administration of kynurenine acid could protect against the neurotoxic and convulsant activities induced by previous quinolinic acid administration in rats (165). Increased kynurenine acid concentrations in the central nervous system have been reported for various inflammatory neurological disorders (159). It has been proposed that inflammatory-related neurotoxicity may reflect a localized imbalance of the quinolinic to kynurenic acid ratio in favor of the neurotoxin. The imbalance has been seen in cerebrospinal fluids of macaques infected with the simian immunodeficiency virus (166), human

patients infected with HIV-1 (167), and brains of mice suffering cerebral malaria (168).

The kynurenine pathway including at least two other compounds relevant to neurodegeneration, namely 3-hydroxykynurenine and 3-hydroxyanthranilic acid, can also produce neuronal damage, although primarily by the induction of free radical formation rather than through action on glutamate receptor (169). The effects of catabolites of tryptophan on other tissues such as lymph tissues will be discussed in the following section.

B. Enzyme Activity, Expression and Tissue Distribution of IDO

IDO is a monomeric heme-containing enzyme that catalyzes the opening of the pyrrole ring of L-tryptophan to yield N-formylkynurenine. The later, an unstable product, is quickly converted to kynurenine (170). IDO uses superoxide anion, a univalent reduced molecular oxygen, for its activity (171). However, typical in vitro IDO activity assay system, purified enzyme uses methylene blue together with ascorbate for its activity (172). Under certain conditions such as in the presence of methylence blue, superoxide anion is not an absolute requirement for the maximal catalytic activity of IDO (172).

The IDO protein is encoded by a single gene with 10 exons and

with 15 kbp of DNA located at human and mouse chromosome 8. The cDNA of human IDO gene has been isolated by two independent research groups. Tone et al. (173) isolated the cDNA of human IDO in a λgt11 library prepared from poly (A) RNA in IFN-γ-treated Hela cells with a monoclonal antibody against IDO. Gupta and co-workers (174) isolated the cDNA of human IDO by differential screening from a cDNA library made with poly (A) RNA fraction in IFN-γ treated human fibroblasts. The nucleotide sequence of the putative IDO cDNA was determined, revealing an open reading frame that encodes a protein of 403 amino acids with a calculated molecular weight of 45,332 Da. However, in vitro translation of the IDO gene indicated that it encodes for a protein with an approximate molecular weight of 42, 000 Da. The open frame is protected by a long, untranslated sequence. No Kozak consensus was detected before the first ATG open reading frame (174).

The cDNA for mouse IDO has also been cloned and sequenced (175). The mouse IDO cDNA encodes a 407 amino acid protein with the molecular weight of 45,693 Da. It reveals a 62% overall homology with that of human IDO amino acids. However, amino acids from 101 to 184 of mouse IDO are highly homologous (89%) with the human counterpart (amino acids from 96 to 181), suggesting an enzymatic active center might be located at this domain.

The IDO gene is well conserved (176). Gene transcription is strictly controlled, responding to specific inflammatory mediators, and confined to a limited range of cell types. So far, several inducers of IDO have been defined. They include type I interferon (IFN-α/β) (177), type II interferon (IFN-γ) (178), lipopolysaccharide (LPS) (179), interleukin-1 (IL-1) (180), and tumor-necrosis factor α (TNFα) (180, 181). Unexpectedly, soluble CTLA-4-immunoglobulin fusion protein was also found to induce IDO expression through the ligation of cell-surface CD80 (B7-1)/CD86 (B7-2) molecule (182).

IDO is differentially regulated by IFN-α and IFN-γ. Induction with IFN-γ was sensitive to cycloheximide or anisomycin (183) and to actinomycin D (184), indicating the need for synthesis of a transactivating factor. However, induction of IDO expression with IFN-α does not require synthesis of such a factor (184). Signal transducer and activator of transcription 1 (STAT1) and IFN-regulatory factor 1 (IRF1) function cooperatively to mediate the induction of IDO expression by IFN-γ (185). The mice that lack either IFN-γ or IRF1 are deficient in IDO expression during infection (186).

IDO is normally distributed at the placenta (187), epididymis, gut, lymph nodes, spleen, thymus, and lungs (188). IDO activity in these tissues was significantly increased by treatment with LPS *in vivo* (189,

190). The function of IDO in the epididymis is unknown, but the other tissues all have extensive mucosal surfaces and/or large lymphoid compartments. The presence of constitutive and inducible IDO expression in these tissues might function as an anti-inflammatory immunosuppressive factor.

C. Early Studies of IDO

The earliest studies of IDO mainly focused on its enzyme characteristics, its expression by induction, and its role in anti-microbial and anti-proliferation.

In 1936, Kotake and Masayama (191) isolated an enzyme that catalyzed conversion of tryptophan to formylkynurenine, and called it tryptophan pyrrolase. This enzyme was later named tryptophan 2,3-dioxygenase (TDO) (EC 1.13.11.11). TDO is found only in the liver, and is induced by the administration of tryptophan. Following the indication that TDO may not be the only enzyme initiating the catabolism of tryptophan by studied *in vivo* (192), in 1963, Hayashi and colleagues isolated a second enzyme that catalyzed the conversion of tryptophan into formylkynurenine (193). As this enzyme can utilize many indoleamine derivatives including L-and D-tryptophan, tryptamine, and 5-hydroxytryptophan as its substrates, this enzyme is called indoleamine 2,3-dioxygenase (IDO). Later studies revealed its presence in a range of

tissues in mice and rats (189, 194), and more recently in human (195).

Early 1970s, Hayaishi et al. reported that the purified IDO is totally inactive, even in the presence of oxygen, and requires the superoxide anion for its activity (196). IDO activity can be initiated by O_2^- added directly as potassium superoxide or generated chemically or enzymatically (172). Evidence for the involvement of O_2^- in cellular IDO activity has been provided by experiments using enterocytes that express IDO constitutively (197). Treatment of these cells with the xanthine oxidase inhibitor, allopurinol, resulted in an inhibition of IDO activity, while addition of the SOD inhibitor diethyldithiocarbamate (DDTC) or the xanthine oxidase substrate, inosine, increased IDO activity.

In the 1980s, interest shifted onto the induction of IDO expression and its biological significance. IDO is markedly induced by interferon-γ and pathological conditions in which IFN-γ production is stimulated (190, 198). This was the first hint that the physiological role of IDO might be connected with the biological effects of interferon-γ. Between 1984 and 1986, Pfefferkon and co-workers reported that the IFN-γ induced suppression of Toxoplama gondii in human fibroblasts is a consequence of the induction of IDO, which depletes tryptophan (199-201). Supplementing the medium with L-tryptophan could overcome the anti-microbial activity of IFN-γ (199, 202). Later on, three separate

research groups reported that IDO induction and tryptophan depletion may also be responsible, at least in part, for the anti-tumor effects of IFN-γ (203-205). That several pieces of evidence from *in vitro* studies with tumor cells, and the fact that the IDO level was significantly higher in the human lung tissue bearing tumor than that in the normal tissue (206), enable Takikawa and co-workers to postulate a hypothesis that tryptophan depletion caused by IDO induction may be an *in vivo* anti-tumor mechanism (207). Being consistent with this idea, they found that a marked induction of IDO occurred in tumor cells (Meth-A cells) undergoing rejection from mice (C57BL/6) (208). The IDO induction was restricted to tumor cells, while there was no change in the IDO activity of host cells infiltrated into the transplantation loci. Furthermore, they demonstrated that the induction of IDO was mediated by interferon-γ (207). Many other studies *in vitro* since that time have further implicated that IDO is associated with biostatic immune response. For example, IDO induction in vascular endothelial cell cultures via IFN-γ results in bacteriostasis of staphylococcal infection (209).

D. IDO and Immune Tolerance of Fetus During Pregnancy

Why the maternal immune system tolerates the presence of a fetus expressing alloantigen derived from the father has intrigued immunologists for many years. Medawar gave three possible explanations for the acceptance of the fetus (210). He suggested that: 1) there exists an

anatomic barrier between mother and fetus, 2) the fetus expresses no antigens, or 3) the maternal immune system is suppressed or modified. It has become evident that one and two, namely the segregation of the fetal and material circulations and the immunological immaturity of fetal tissue, do not pertain during pregnancy. The maternal-fetal interface is not an impassable barrier. Fetal cells are readily detectable in maternal circulation. Meanwhile, fetal tissue expresses MHC class I and class II and is antigenically mature (211). Research has therefore focused on the third hypothesis, that the maternal immune system somehow ignores potentially immunogenic fetal tissue.

Over the years, there have been many discoveries that have allowed us to refine our views of immune regulation during pregnancy. Expression of non-polymorphic class I molecules (HLA-G), regulatory cytokines (IL-10 and TGF-β) and Fas ligand (FASL/CD95L) all contribute to suppress T cell activation at the maternal-fetal interface in some way (212). However, the contributions by these factors in preventing fetus rejection still lack experimental evidence. In 1998, David Munn and co-workers reported a novel mechanism for maternal T cell tolerance to paternal MHC (213). The new findings add IDO to the list of potential immunosuppressive factors in pregnancy. In a series of remarkably clear experiments, the authors showed that the systemic administration of an IDO inhibitor (1-methyl-tryptophan) resulted in a T-cell mediated

rejection of allogeneic concepti. Syngeneic concepti were not affected, leading the authors to hypothesize that IDO expression at the feta-maternal interface is necessary to prevent the rejection of fetal allograft. A link between IDO expression and pregnancy success had been reported previously in humans (214), but the immunological implications of the data generated by Munn and co-workers were provocative and introduced a new area of research in reproductive immunology.

The cellular localization of IDO in placenta has also been determined (215-218). It was clear that IDO is expressed in syncytiotrophoblasts, villous endothelial cells, invasive extravillous trophoblasts, and local macrophages.

How IDO allows for a successful pregnancy is uncertain. It appears that IDO might prevent allogeneic reactions, in addition to controlling complement (219). It is known that IDO can deplete tryptophan in the microenvironment and produce catabolites that regulate the cellular immune response as discussed in detail in the section of " IDO and Immune Regulation". It has been shown that the depletion of tryptophan by IDO in macrophages could prevent T cell proliferation (220). T cells activated in a tryptophan-deficient environment were arrested in the mid-G1 phase of the cell cycle (220). The catabolites of tryptophan such as kynurenine, 3-hydroxykynurenine, and 3-

hydroxyanthranilic acid have also been shown to prevent activation and proliferation of T cells, B cells, and NK cells in vitro (221). In addition to a local effect, IDO expressed in maternal dendritic cells and macrophages might also participate in the immune regulation through inhibiting lymphocyte proliferation in local lymph nodes during pregnancy (222).

Although IDO has been strongly indicated in the response for fetal immune tolerance during pregnancy, mice in which the gene for IDO has been deleted have normal pregnancy outcomes (223), implying that compensatory or redundant immunosuppressive mechanisms protected allogenic fetuses during gestation in IDO-deficient mice. Consistent with this notion, treatment with IDO inhibitor did not affect allogeneic pregnancy rates when both parents were IDO-deficient (223). Processes that compensate for specific gene defects have been described in many mouse models. Except IDO, the placenta also expresses TDO (277). It is possible in IDO-deficiency mice, TDO might compensate for lack of IDO activity during gestation. If so, treating pregnant mice with 1-methyl-tryptophan should not block tryptophan degradation since this inhibitor is specific for IDO rather than TDO. In conclusion, IDO activity is a key mechanism that protects the allogenic fetus in normal mice, but it is not the sole mechanism, since other mechanisms can compensate for loss if IDO activity during gestation.

IDO and Tumor Tolerance

The immune system in the body can discriminate between a range of stimuli, allowing some to provoke immune responses, which leads to immunity, and preventing others from doing so, which leads to tolerance. In 1957, Burnet and Thomas postulated the existence of tumor immune surveillance: the immunological resistance of the host against the development of cancer (224). This concept has been challenged for decades primarily because of experimental data showing that nude mice, which have T-cell defects, do not have an increased incidence of tumor development (225-226). However, during the 1970s and 1990s, experimental data from several groups seemed to support the existence of tumor surveillance, and by 2003, an accumulation of new evidence proved that the immune system is able to recognize and defeat tumors (227-232).

Cellular and humoral immune responses against tumors have been detected spontaneously in the tumor-bearing host (233). On the basis of their expression pattern, the following two groups of tumor antigens have been identified: true tumor-specific antigen and tumor-associated antigen (TAA) (234). Meanwhile, T cells specific for these antigens have been shown to be present within cancer patients (235).

Since tumors contain tumor antigens and these antigens can stimulate a host immune response, why do tumors create a pathological

state of tolerance towards their own antigens? Regarding this issue, two hypotheses have been postulated: 1) T cells are ignorant, and 2) tumor microenvironment confers protection from a T cell-mediated rejection. With regard to the latter possibility, some tumor cells have been shown to express Fas ligand and therefore may induce apoptosis in Fas-expressing activated T cells. This mechanism, however, may not be relevant in most tumors (236, 237). Other tumor cells have been shown to secrete the immunosuppressive cytokine TGF-β, but once again this cytokine is not expressed by many tumors.

In considering the role of IDO in the regulation of immune responses, Mellor and Munn recently has hypothesized that IDO may be a tumor microenvironment factor that could contribute to the evasion by tumor from T cell-mediated rejection (238). To support this hypothesis, Uyttenhove et al. (239) studied the role of IDO in tumors by using the P185 mouse mastocytoma cell line. P185 normally forms lethal tumors in naïve syngeneic hosts, but it also expresses well-defined tumor antigens that facilitate the rejection of tumors by pre-immunized hosts. In this study, Uyttenhove et al. showed that transfecting IDO into P815 tumors could prevent their rejection from pre-immunized hosts, enabling the tumors to circumvent to a normally protective immune response. This immunosuppressive effect of IDO could be reversed by the administration of the IDO inhibitor 1 methyl-tryptophan. This finding is highly consistent

with Friberg et al.'s observation that 1- methyl-tryptophan significantly delays tumor outgrowth in a model of Lewis Lung carcinoma (240). These findings are in marked contrast to the previous hypothesis regarding the role of IDO expression by malignant cells, which suggested that IDO serves as an interferon-inducible autocrine growth inhibitor of tumors (241).

How does IDO expression on tumor cells cause immune suppression on host T cells? Recent studies suggest that T cells may be particularly sensitive to the negative effects of low tryptophan levels combined with tryptophan metabolite accumulation. Under these conditions, T cells fail to proliferate, and undergo cell cycle arrest in mid-G1 phase as described above. Although IDO expression by tumor cells could stall surrounding T cells by creating a local tryptophan sink, tumor cell proliferation may continue relatively unimpaired. In addition, the tumor could recruit a population of host cells that express IDO, a process conceptually analogous to the host-derived stroma and neovascularization that are also recruited by the tumor. A study by Munn et al (242) has provided the evidence that tumor-draining lymph nodes also express IDO. The ability to attract host-derived antigen presenting cells expressing IDO could confer a significant advantage on the tumor, since these cells could directly suppress an immune response in lymph nodes.

F. IDO and Immune Regulation

In vivo, endogenous IDO has been implicated in maternal tolerance toward the allogeneic fetus and tumors as described above. IDO was also been implicated in tolerance to self antigens in non-obese diabetic (NOD) mice (243); as a downstream effector mechanism for the tolerance-inducing agent CTLA4-Ig (244); as a protective negative regulator of experimentally induced autoimmune disorders (245, 246); and as an effector of asthma in mice (247) and humans (248). In humans, IDO expression has been observed by immunohistochemistry in placenta, tumor-draining lymph nodes and primary tumors as described above. Both direct and indirect evidence indicate that IDO is widely expressed throughout the immune system, and more specifically, it is localized to a subset of cells with a macrophage or dendritic cell morphology (249, 250). These IDO-expressing cells are found at several sites of immune tolerance or privilege, including the thymus, mucosa of gut, epididymis, placenta, and the anterior chamber of the eyes (188-189, 215, 249, 251). In these tissues, constant tryptophan degradation and some of its breakdown products have been implicated in the control of cellular gluconeogenesis, free radical scavenging, and serotonin synthesis (a vasoconstrictor), which are useful to defend against potential tissue inflammation (252).

To study the role of IDO in immune regulation, many researchers have focused on IDO activation in antigen presenting cells (APCs),

particularly in macrophages and dendritic cells. This is because these cells play an important role in mediating immunosuppression in various disorders, such as viral infections and autoimmunity (253).

Dendritic cells (DCs) are potent T cell stimulators, and act as sentinels against the invasion of pathogens (254). DCs can regulate the generation of Th1 and Th2 cell immunity (255). DCs are also important for the induction of peripheral tolerance (256). Unlike DCs, macrophages are capable of performing a dual role: 1) they phagocytose invading microorganisms and present processed antigens to T cells at sites of inflammation; and 2) they also phagocytose apoptotic and senescent cells in healthy tissues. Thus, macrophages present both foreign and self-antigens to CD4 T cells. It has been known that both DCs and macrophages can express IDO, which suppress T cell proliferation both in vitro and in vivo (213, 220, 257). Indirect evidence supporting that IDO expression in antigen presenting cells mediates immune regulation, comes from pharmacologic blockade of the regulatory properties of murine CD8α+ DCs in T cell mediated responses elicited in vivo (244, 258), and come from studies with IDO-transfected murine tumor cell line used as T cell stimulators and with cloned Th1 T cells used as responders (259, 260). In addition, a study conducted by Fallarino et al. (261) showed that mouse CD4+ CD25+ T cells, either resting or induced to overexpress CTLA-4 by treatment with antibody to CD3, initiated tryptophan

catabolism in dendritic cells through a CTLA-4-dependent mechanism, indicating that regulation of immunosuppressive tryptophan catabolism in dendritic cells might represent a major mechanism of action of regulatory T cells.

The downstream molecular mechanism through which IDO mediates immune suppression is still not clear. Conceptual possibilities include a direct effect on T cells, mediated either by tryptophan depletion or by cytotoxicity of tryptophan metabolites, and the effect of IDO on the antigen presenting cells.

A number of studies have demonstrated that excess tryptophan could reverse IDO-mediated inhibition of T cell proliferation (220, 245, 262-264). Consistent with these studies, *in vitro*-activated human and mouse T cells undergo cell-cycle arrest when deprived of tryptophan (220, 265). In other models, however, toxic metabolites of tryptophan seem to play a key role in mediating the immunosuppressive effects of IDO. Mouse thymocytes and mouse CD4+ cells are sensitive to apoptosis by the addition of tryptophan metabolites, such as quinolinic acid and 3-hydroxy-anthranilic acids (266). In this study, they found that Th1 but not Th2 cells are sensitive to metabolite-induced apoptosis, raising the possibility that IDO might alter the Th1/Th2 balance. This result was highly consistent with a recent study showing that IDO expressed by eosinophils

might mediate Th1/Th2 selection (248). The argument for this mechanism is that concentrations of tryptophan metabolites used in these experiments are much higher compared to the physiological levels discussed in the section of "Tryptophan Metabolism".

It is not known how T cells but not other primary cells and tumor cells sense the depletion of tryptophan. Regarding the mechanism of depleted tryptophan mediating immune suppression, two amino-acid sensitive signaling pathways have been postulated: the GCN2 stress-kinase pathway (267) and mammalian target of rapamycin (mTOR) signaling pathway (268).

The mTOR signaling pathway is required for the normal initiation of ribosomal translation. T cells seem particularly sensitive to inhibition of this pathway, as shown by the clinical use of rapamycin (an mTOR inhibitor) as a T- cell immunosuppressant (269). However, the findings from Fox and colleagues (270) indicate that inhibitors of mTOR such as rapamycin did not recapitulate the profound proliferative arrest as seen with IDO-mediated suppression.

GCN2 is one of a family of four related kinases (GCN2, PERK, HRI, and PKR), which share as their only known substrate the alpha subunit of translation initiation factor 2 (eIF2α). GCN2 and its family

members target a similar downstream pathway, referred to as the integrated stress response (ISR) pathway (271). The consequence from activating the ISR pathway depends on the nature of the stress, the specific initiating kinase, and the cell type involved. The result may be cell cycle arrest, lineage-specific differentiation, metabolic adaptation, or cell death (272-275).

In a recent study (267), Munn and co-workers showed that IDO expressing plasmacytoid DCs activate the GCN2 kinase pathway in responding T cells. In this study, they found that T cells with a targeted disruption of GCN2 were not susceptible to IDO-mediated suppression of proliferation *in vitro*. *In vivo*, proliferation of GCN2-knockout T cell was not inhibited by IDO-expressing DCs from tumor-draining lymph nodes. IDO induced profound anergy in responding to wild-type T cells, but GCN2-knockout cells were refractory to IDO-induced anergy

A third mechanism through which IDO might alter the biology of IDO-expressing APCs, has been proposed. A study conducted by Grohmann et al. (276) showed that pre-activation of CD8α+ DCs *in vitro* with IFN-γ rendered them tolerogenic when subsequently injected *in vivo*. The addition of IDO inhibitor, 1-methyltryptophan, during pre-activation of CD8α+ DCs would block the development of tolerogenic activity after cells were injected *in vivo*. The effect of IDO seemed to be exerted on the

DCs themselves, rather than on the responding T cells, since the IDO inhibitor was not present when the DCs were subsequently transferred into the recipient host. Based on this data, they postulated a novel mechanism that IDO might functionally alter the DCs, either by decreasing its APC function or by upregulating expression of suppressive ligands such as B7-1/B7-2, FasL, or by triggering the secretion of immunoregulatory cytokines such as IL-10 and TGF-β, based on this result. However, further experiments are needed to clarify this notion.

IX. Thesis Aims and Experimental Rationale

Skin substitute transplantation in which the outcome is the complete regeneration of a morphologically normal organ remains both an experimental and clinical challenge. With the anatomical and physiological improvements of cultured skin substitutes, it is hoped that one day, they will become more homologous to native skin. Improved homology may reduce the stringency for clinical use of cultured skin substitutes and accomplish the efficacy of skin autograft. Autologous materials have disadvantages in that they take many days to prepare in culture, are susceptible to infection, and are expensive to produce. Allogeneic or xenogeneic materials show promise but face the possibility of immune rejection. Several immunosuppressive genes, which modify the

donor organ or cells, have shown putative results to control graft rejection *in vitro* or *in vivo*. In the future, non-rejectable and non-expensive skin substitutes, which could be modified by chimeras or immunosuppressive genes, will provide great benefits as a form of permanent wound coverage for extensive burn patients and chronic skin ulcers patients.

Among these immunosuppressive genes, IDO has shown promising results in the inhibition of the immune response *in vitro* and *in vivo*. IDO has also been indicated in playing a key role in the immune tolerance of allogeneic fetus and tumors. However, its mechanism in the inhibition of immune response is still not completely understood. Meanwhile, the novel immunosuppressive gene has yet to be applied to organ or cell transplantation. Therefore, its efficacy in immunosuppressive function *in vivo* needs to be further studied. Here, we therefore hypothesize that IDO expression can function as a local immuno-suppressive factor to prevent allogenic engraftment by suppressing the immune cell proliferation. To address this hypothesis, the following specific aims will be examined:

The specific aims of the current study are to:

generate a recombinant adenovirus containing human IDO gene for skin cell gene modification

evaluate the efficacy and toxicity of transfection in skin cells by

recombinant adenovirus.

study the possible mechanisms of IDO in immune suppression in vitro

evaluate the effect of grafting skin substitutes containing IDO genetically modified skin cells on wound healing and neovascularization

evaluate the effect of IDO in prevention of immune rejection in xenograft model.

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Chapter 2

Expression of Indoleamine 2,3-dioxygenase in Dermal Fibroblasts Functions as a Local Immunosuppressive Factor

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I. INTRODUCTION

Extensive skin loss from a variety of conditions such as severe thermal injury is associated with significant functional morbidity and mortality (Cairns et al, 1993). In recent years, however, the overall mortality rate has been improved for patients suffering from burns due in part to the significant biotechnological advancements in skin replacement for wound closure (Cairns et al, 1993). *In vitro,* cultivation of keratinocytes with the support of a feeder layer of lethally irradiated 3T3-cells was initially introduced by Rheinwald and Green (1975). These investigators were later able to grow keratinocytes to confluence, which was suitable for grafting (Gallico et al, 1984). Although sheets of autologous keratinocytes are currently used in some burn centers to treat patients with large thermal injury (Thivolet et al, 1986; Morthenn et al, 1982), this has not been a routine procedure due to: firstly sheets of keratinocytes prepared from layers of cultured keratinocytes without matrix are very fragile and difficult to cultivate, secondly the rate of graft-take is relatively low (50-60%), thirdly patients with large injuries do not have enough of uninjured skin to be used for cell culture, lastly generating another wound increases the risk of an infection and development of hypertrophic scarring. Considering the fact that all of these factors are pervasive medical problems with far-reaching clinical and economic implications, utilizing an allogeneic and readily available skin substitute seems logical to overcome these problems. Therefore, a series of experiments have been designed and conducted by our laboratory to examine the benefit of using allogeneic

skin cells expressing indoleamine 2,3-dioxygenase as a local immunosuppressive factor.

Indoleamine 2,3-dioxygenase (IDO) is a monomeric heme-containing enzyme that catalyzes the opening of the pyrrole ring of L-tryptophan to yield N-formylkynurenine, which rapidly degrades to give kynurenine (Higuchi and Hayaishi, 1967). Interferon-gamma (IFN-γ) is a strong inducer of IDO expression in cultured fibroblasts (Dai and Gupta, 1990a), macrophages (Carlin etal, 1989), dendritic cells (Hwu et al, 2000), and many cancer cell lines (Taylor and Feng, 1991). IDO is also induced rather poorly by LPS (Yoshida and Hayaishi, 1978), interferon-α and interferon-β (Bianchi et al, 1988). It is demonstrated that IDO activity significantly increases in certain pathophysiologic conditions such as transplanted tumor cells (Takikawa et al, 1990), viral transfected lung (Yoshida et al, 1979), and viral transfected epithelial cells (Jacoby and Choi, 1994). The role of IDO in the survival of fetal allograft during pregnancy has also been explored. Munn, et al (1998) reported that the expression of IDO in the placenta is crucial in the prevention of immunological rejection of the fetal allograft. These investigators suggested that proliferation of infiltrated T cells was inhibited by IDO, as it generates a tryptophan deficient environment in the placenta. In vitro studies have also demonstrated that IDO expression by macrophages (Munn et al, 1999) and dendritic cells (Hwu et al, 2000) inhibits bystander lymphocyte proliferation.

Considering the protective role of IDO in maternal T cell mediated rejection of allogenic fetuses (Munn et al, 1998; Mellor et al, 2001) and the success of IDO in prolonging the survival of pancreatic islet cells (Alexander et al, 2002), we hypothesized that IDO expression may function as a local immune suppressive factor to protect allogenic skin substitutes. In this study, we, therefore, constructed and used recombinant adenoviral vectors bearing either GFP as a marker or GFP-IDO genes to infect dermal fibroblasts. Co-culturing IDO genetically modified fibroblasts with different types of immune cells, we demonstrated a significant increase in damage of bystander human PBMC, $CD4^+$, $CD8^+$ and B cell-riched lymphocytes, CD4 positive Jurkat cells, and THP-1 cells relative to those of controls. This bystander effect proved to be due to IDO inducing a tryptophan deficient cell culture environment. In addition, since IDO expression seems to affect only infiltrated immune, but not non-immune cells in these tissue environments, we also hypothesized that skin cells, such as keratinocytes and fibroblasts, survive and proliferate in IDO-generating tryptophan deficient environment. Using the same approach, our study demonstrated that fibroblasts would survive even after 15 days of culture in a low tryptophan environment. Both keratinocytes and endothelial cells, co-cultured with IDO-expressing cells, were also viable up to 5 days examined. These results suggest that the expression of IDO by dermal fibroblasts could function as a local immunosuppressive factor for prevention of rejection of grafted skin substitutes.

III. MATERIALS AND METHODS

A. Immune and Non Immune Cell Cultures

Following receiving informed consent, skin punch biopsies were obtained from patients undergoing elective reconstructive surgery, under local anaesthesia, according to a protocol approved by the University of Alberta Hospitals Human Ethics Committee. Biopsies were collected individually and washed three times in sterile Dulbecco's modified Eagle's medium (DMEM) (GIBCO) supplemented with antibiotic-antimycotic preparation (100u/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B) (GIBCO). Cultures of fibroblasts were established as previously described (Karimi-Busheri et al, 2002). Upon reaching confluence, the cells were released by trypsinization, split for subculture at a ratio of 1:6, and reseeded onto 75-cm^2 flasks. Fibroblasts at passages 3-7 were used in either monoculture or fibroblast/fibroblast co-culture system.

To establish cultured keratinocytes, the procedure of Rheinwald and Green (Rheinwald and Green, 1975) was used. Human foreskin keratinocytes were grown using serum-free keratinocyte medium (KSFM, GIBCO) supplemented with bovine pituitary extract (50 μg/ml) and EGF (5μg/ml).

Human umbilical vein endothelial cells (HUVEC) were isolated by collagenase digestion (Narumiya et al, 2001) and grown on gelatin-coated flask in

M199 medium (GIBCO) supplemented with 20% FBS and endothelial cell growth supplement (ECGS) (VWR) at a final concentration of 10 μg/ml.

The Jurkat cells (ATCC) and THP-1 (ATCC) were maintained in RPMI 1640 containing 10% FBS and 2 mM glutamine. Human PBMC were isolated from donor blood by Ficoll-paque^{TM} Plus (Amersham/Pharmacia Biotech) and cultured in RPMI 1640 containing 10% FBS and 2 mM glutamine. $CD4^+$, $CD8^+$ and B cell-riched lymphocytes were purified by RosetteSep Cell Enrichment Kit (Stem Cell Technologies). In brief, 4 ml of whole blood was incubated with the antibody-based enrichment cocktail at room temperature for 20 minutes. The RosetteSep antibody cocktail crosslinked unwanted cells in human whole blood to multiple red blood cells, forming immunorosettes. Following a standard buoyand density separation, either $CD4^+$, $CD8^+$ or B cell-riched lymphocytes were harvested, washed by PBS, and cultured in RPMI 1640 containing 10%FBS and 2 mM glutamine. All these cells were cultured at 37°C in an atmosphere of 5% CO_2 and 95% air. All media contained 100 units /ml penicillin and 100 units/ml streptomycin.

B. Construction of IDO Adenoviral Vectors and Measurement of IDO Enzyme Activity

Full length human IDO gene was kindly provided by Dr J.M. Carlin (Department of Microbiology, Miami University, Oxford, OH, USA). Following amplification by PCR, the IDO gene was subcloned into a shuttle vector containing a green fluorescent protein (GFP) gene according to manufacturer's instructions (Q-

Biogene). The cloned plasmid was then homologously recombined with adenoviral plasmid in *E. coli.*, BJ5183, by electroporation. The success of IDO insertion into adenoviral plasmid was confirmed by restriction endonuclease mapping. Plasmid DNA was amplified in competent DH5α bacteria and purified by CsCl gradient in an ultracentrifuge. Adenoviral vectors carrying either GFP alone or GFP plus IDO gene were linearized by PacI digestion and used to transfect 293A package cells using Fugene-6 transfection reagent (Roche). Transfected cells were monitored for GFP expression and after three cycles of freezing in an ethanol/dry ice bath and rapid thawing at 37°C, the cell lysates were used to amplify viral particles in large scale. The viral titre and multiplicity of infection (MOI) was determined in a 96-well plate according to the manufacturer's instructions. To further confirm the success of IDO gene transfection, the 293 transfected and untransfected cell lysates were prepared, DNA was extracted and the presence of IDO gene was confirmed by PCR analysis using sense (5'-GACTACAAGAAAGAGTACCA-3') and antisense (5'-TTGGGTTA CATTAACCTTCC-3') IDO gene primers.

The expression and biological activity of the IDO was evaluated by microscopic evaluation of GFP positive cells and measuring the level of tryptophan and its degraded product, kynurenine, present in conditioned medium derived from IDO and control vector transfected cells. The conditioned medium related to the same number of cells was deproteinized by adding 100 μl of acetone to 50 μl of cell culture medium. It was vortexed for 30 seconds, cooled on ice for 5 min and centrifuged at 13000 rpm at 4 ^{0}C for 15 min. The supernatant of each sample received 10 μl of 0.25

M $HClO_4$ and IS (4-amino-hippuric acid) solution and then the mixture was incubated at 25 ^{0}C for 20 min. The mixture was evaporated until dry under a vacuum. 100 μl of 14 %BF_3-Propanol was then added and the mixture was heated at 90 ^{0}C for 2 hrs. The vacuum dried material was dissolved in 40 μl of 20 % methanol in water and analyzed by liquid chromatography with electrospray mass spectrometry (LC/MS) using a Hewlett Packard series 1100 mass selective detector controlled by 1100-MSD Chem. Station. The mass spectrometer was operated in positive ion mode. Tryptophan and kynurenine quantitative analyses were performed in the selected ion-monitoring mode.

C. Detection of IDO Protein by Western Blotting

For detection of IDO expression, non- (C), Ad-GFP- (V) or Ad-GFP-IDO- (IDO) tranfected cells were harvested after 72 hr post infection and washed twice with PBS. Cells were then lyzed in lysis buffer [50 mM Tris-HCl, pH 7.4, 10 mM EDTA, 5 mM EGTA, 0.5% NP40, 1% Triton X-100, and protease inhibitor cocktail (Sigma)]. Cell lysate was centrifuged at 14,000 rpm for 15 min. The protein concentration of supernatant was measured with a BCA protein assay kit (Pierce). Equal amounts of total protein were separated by 12% SDS-PAGE and immunoblotted with our recently raised rabbit polyclonal IDO antibody at final concentration of 1:5000. Horseradish peroxidase-conjugated goat anti-rabbit IgG served as a secondary antibody for the enhanced chemiluminescence detection system (ECL; Amersham).

D. Viral Infection and Cell Co-culture System

Human fibroblasts, keratinocytes, and endothelial cells were separately infected with either Ad-IDO or control viral vectors for 30 hrs at a MOI of 2000, 100 and 100, respectively. The expression of IDO was confirmed by: 1) monitoring GFP expression under fluorescence microscopy (Nikon, HB-1010 AF), 2) measuring the level of kynurenine in conditioned medium and, 3) the detection of IDO protein expression using western blot. Infected cells were then washed twice with PBS to remove the viral particles and then sub-cultured in 6-wells plates for co-culture assays. Fibroblasts and immune cells were co-cultured into a two-chamber culture plate in which 0.4 μm insert (Millipore) separates the upper (fibroblasts) chamber from the bottom (immune cells). A RPMI 1640 culture medium containing 10% FBS and 2 mM glutamine, a suitable culture medium for both immune cells and fibroblasts, was used in this co-cultured assay. Immune cells were harvested at the different time points. Similarly, using a two-chamber co-culture system, either Ad-GFP, or Ad-IDO-GFP infected cells were co-cultured with non-infected either keratinocytes or fibroblasts or endothelial cells for different durations. Cells (non-infected cells) from bottom chambers were harvested, stained and subsequently evaluated by FACS analysis.

E. Interferon Treatment

In another approach to induce IDO expression in dermal fibroblasts (Sarkhosh et al, 2003), cells were treated with 1000 units/ml of IFN-γ (Gibco) or vehicle alone for 40 hrs to stimulate the expression of IDO to its maximal level. The cells were then

washed twice with PBS to remove the IFN-γ and co-cultured with Jurkat cells for another 4 days. The IDO activity in fibroblasts induced by interferon was monitored by measuring the level of kyrurenine in conditioned medium as previously described.

F. PI staining and Flow-cytometric Analysis

Harvested cells were stained with 10 μg/ml of PI (Sigma) for 10 min. Stained cells were centrifuged and washed twice with fresh medium. Fluorescence microscopy (Nikon, HB-10101AF) was used to monitor the pattern of PI stained cells and flow cytometry (FACS) was used to quantify the number of PI positive cells.

G. Terminal Deoxynucleotidyl Transferase (TdT)-Mediated dUTP-Biotin Nick End Labelling (TUNEL)

Cyto-spun cells were fixed with acetone:methanol (1:1) for 10 min at room temperature, washed three times with PBS, then subjected to TUNEL assay. The fixed cells were incubated in TdT buffer containing 30 mM Trizma base, pH 7.2, 140 mM sodium cacodylate, and 1 mM cobalt chloride for 10 min at room temperature. A mixture of bio-16-dUTP (Sigma) at 16.5 μM dATP (Boehringer Mannheim), and TdT enzyme at 5 U/ml (Boehringer Mannheim) in TdT buffer was added and the reaction was allowed to proceed for 60 min at 37°C. The reaction was terminated by addition of 2X SSC (300 mM sodium chloride plus 30 mM sodium citrate). After washing the cells three times in ddH_2O, endogenous peroxidase activity was quenched in a 3% H_2O_2 solution for 10 min at room temperature. Cells were rinsed with PBS and non-specific binding was reduced by incubation of cells with blocking buffer (3% skim

milk powder in PBS containing 0.5 % Tween 20) for 15 min. The cells were incubated for 30 min at room temperature with ExtrAvidin peroxidase (Sigma) diluted 1:50 in blocking buffer. Cells were washed 3 times with ddH_2O, and then stained with aminoethyl carbizole at room temperature until a red colour appeared.

H. Cell Proliferation of IDO Expressing Skin Cells

[^{3}H] thymidine incorporation was employed to determine and compare the proliferation rate between IDO and non- IDO expressing cells. Fibroblasts and keratinocytes were infected with either nothing (control), Ad-GFP (Vector) or Ad-GFP-IDO (IDO) for 30 hrs. Free viral particles were removed by washing with PBS and cells were allowed to grow until day 3 or 6. Cells then received 1 μCi/ml of [^{3}H] thymidine (Amersham/Pharmacia Biotech) for 16 h, harvested and washed 3 times with PBS. The radioactivity was determined by scintillation counting.

I. Statistics

All values are presented as mean ± SD. The comparisons of cell proliferation between IDO expressing cells and non-IDO expressing cells were assessed using the non-parametric Fisher's exact test. A p-value of < 0.05 was considered as statistically significant.

IV. RESULTS

A. Adenoviral Vector Infected Cells Expressed IDO and GFP Proteins

To distinguish the IDO transfected cells from untransfected cells and to evaluate the level of IDO expression in these cells, we employed an adenoviral vector bearing the GFP gene, as a reporter gene, to construct a human IDO plus GFP recombinant adenoviral vector. As shown in the schematic diagram Fig. 1A, the CMV5 promoter drives the expression of either GFP in control or IDO plus the GFP in the recombinant IDO vector. Recombinant human IDO gene and its correct orientation has been confirmed by serial restriction enzyme digestions and mapping (data not shown). A PCR analysis of the viral DNA product confirmed the successful transfection of IDO recombinant gene in packaging 293 cells (Fig.1B). Microscopic evaluation of Ad-GFP (Fig.1Ca) and Ad-IDO-GFP(Fig.1Cb)l infected 293 cells showed a bright green fluorescent stained, indicating GFP expression in these cells.To further validate the biological activity of IDO protein, the levels of tryptophan and its IDO generated degraded product, kynurenine, in conditioned medium derived from Ad-IDO infected and non-infected 293 cells, was evaluated by LC-MS. As shown in Fig. 2, the level of tryptophan (Panel A) was almost undetectable in conditioned medium obtained from Ad-IDO infected cells relative to that of either non-infected or Ad-GFP infected controls; while the level of kynurenine (Panel B) was more than 20 fold higher in the same conditioned medium.

In a similar experimental setting, fibroblasts, keratinocytes and endothelial cells were separately infected with mock or IDO recombinant adenovirus and evaluated for efficacy of infection. As shown in Fig. 3A, after 72 hr post infection, more than 90% keratinocytes and endothelial cells were successfully

transfected and were positive for GFP protein. However, fibroblasts had lower efficacy of Ad-GFP- IDO transfection compared to the other two cell types used. The number of GFP positive fibroblasts was about 30-50% as evaluated by FACS analysis (data not shown). To confirm these findings, the expression of IDO protein in Ad-GFP-IDO infected fibroblasts, keratinocytes, and endothelial cells harvested at 72 hr post infection was analyzed by western blot using rabbit polyclonal anti-human IDO. As shown in Fig. 3B, an anti-IDO antibody immunorecognized a 42 kDa band in IDO recombinant adenovirus infected cells. The size of protein was consistent with the expected molecular weight of human IDO (Dai and Gupta, 1990b). Consistent with IDO protein expression, a significant increase in the levels of kynurenine, used as an index for IDO activity, was found in only Ad-GFP-IDO infected fibroblasts and keratinocytes (data not shown).

B. IDO Expression Mediates Bystander Immune Cell Apoptosis

To examine the mediating role of IDO expression in bystander immune cell damage, different types of immune cells such as human PBMC, $CD4^+$, $CD8^+$ and B cell riched cells as well as $CD4^+$ Jurkat cells, THP-1 monocytes as non-IDO expressing bystander cells were employed. In these experiments, IDO infected human dermal fibroblasts were used as IDO expressing cells, and non-adenoviral infected and Ad-GFP infected fibroblasts were used as controls. After 30h of fibroblast infection and following confirmation of IDO and GFP expression on day 4 postinfectection, cells were co-cultured with immune cells. After 3-5 days, bystander

co-cultured immune cells were then harvested and evaluated for PI staining by FACS analysis. As shown in Fig. 4A, almost all immune cells used are sensitive to IDO induced low tryptophan environment. The highest number of PI positive cells was found in the $CD4^+$ lymphocyte population (30%) relative to either PBMC (18%), $CD4^+$ (16%) and B cell rechied (16.4%) population. The number of PI positive in either of these immune cell types was the same when they were co-cultured with either non- or AD-GFP infected cells. Similarly, the number of PI positive Jurkat and THP-1 cells was more than 16 fold (82.7% vs 5.2%) and 8 fold (35.8% vs 3.9%), respectively, higher in cells co-cultured with Ad-IDO infected fibroblasts compared to those co-cultured with mock infected fibroblasts (Fig. 4B).

To further demonstrate that IDO induction is the main cause of bystander immune cell damage, IFN-γ as a strong IDO inducer (Dai and Gupta, 1990) was used to treat dermal fibroblasts as a source of IDO induction. The induction of IDO mRNA expression was confirmed by Northern analysis; while the activity of IDO was monitored by measuring the level of kynurenine in condition medium by LC-MS. After confirmation of IDO induction and activity, fibroblasts were co-cultured with CD4+ Jurkat cells for another 4 days. Unattached Jurkat cells were then harvested and stained with PI. The number of PI positive cells were evaluated by FACS analysis. As shown in Fig. 4C, the number of PI positive Jurkat cells co-cultured with IFN-γ pretreated fibroblasts increased more than 25 fold (77 % vs 3 %) relative to those co-cultured with IFN-γ untreated fibroblasts. This result thus provides further evidence that IDO expression causes bystander immune cell damage.

To investigate whether the mediating role of IDO in bystander immune cell damage is due to apoptosis, PI staining; TUNEL assay and nuclear fragmentation were used. The results of the microscopic PI stained cells revealed a typical nuclear fragmentation, a hallmark of apoptosis, in Jurkat cells co-cultured with IDO expressing fibroblasts for 3 days (Fig. 5 A). Consistent with PI staining results, the finding of TUNEL assays demonstrated a large number of apoptotic Jurkat cells co-cultured with either IFN-γ pre-treated fibroblasts (Fig. 5Bb) or Ad-IDO infected fibroblasts (Fig. 5Cc). In contrast, no sign of apoptotic cells was found in Jurkat cells co-cultured with either non-infected cells (Fig. 5Ba and Fig.5Ca) or Ad-GFP infected cells (Fig. 5Cb). Furthermore, a typical DNA ladder, as another index for apoptosis, was also seen in Jurkat cells co-cultured with IDO expressing fibroblast (data not shown). These results collectively suggest that IDO expression is responsible for the apoptosis of bystander immune cells in our co-cultured system.

C. Addition of Tryptophan and IDO Inhibitor Restored Immune Cell Damage Induced by IDO.

To test the possible mechanism through which IDO mediates bystander cell damage, we examined the effect of tryptophan and its degraded product, kynurenine to see whether the expression of IDO induced immune cell damage is due to kynurenine toxicity or tryptophan depletion. The results showed that an addition of kynurenine at various concentrations ranging from 40 to 400 μM to the conditioned medium of cultured THP-1 cells and Jurkat cells has no cytotoxic effect on these

cells. However, an addition of L-tryptophan to conditioned medium of either of these cell types markedly decreased bystander cell damage. As shown in Fig. 6, the number of PI positive Jurkat cells and THP-1 co-cultured with IDO expressing fibroblasts markedly reduced from 83 % to 13% and 33% to 3%, respectively. To confirm these results, a specific IDO inhibitor, 1-methyl D-tryptophan, was added to conditioned medium at a concentration of 800μM. As expected, the number of PI positive Jurkat and THP-1 cells co-cultured with IDO expressing fibroblasts markedly reduced from 82% to 25% and from 33% to 5.4%, respectively.

D. Skin Cells and Endothelial Cells are Resistant to IDO Induced Low Tryptophan Culture Environment

To assess the mediating role of IDO expression in bystander primary skin cells and endothelial cells, either Ad-GFP or Ad-GFP-IDO infected keratinocytes, endothelial cells (Fig. 7A) or fibroblasts (Fig. 7B) were co-cultured with its corresponding non-infected cells, respectively. Cells from the bottom chambers (non-infected cells) were harvested and stained with PI. Positive cells were determined by FACS analysis. As shown in Fig. 7A, only 2.6% of keratinocytes grown in IDO induced tryptophan depleted medium were PI positive after 5 days of culture. Similarly the number of PI positive endothelial cells (Fig. 7A) and fibroblasts (Fig. 7B) grown in the same environment for 5 days was less than 3.5%. When this duration was extended to 15 days for dermal fibroblasts, no significant difference in numbers of PI positive fibroblasts between Ad-GFP and Ad-GFP-IDO infected cells was found (Fig. 7B). These results reveal that primary

skin cells such as keratinocytes and fibroblasts as well as endothelial cells are resistant to IDO generated low tryptophan environment, at least, for the indicated durations examined.

E. IDO does not Alter Proliferative Capacity of Infected Fibroblasts and Keratinocytes

To examine whether IDO expression influences the proliferative capacity of IDO expressing cells, either fibroblasts (Fig. 8A) or keratinocytes (Fig. 8B) were infected with either nothing (column C), Ad-GFP (column V) or Ad-GFP-IDO (column IDO). Cell proliferation was then evaluated by [^{3}H] thymidine incorporation after 3 and 6 days post infection. As shown in Figure 8A, infected fibroblasts showed less [^{3}H] thymidine incorporation at both time points examined relative to those of non-infected cells (column C, $p< 0.05$). Similarly, infected keratinocytes (Fig 8B) also showed less [^{3}H] thymidine incorporation at day 3 ($p<0.05$). However, the rate of cell proliferation between infected and non-infected keratinocytes did not show a significant change at day 6 ($p>0.05$). Compared with Ad-GFP infected either fibroblasts or keratinocytes, proliferation of Ad-GFP-IDO infected fibroblasts was higher at both time points ($P<0.05$) and higher for IDO expressing keratinocytes at day 3 ($p<0.05$). These results may imply that adenovirus, but not IDO expression, is responsible for the suppression of IDO expressing fibroblast and keratinocyte proliferation.

DISCUSSION

Considering the fact that an ultimate goal of any tissue engineering related study is to explore the possible approaches through which the clinical complications of both non-healing and over-healing wounds are improved, there is a need to develop a non-rejectable and readily available skin substitute. This skin substitute would function as a wound coverage to prevent or reduce heat and fluid loss, and to prevent wound infection. The skin substitute should also function as a wound coverage through which dermal-epidermal wound healing modulating factors are released locally to facilitate granulation tissue formation, re-epithelialization and improve closure of non-healing wounds, such as those seen in the diabetic patients. Different approaches have been attempted for temporary or permanent coverage of wounds. Synthetic dressings (e.g. Integra and Dermagraft artificial skin) and biological dressings (e.g. Alloderm and Apligraf artificial skin) have been developed for the last 30 years (Philips 1998). Two products possessing allogeneic components are commercially available. Dermagraft consists of living allogeneic dermal fibroblasts grown on degradable scaffold. Apligraf is the living allogeneic bilayered construct containing keratinocytes, fibroblasts and bovine collagen. Although, these skin substitutes have not been shown to be immunogeneic by limited clinical data, the potential risks of acute and chronic rejection of allograft need to be further evaluated (Briscoe, et al, 1999; Badiavas, et al, 2002). Previous studies have shown that even in the absence of passenger leukocytes and langerhans cells, cultured allogeneic skin cells used as wound a coverage were rejected (Rouabhia et al, 1993; Aubock et al, 1988; Phillips, 1991). Furthermore, a recent study by Lamme et al (2002)

demonstrated that allogeneic fibroblasts in a dermal skin substitute induce inflammation and scar formation. Engineered skin substitutes using the patient's skin cells are far more desirable. However, it is difficult to perform an autologous engraftment for patients who suffer from extensive skin loss from a variety of conditions such as large and severe thermal injury due to limited amount of uninjured tissue. This is also true for diabetic, elderly and immune-compromised patients who suffer from non-healing complications. Therefore, exploring an allogeneic, non-rejectable and readily available skin substitute may provide a better means of improving wound healing. As rejection is a major obstacle in any type of grafting, the current study seeks a novel approach through which local induction of immunosuppressive factors, such as indoleamine 2, 3-dioxygenase (IDO), a tryptophan catabolizing enzyme, generates a tryptophan-deficient microenvironment in which immune cells are unable to survive.

As a part of a series of experiments, here, we constructed two human adenoviral vectors bearing either GFP as a reporter gene or IDO plus GFP genes and demonstrated that dermal fibroblasts can successfully be infected and the resultant IDO is catalytically active in degradation of tryptophan, an essential amino acid required for protein synthesis. In a co-culture system, the IDO expression in dermal fibroblasts generated a tryptophan deficient environment in which the majority of immune cells such as PBMC, $CD4^+$, $CD8^+$, B cells, Jurkat cells and THP-1 monocytes were unable to survive. As the same adenoviral vector (Ad-GFP vector) was used as a negative control, any alteration in immune cell damage should be due to the expression of IDO itself. The number of PI

positive Jurkat cells co-cultured with IFN-γ pretreated fibroblasts increased more than 25 fold relative to those co-cultured with IFN-γ non-treated fibroblasts. This result provides further evidence that it is IDO expression and not adenoviral components that causes bystander immune cell damage.

A role for cell expressing IDO in inhibiting immune responses *in vivo* is consistent with that previously reported in murine pregnancy, grafted IDO expressing islets and IDO expressing tumor cells injected into mice (Munn et al, 1998; Alexander et al, 2002; Mellor et al, 2002). In this regard, it has been hypothesized that IDO expression by either trophoblasts and macrophages in placenta, or IDO transfected islets and tumor cells, helps to maintain peripheral tolerance through regulating allogeneic T cell response. Indeed, *in vitro* studies showed that monocyte-derived macrophages exposed to macrophage colony-stimulating factor (MCSF) acquire the capacity to suppress T cell proliferation by inducing IDO expression (Munn et al, 1999). Similarly, Hwu et al (2000) reported that the proliferation of OKT3-stimulated autologous T cells is also inhibited by IDO producing dendritic cells. These, as well as similar findings obtained with IDO recombinant adenovirus transduced dendritic cells (Terness et al, 2002) support the hypothesis that IDO expression by antigen-presenting cells is responsible for suppression of "unwanted" T cells. However, during pregnancy, IDO expression is restricted to the maternal-fetal interface (Sedlmaryr et al, 2002). Similarly, the expression of IDO in genetically modified islets and tumor cells is also found locally. These findings suggest that local expression of IDO by skin cells is likely to function as a local immunosuppressive

factor for infiltrated immune cells at the wound site. Others have suggested that tryptophan deprivation causes activated T cell apoptosis induced by expression of FasL (Lee et al, 2002). It is also reported that a tryptophan metabolite, 3-hydroxyanthranilic acid induces THP-1 and U937 cell apoptosis (Morita et al, 2001). Collectively, these results indicated that bystander immune cell damage induced by IDO maybe a key contributor for IDO mediating immune tolerance.

To gain insights into the mechanisms of IDO-induced apoptosis of immune cells, we evaluated the effects of an addition of tryptophan and its catabolite, kynurenine, in conditioned medium and demonstrated that an addition of tryptophan restored the bystander effects of IDO expression for co-cultured immune cells. In contrast, an addition of kynurenine to conditioned medium failed to induce immune cell damage. This is consistent with previous studies indicating that depletion of tryptophan is involved in human IFN-γ mediated apoptosis (Konan and Taylor, 1996). Furthermore, we have demonstrated that IDO inhibitor at a concentration previously reported (Sarkhosh et al, 2003) also prevents the mediating effect of IDO in bystander cell damage. Collectively, these results suggest that depletion of tryptophan, but not kynurenine toxicity, is the main mechanism by which IDO expression induces bystander immune cell damage.

Although, we have demonstrated that IDO mediated activated immune cell damage via tryptophan depletion of culture environment, the degree of cell sensitivity to low tryptophan environment seems to vary from one cell strain to another. As

shown in figure 4, the number of PI stained $CD4^+$ Jurkate cells co-cultured with IDO expressing fibroblasts was markedly higher than those of THP-1 monocyte cells. Similarly, $CD4^+$-riched lymphocytes were more sensitive to a tryptophan deficient environment compared to $CD8^+$ and B cell-riched lymphocyte population. It is not clear why there is a great variation in sensitivity of these immune cells to low tryptophan environment. It seems that high proliferating immune cells such as Jurkat cells are more sensitive to tryptophan deficient environment than other cells. It should be emphasized that primary skin cells such as dermal fibroblasts, keratinocytes and endothelial cells seem to be more resistant to low tryptophan than immune cells (Fig. 7). As the success of making a non-rejectable skin substitute depends on skin cell survival in IDO generated low tryptophan environment, it would be encouraging to see that primary skin cells, but not immune cells, are resistant to IDO induced tryptophan deficient environment. Consistent with this data, our preliminary *in vivo* experimental data using a fibroblast embedded collagen as wound coverage clearly showed that the tryptophan deficient environment generated by IDO is not toxic to the normal wound healing processes. Surprisingly, grafting IDO-expressing fibroblasts embedded in a collagen gel would accelerates wound healing and closure compared to untreated wounds, untreated fibroblasts and adenoviral vector infected fibroblasts embedded collagen gel. The feasibility of usage of IDO transfected skin cells embedded collagen gel as wound a coverage is under our current investigation.

In conclusion, we constructed an adenoviral vector bearing IDO gene and for the first time demonstrated that dermal fibroblasts expressing IDO generated a

tryptophan deficient microenvironment in which majority of immune cells were unable to survive. Further we also showed that primary skin cells are less sensitive to IDO generated tryptophan deficient environment. This differential sensitivity seen between immune and primary cells would make our attempt easier in preparing a non-rejectable allogeneic skin substitute to be used not only as a wound coverage, but also as a rich source of wound healing promoting factors. Thus, the finding of this study just initiates a new approach through which the feasibility and benefit of a local immunosuppressive factor such as IDO in development of a non-rejectable skin substitutes is evaluated.

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VI. FIGURES AND FIGURE LEGENDS

Figure 1

Schematic Diagram of Constructed Human IDO, Its PCR Gene Product and Microscopic Appearance of GFP Expression. IDO was initially cloned into a shuttle vector, pAdenoVator-CMV5-IRES-GFP, The constructed plasmid was then linearized by PmeI restriction endonuclease and inserted into an adenoviral backbone plasmid, pAdenoVator △E1/ △E3, and used to transform E. coli BJ5183 cells (Panel A). Finally the linearized recombinant IDO plasmid was used to transfect adenoviral packaging 293 cells using Fugene-6 transfection kit. After 10 days, a high titer of IDO adenoviral particles was confirmed in 293 cells and the conditioned medium of transfected cells was used to infect the other cell strains. Panel B shows the PCR product detected in IDO infected 293 cells using PCR procedure and specific IDO primers described in the Materials and Methods. Panel C shows microscopic appearance of GFP-expression in 293 cells infected at a MOI of 5 with either Ad-GFP (Panel Ca) or Ad-GFP-IDO (Panel Cb) for 3 days.

Figure 2

Tryptophan and Kynurenine Levels in Conditioned Medium of Cultured 293 Cells with or without Recombinant Adenoviral Infection. To examine the IDO enzyme activity, the conditioned medium derived from either non-infected, Ad-

GFP, or Ad-IDO-GFP infected cells was collected and evaluated for the levels of either tryptophan (Panel A) or kynurenine (panel B) using the LC/MS procedure described in the Materials and Methods.

Figure 3

A- GFP Expression in Fibroblasts (upper panel), Keratinocytes (medium panel), and Endothelial Cells (lower panel) after 72 hrs Infection with either Ad-GFP (left panel) or Ad-IDO-GFP (right panel). Fibroblasts, keratinocytes, or endothelial cells were infected with either Ad-GFP or Ad-IDO-GFP at indicated MOI of 2000, 100, 100, respectively. The extent of infection was monitored by GFP expression under fluorescence microscopy. Magnification 100×.

B- Western Blot Analysis of IDO Expression. Fibroblasts, keratinocytes, or endothelial cells were infected with either Ad-GFP, or Ad-IDO-GFP for 72 hrs. Non-infected and infected cells were harvested, and cell lysates were fractionated by SDS-PAGE. IDO protein was detected using a polyclonal antibody from rabbit immunized with a purified recombinant IDO protein. Arrow shows a 42 kDa band corresponding to the IDO protein.

Figure 4

FACS Analysis of PI Positive Bystander Immune Cells.

A-Non-viral infected (control) and pre-infected fibroblasts with either an empty vector (Ad-GFP) or IDO adenoviral vector (Ad-GFP-IDO) were co-cultured with either human PBMC, $CD4^+$, $CD8^+$ or B cell-riched immune cells for 5 days, respectively. The immune cells were then harvested and stained with 10 μg/ml of PI for 10 min and analyzed by FACS.

B-Non-viral infected (control) and pre-infected fibroblasts with either an empty vector (Ad-GFP) or IDO adenoviral vector (Ad-GFP-IDO) were co-cultured with either CD4 + Jurkat cells or THP-1 monocytes for 3 days, respectively. The bystander immune cells were then harvested and stained with 10 μg/ml of PI for 10 min and analyzed by FACS.

C-CD4 + Jurkat cells were co-cultured with either non-treated fibroblasts or INF-γ pre-treated fibroblasts for 4 days. Jurkat cells were then harvested and stained with 10 μg/ml of PI for 10 min and analyzed by FACS.

Figure 5

PI Positive Cell Microscopic Appearance and Apoptosis of Bystander Immune Cells. To evaluate nuclear fragmentation of apoptotic bystander immune cells (Panel A), IDO expressing cells were co-cultured with Jurkat cells for 3-4 days. Jurkat cells were then harvested and stained with 10 μg/ml of PI. The PI positive cells were visualized under fluorescent microscope (original

magnification × 400). Panels Aa and Ab are Jurkat cells co-cultured with either INF-γ treated fibroblasts or Ad-GFP-IDO transfected fibroblasts, respectively.

For TUNEL assay, Jurkat cells were co-cultured with either non-treated fibroblasts (Panel Ba) or IFN-γ pre-treated fibroblasts (Panel Bb) for 4 days. Junkat cells were harvested and then subjected to TUNEL assay to determine the apoptotic cells. In a similar experiment (Panel C), Jurkat cells were co-cultured with either non-infected (a), Ad-GFP infected (b), or Ad-GFP-IDO infected (c) fibroblasts for 3 days and then subjected to TUNEL assay.

Figure 6

Addition of Tryptophan Prevents the IDO Induced Apoptosis of Bystander Cells. Jurkat cells (Panel A) or THP-1 monocytes (Panel B) were co-cultured with Ad-IDO infected fibroblasts in the presence (right panel) and absence (left panel) of 250 μM of L-tryptophan for 3 days. The immune cells were then harvested, stained with 10 μg/ml of PI, and the number of PI positive cells was determined by FACS analysis.

Figure 7

FACS Analyses of PI Positive Bystander Skin Cells and Endothelial Cells. Either keratinocytes (panel A, upper panel) or endothelial cells (panel A, lower panel) were co-cultured with either Ad-GFP (left) or Ad-IDO-GFP (right) infected cells for 5 days in a two-chamber co-culture system. The non-infected

cells from bottom chambers were harvested and stained with PI. FACS analysis was used to determine the number of PI positive cells. In the similar experimental conditions, Foreskin human fibroblasts (panel B) were co-cultured with pre-infected fibroblasts by either Ad-GFP control (left) or Ad-IDO-GFP (right) for 5, 10, and15 days. The non-infected fibroblasts were harvested and stained with PI for FACS analysis.

Figure 8

Effect of IDO on Proliferative Capacity of Fibroblasts and Keratinocytes. Fibroblasts (panel A) were infected with either Ad-GFP (V), or Ad-IDO-GFP (IDO) at MOI of 2000. After 30 hrs, free viral particles were removed by washing with PBS and incubated up to day 3 or 6 post infection. After addition of 1 μci/ml of [^{3}H] thymidine, cell proliferation was determined and compared to the proliferation of non-infected control cells (C). Similarly, keratinocytes (panel B) were infected with either Ad-GFP or Ad-IDO-GFP at MOI of 100 for 30 hrs. Cell proliferation was determined at day 3 and day 6. Each graph is a representative experiment of four repeats. Values presented are means ± SD for 4 samples. * $p<0.05$, control (C) vs either vector alone (V) or Ad-GFP-IDO (IDO).

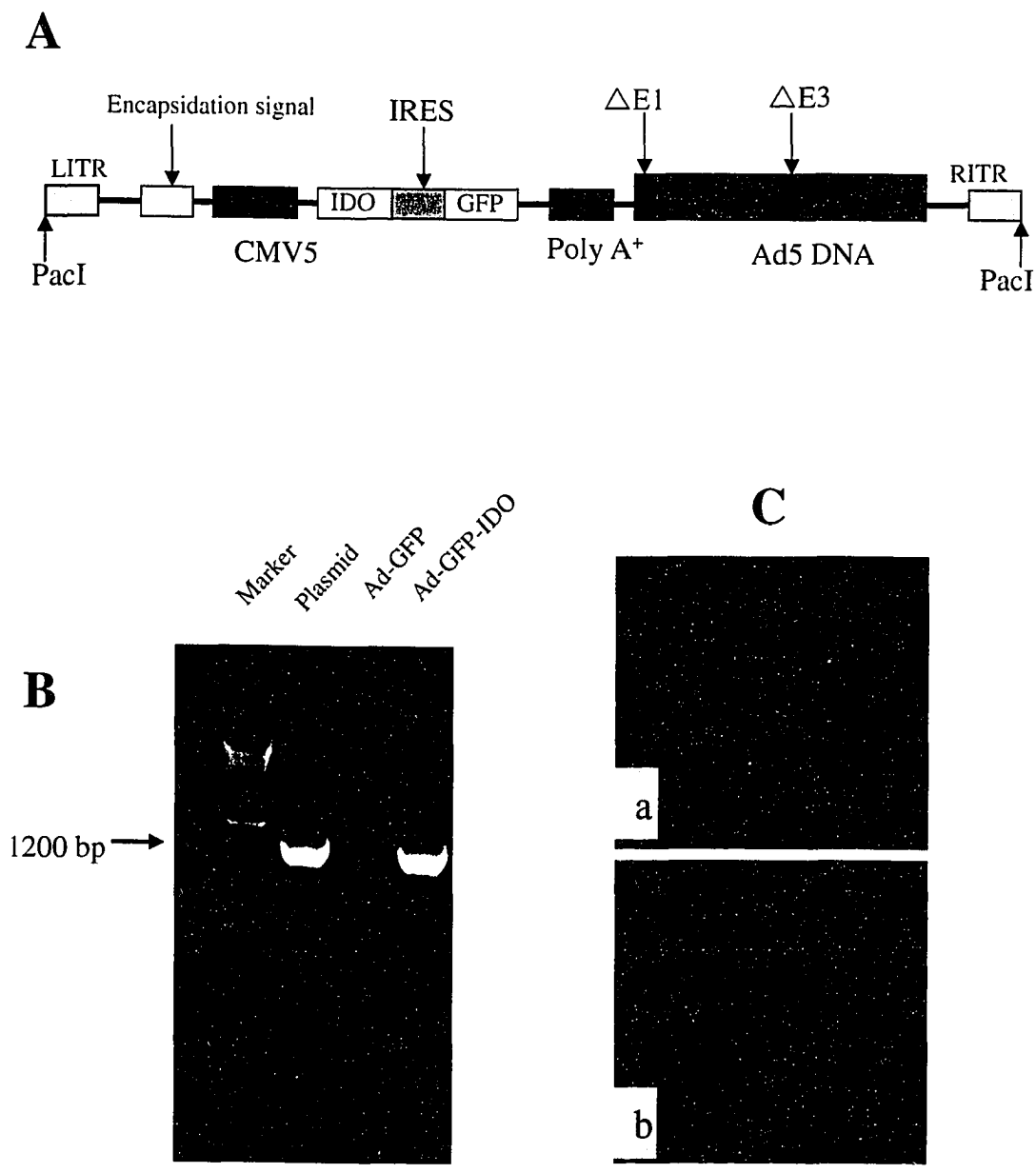
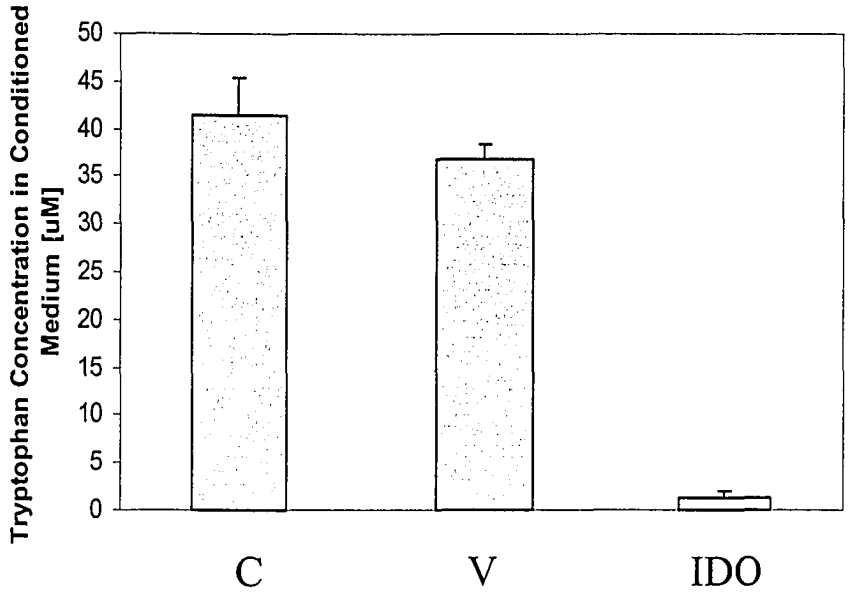


Fig. 2-1

A



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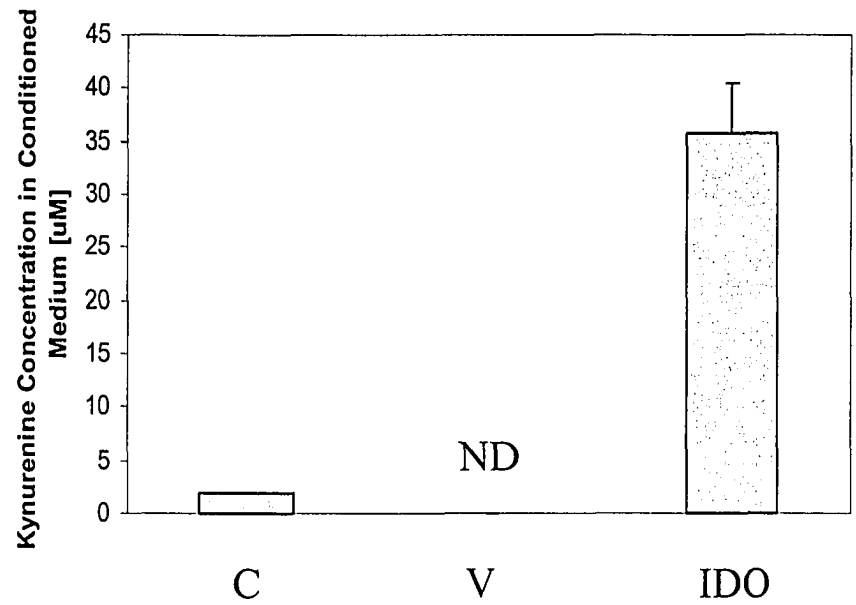


Fig. 2-2

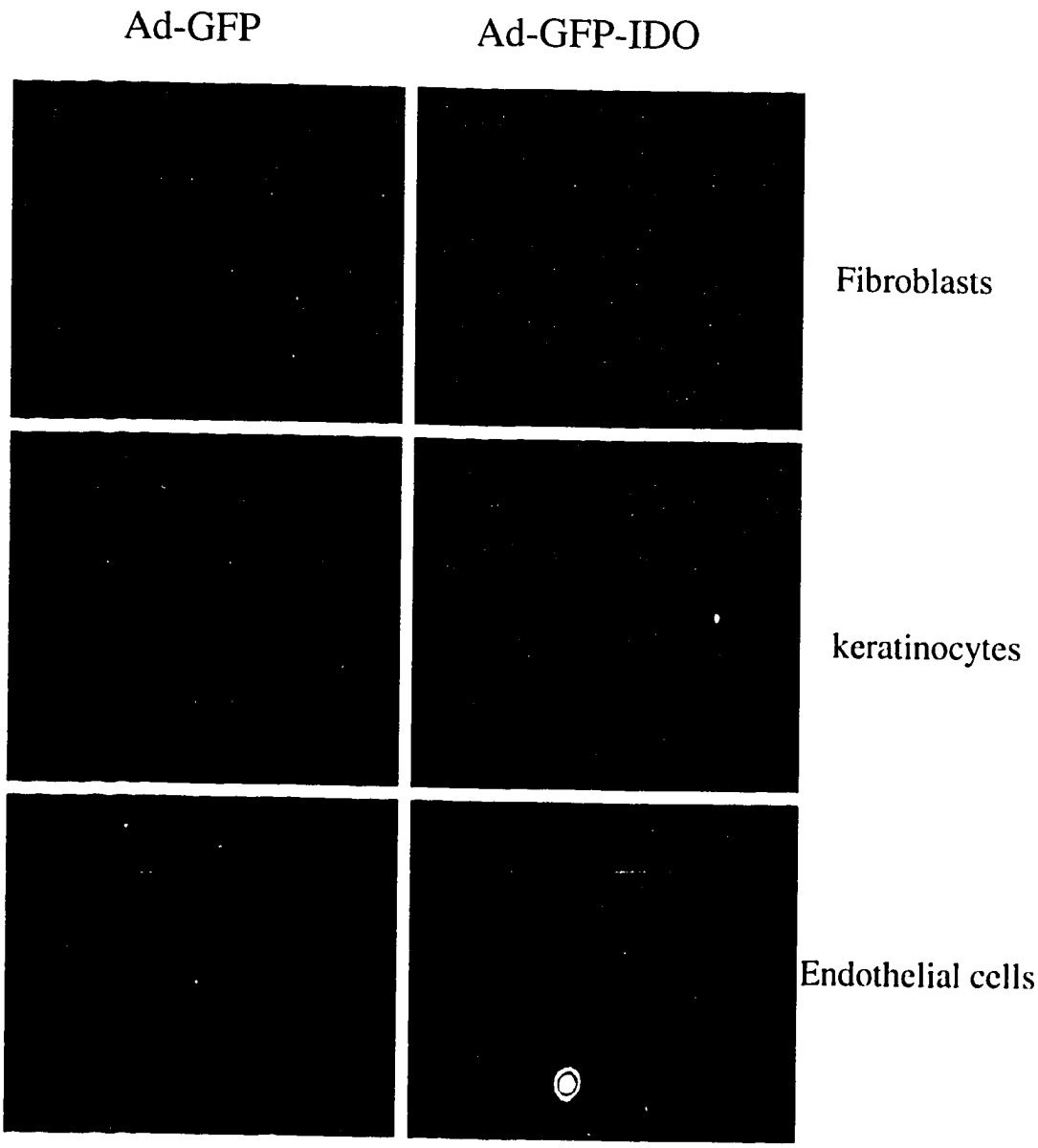


Fig. 2-3A

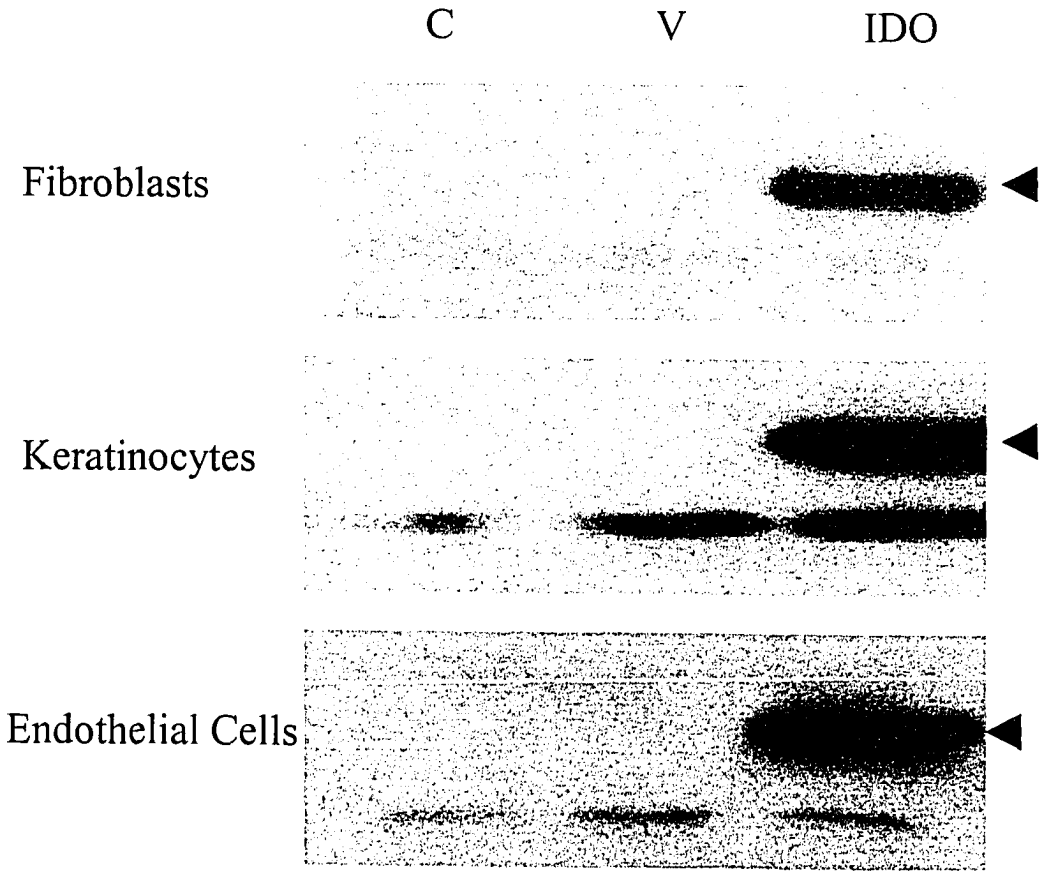


Fig. 2-3B

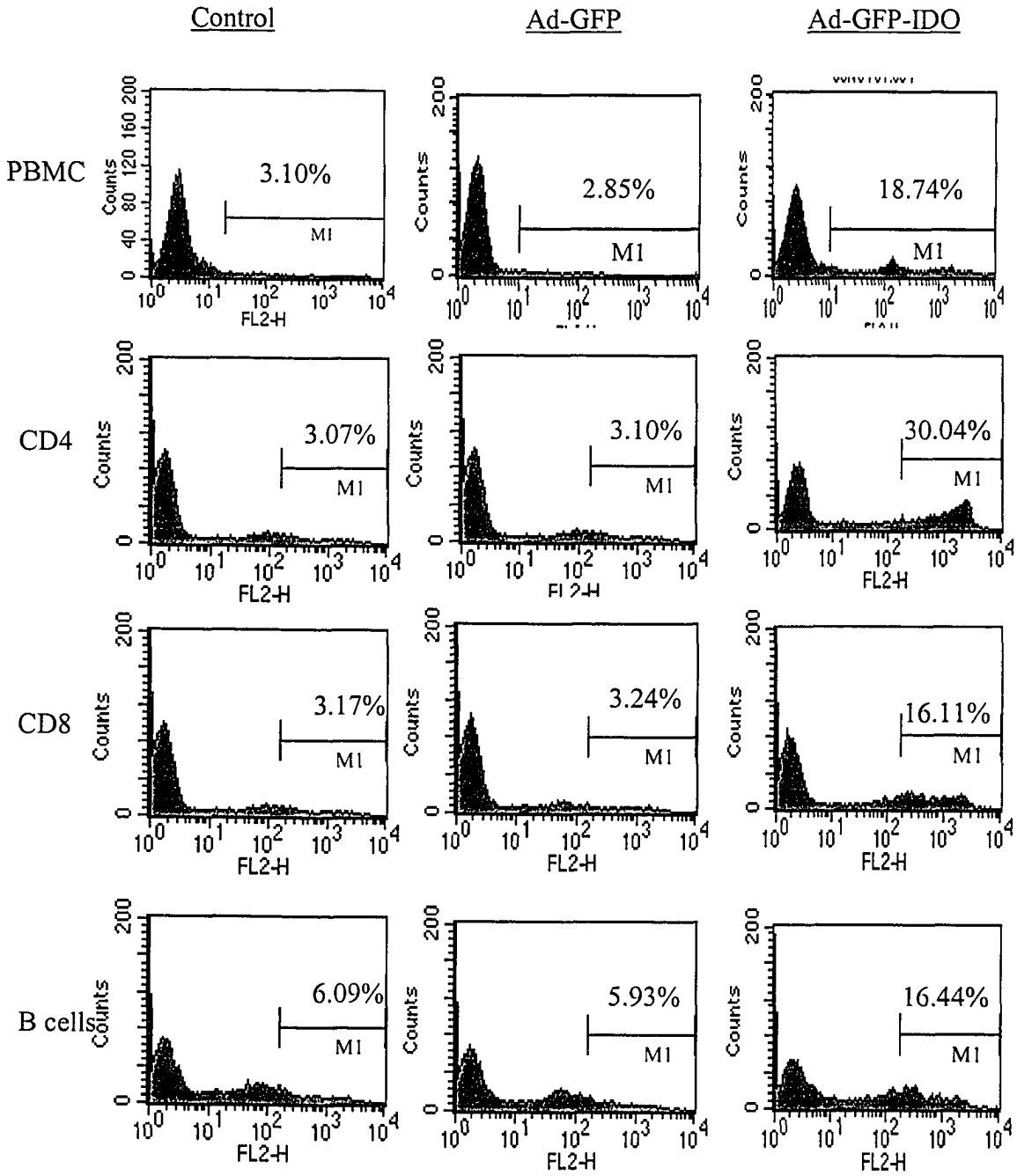
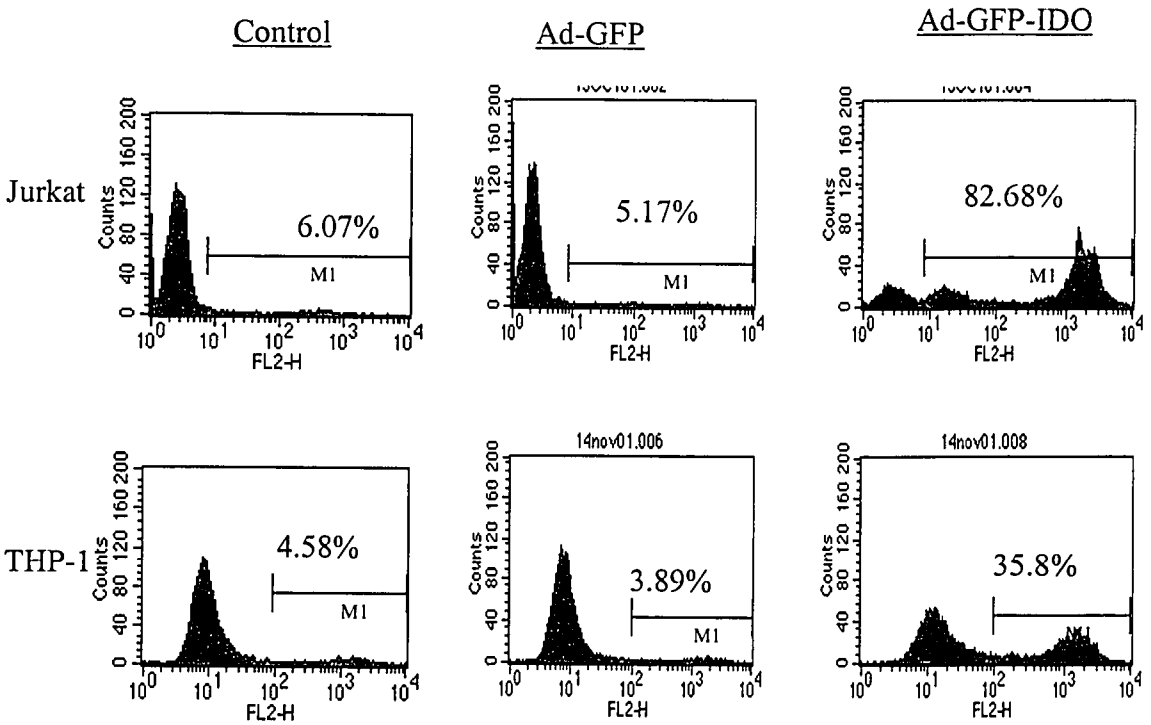


Fig. 2-4A

B



C

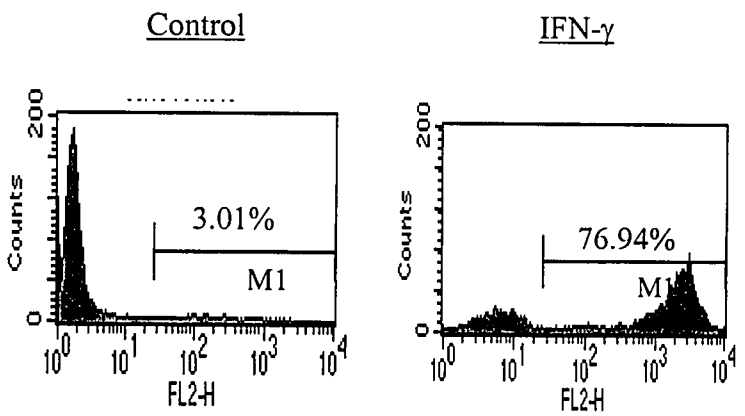


Fig. 2-4B and 2-4C

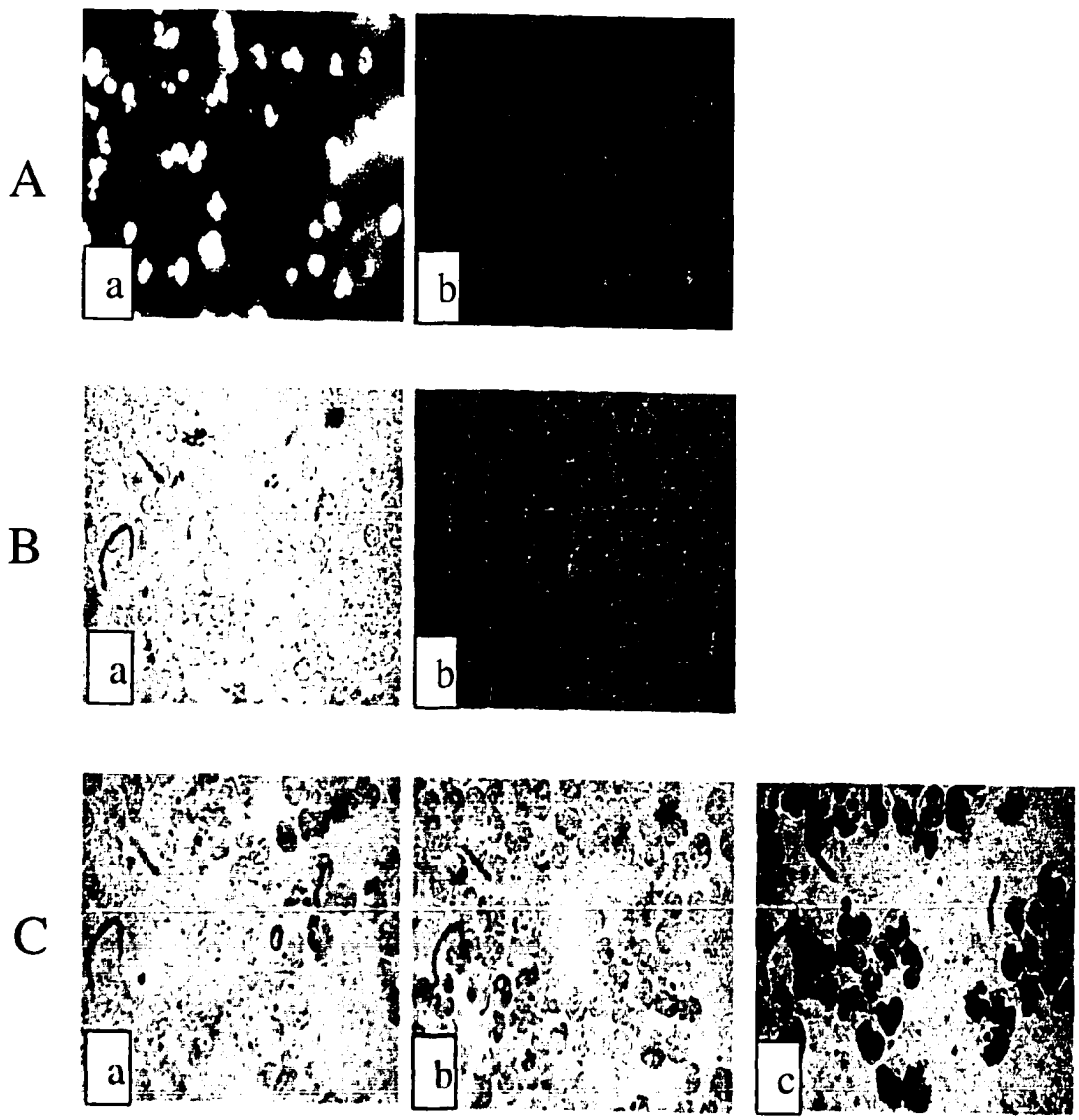


Fig. 2-5

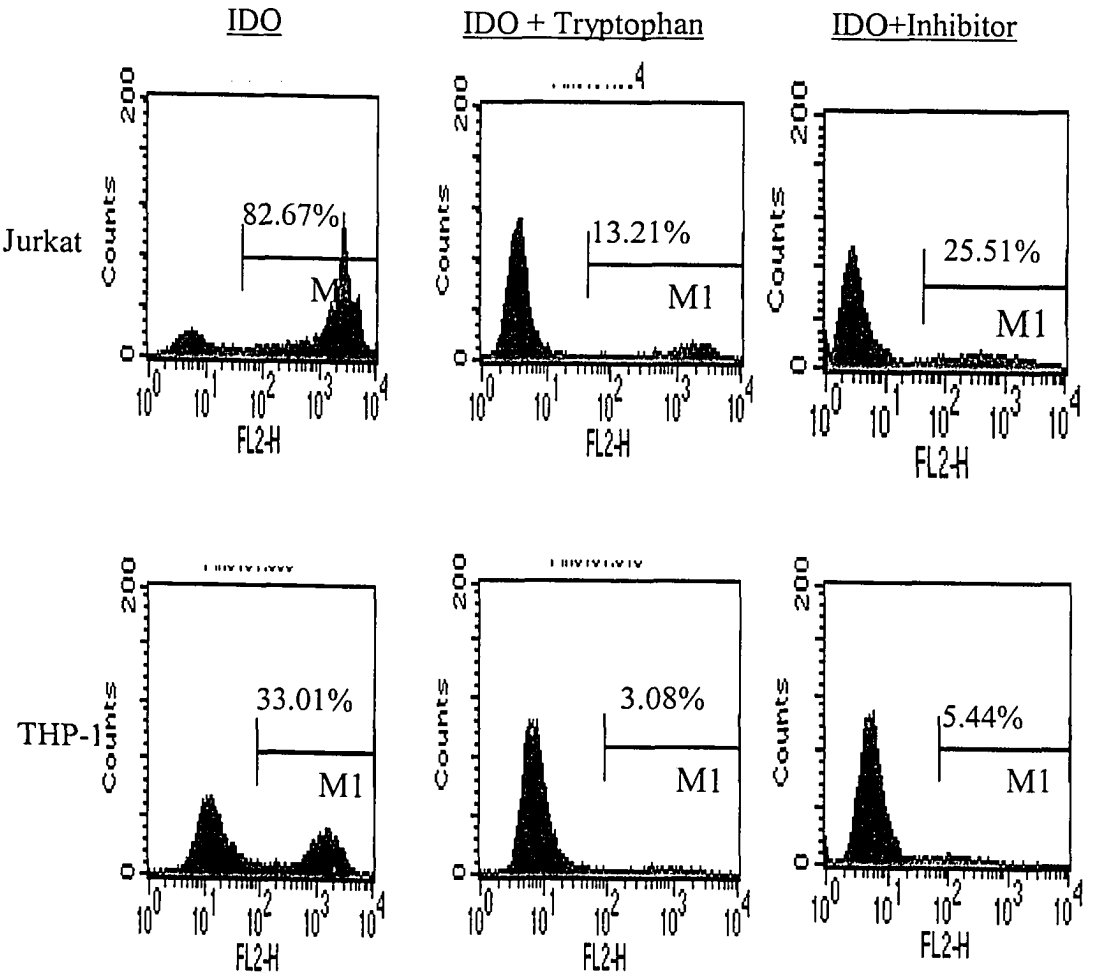


Fig. 2- 6

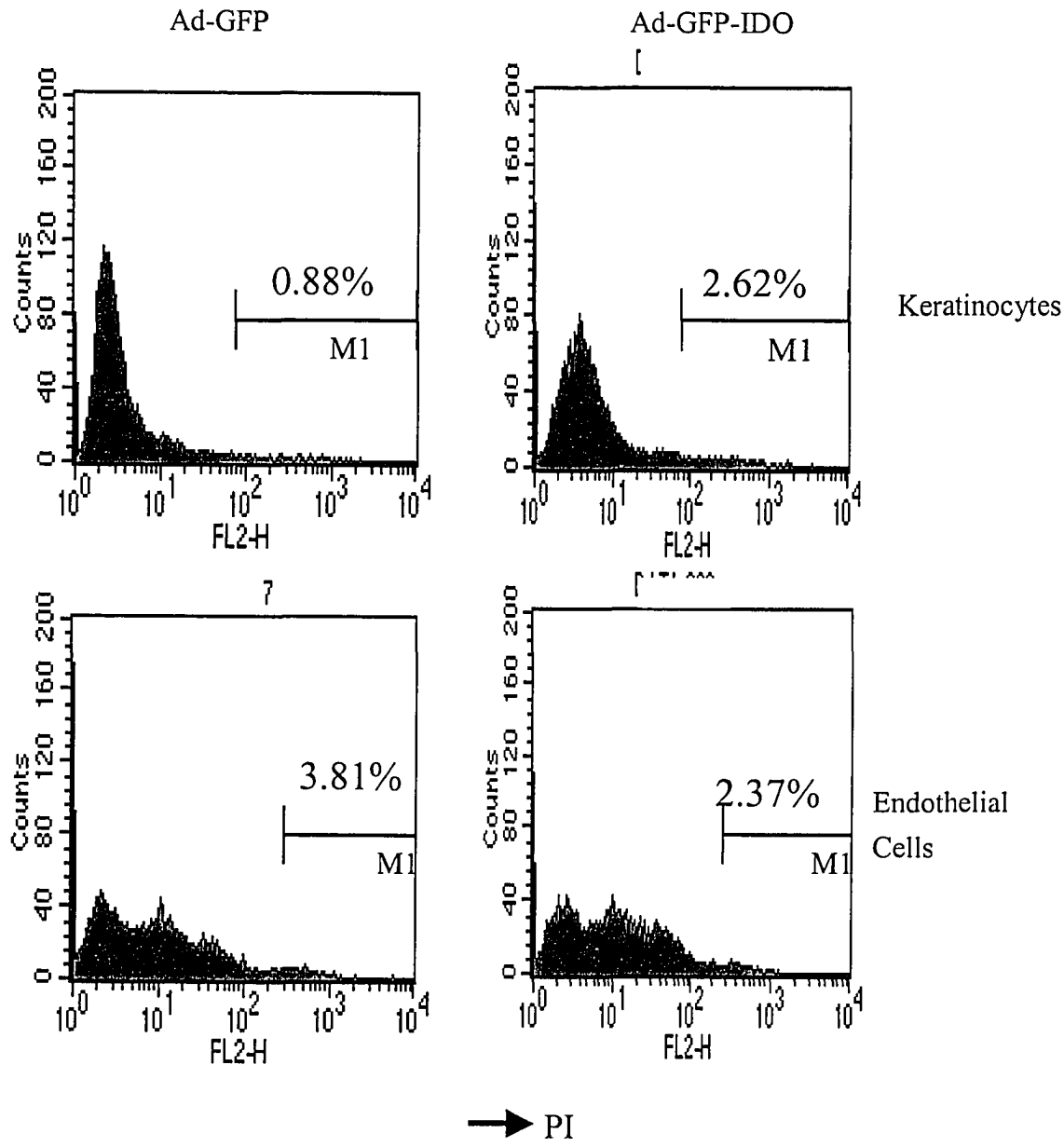


Fig. 2-7A

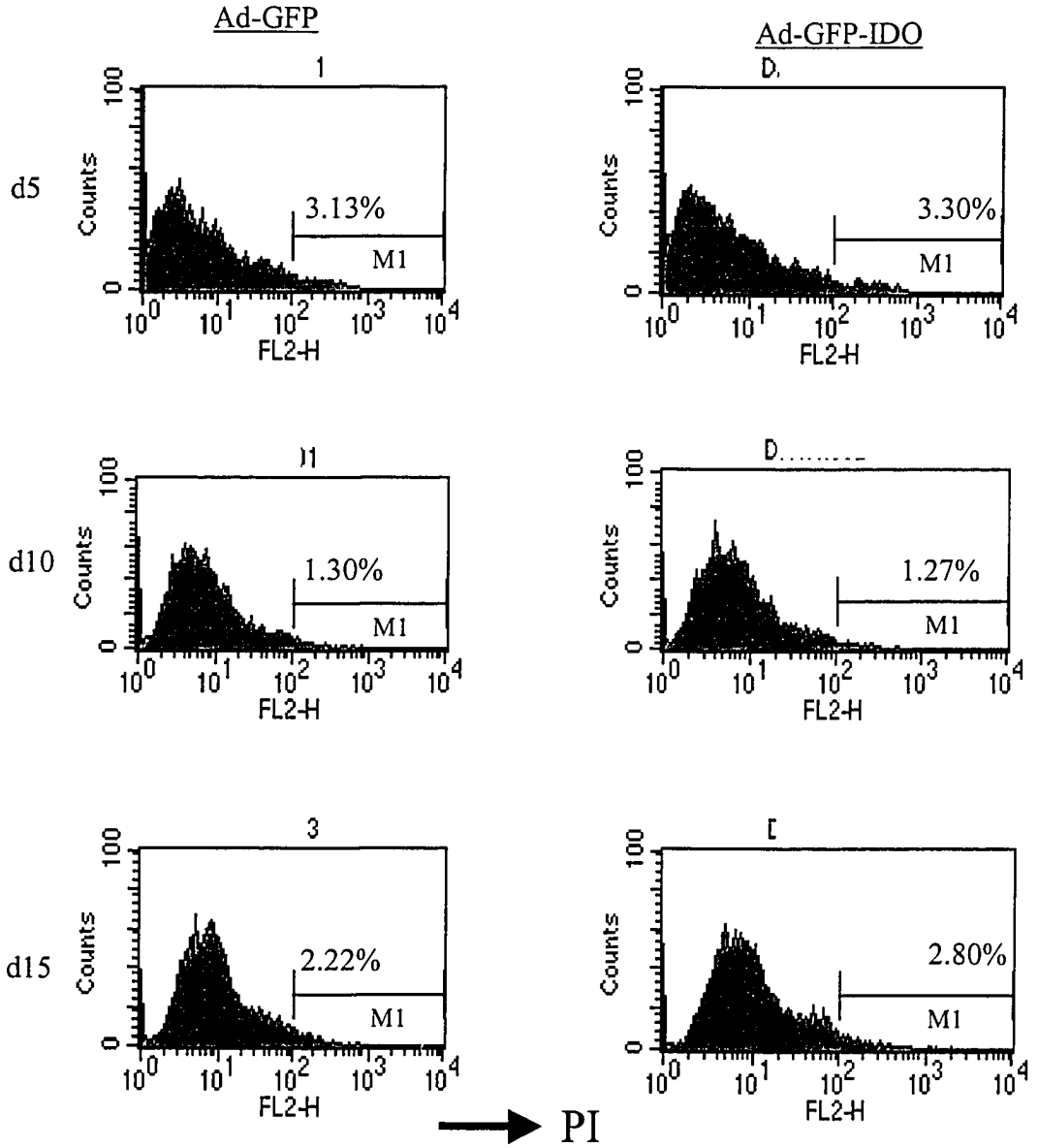
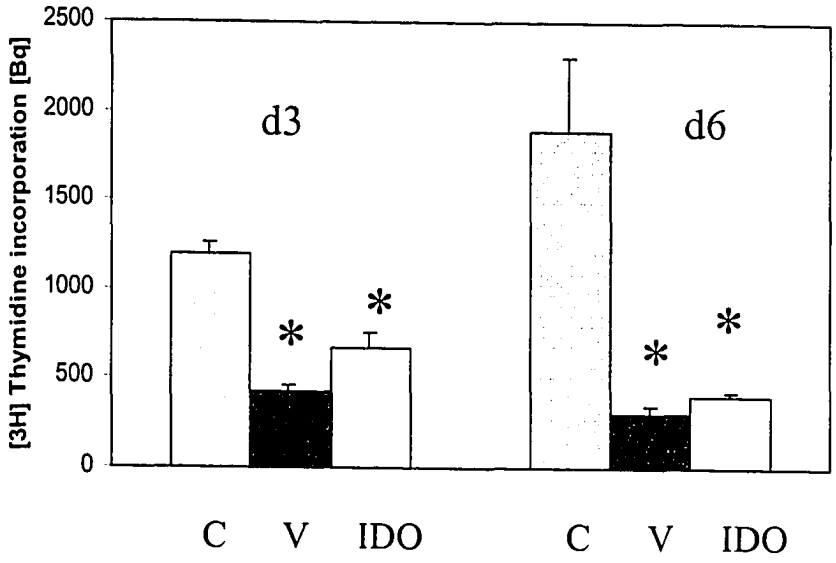


Fig. 2-7B

A



B

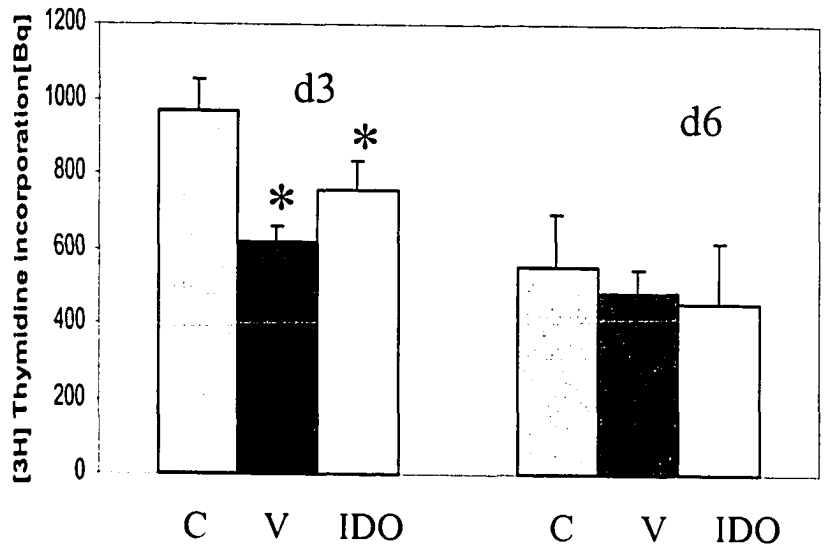


Fig. 2-8

Chapter 3

Cell Surface Expression of MHC Class I Antigen is Suppressed in Indoleamine 2,3-Dioxygenase (IDO) Genetically Modified Keratinocytes: Implications in Allogeneic Skin Substitute Engraftment

A version of this chapter has been published in: Li Y, Tredget EE, and Ghahary A. Cell surface expression of MHC class I antigen is suppresses in indoleamine 2,3-dioxygenase genetically modified keratinocytes: implications in allogeneic nskin substitute engraftment. Human Immunol. 2004; 65:114-123

II. INTRODUCTION

The acceptance of allografts without the need for long-term nonspecific immunosuppressive drug usage has long been the main goal of transplantation. As compared to the systemic use of immunosuppressive drugs, local expression of immunomodulatory or graft-protecting molecules has the potential to eliminate problems associated with increased incidence of malignancy and susceptibility to opportunistic infections. Several local immunosuppressive factors including IL-10 [1], TGF-beta [2], and FasL [3] have been studied for this aim. Despite advances made in the past decade, these molecules have low therapeutic efficiency. Thus, a more specific and potent local immunosuppressive molecule to protect grafted tissues is still desirable.

The absence of a harmful maternal immune response against the fetal allograft during pregnancy is one of the most intriguing models of immune tolerance. Many hypotheses have been proposed to explain this [4]. Recent evidence obtained in mouse models [5] indirectly suggested that expression of indoleamine 2,3-dioxygenase (IDO), a tryptophan catalyzing enzyme, present in the interface between fetus and mother during pregnancy is required to prevent rejection of the allogeneic fetus by maternal T cells. These studies suggested that proliferation of infiltrated T cells was inhibited by IDO generating a tryptophan deficient environment in placenta. Indeed, *in vitro* studies demonstrated that IDO expression by macrophages [6] and dendritic cells [7] inhibits bystander

lymphocyte proliferation. However, the mechanisms of immune tolerance induced by IDO at a local environment surrounding the placenta are not fully understood.

The importance of T cells in initiating and amplifying immune rejection has been demonstrated in a number of experimental models [8-10]. T cell mediated events are initiated by recognition of antigen presented in the context of appropriate major histocompatibility complex (MHC) molecule [11]. Antigen presentation by MHC class I targets destruction of foreign or pathogen infected cells by CD8+ cytotoxic T lymphocytes (CTL). It has been hypothesized that maternal tolerance to the fetus may be primarily achieved by the lack of human leukocyte antigen (HLA) MHC class I antigen expression on the fetal trophoblasts [12]. Interestingly, these cells also express IDO [5].

HLA class I is a complex molecule, consisting of a heavy chain (encoded by the HLA-A, -B, and –C loci on chromosome 6) and a light chain (encoded by β2 microglobulin on chromosome 15). Down-regulation or loss of HLA expression can lead to an impaired T-cell recognition and, hence, a blunted immune response. HLA class I molecules can be lost at any step required for their synthesis, transport, or expression on the cell surface [13-14]. In addition to the impaired expression during pregnancy, MHC class I molecules are known to be affected by pathological events such as viral infection and malignant transformation.

Regulation of MHC class I antigen by IDO is not well characterized. Therefore, it is of special interest to know whether immunosuppressive effects of IDO are related to the impaired MHC class I expression. Several reports raises the possibility that down-regulation of MHC class I may actually mediate some of the immunomodulatory effects of IDO in a local microenvironment. For instance, up-regulation of IDO expression [5] and absence of MHC class I [12] in trophoblasts have been suggested to protect the fetus from maternal immune rejection. IDO is normally expressed at the anterior chamber of eye, an immunologically privileged site [15] as well as the brain [16-17], where MHC class I expression is absent or low [18-19]. Furthermore, induction of IDO expression [20-23] and down-regulation of MHC class I [24-26] are involved simultaneously in the immune response to infections of human immunodeficiency virus (HIV), poliovirus, and cytomegalovirus (CMV). These and other observations led us to the hypothesis that the expression of IDO might affect the MHC class I synthesis and its expression on the cell surface. In view of the immunosuppressive effects of IDO, we have constructed a vector that contains the human IDO gene in a non-replicated adenovirus. We tested the effect of IDO on MHC class I expression, and revealed that IDO down-regulated the expression of MHC Class I in transfected human foreskin keratinocytes. Further analysis revealed that the down-regulation of MHC class I expression by IDO was significantly attenuated by the addition of tryptophan or an IDO inhibitor in conditioned medium.

III. MATERIALS AND METHODS

A. Cell Culture

Human foreskin keratinocytes were established as previously described [27]. Cells were cultured in serum-free keratinocyte medium (KSFM, Invitrogen Life Technologies, CA, USA) supplemented with bovine pituitary extract (50 μg/ml) and EGF (0.2 ng/ml). These cells at passage 2-5 were used. HeLa cells were cultured in DMEM medium (Invitrogen Life Technologies, CA USA) containing 10% FBS (HyClone Laboratories, Inc., Utah, USA). All media were supplemented with antibiotic-antimycotic preparations (100u/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B) (Invitrogen Life Technologies, CA, USA).

B. Adenovirus Vector Construction

Human IDO gene was kindly given to us by Dr J.M. Carlin (Department of Microbiology, Miami University, Oxford, OH, USA). Following its amplification by PCR, the IDO gene was subcloned into a shuttle vector containing a green fluorescent protein (GFP) gene according to manufacturer's instructions (Q-Biogene, CA, USA). The cloned plasmid was then homologously recombined with adenoviral plasmids in E. coli., BJ5183, by electroporation. The success of IDO insertion into adenoviral plasmid was confirmed by restriction endonuclease digestion. Plasmid cDNA was amplified in competent DH5α bacteria and purified in CsCl gradient in an ultracentrifuge. Adenoviral vectors carrying either GFP

alone (Ad-GFP) or GFP plus IDO gene (Ad-GFP-IDO) were then linearized by PacI digestion and used to transfect 293A package cells using Fugene-6 transfection reagent (Roche Applied Science, Laval, QC, Canada). Transfected cells were monitored for GFP expression and after three cycles of freezing in ethanol/dry ice bath and rapid thawing at 37°C, the cell lysate were used to amplify viral particles in large scale. The viral titer was determined in a 96-well plate according to the manufacturer's instructions.

C. Transfection And GFP Detection

Recombinant Adenoviruses were used to transfect keratinocytes and HeLa cells at a MOI of 100. Free viral particles were removed from cultured medium 30 hrs after transfection. Cells were fed with fresh medium and harvested in indicated time points. The success of transfection was determined by flow cytometry measuring the GFP protein expression and fluorescent microscopy using a Nikon inverted microscope equipped with an FITC filter to view GFP. Images were captured using a digital camera.

D. Kynurenine Measurement

The biological activity of IDO was evaluated by measuring the levels of the tryptophan degraded product, L- kynurenine, present in conditioned medium derived from either non-viral, IDO or control vector transfected cells. The amount of L-kynurenine was measured by a previously established method [28]. Briefly, about 2 ml of conditioned medium were collected from 5×10^5 cells at 72 hrs post transfection, proteins were precipitated by trichloroacetic acid, after

centrifugation, 0.5 ml of supernatant was incubated with equal volume Ehrich's reagent (Sigma Chemicals, Oakville, ON, Canada) for 10 min at room temperature. Absorption of resultant solution was measured at 490 nm by spectrophotometer within 2 hrs. The values of kynurenine in conditioned medium were calculated by a standard curve with defined kynurenine (Sigma Chemicals, Oakville, ON, Canada) concentration (0-100μM).

E. Antibodies

Polyclonal antibodies against human IDO were raised in rabbits by Washington Biotechnology Inc. (Baltimore, MD, USA) using purified human recombinant IDO as antigen. PE-conjugated mouse anti-human HLA-ABC monoclonal antibody was obtained from BD PharMingen Canada Inc. (Mississauga, ON, Canada).

F. Western Blot Analysis Of IDO

Cell extracts were prepared from 2×10^6 cells in lysis buffer [50 mM Tris-HCl (pH 7.40), 150 mM NaCl, 1 mM EDTA, 1mM EGTA, 0.025% NaN_3, 1% TritonX-100, 0.5% Igepal CA-630 (Sigma Chemicals, Oakville, ON, Canada), and protease inhibitor cocktail (Sigma Chemicals, Oakville, ON, Canada)]. Extracts were centrifuged at 14,000 RPM for 10 min. The total protein contents of supernatants were determined by BCA assay (Pierce, Rockford, IL, USA). A total of 100 μg of protein per sample was run on SDS-PAGE. Proteins were then transferred to PVDF membrane with Mini Trans-Blot Cell (Bio-Rad, Hercules,

CA, USA). Immunoblotting against IDO was carried out. Blots were initially incubated with rabbit anti-human IDO polyclonal antibody at a concentration of 1: 5000, subsequently with HRP-conjugated secondary antibody (Bio-Rad, Hercules, CA, USA). Protein bands were visualized by an ECL detection system (Amersham Biosciences Inc. QC, Canada).

G. Flow Cytometric Analysis

The expression of MHC class I antigen on the cell surface was determined by flow cytometry (FACScan, Becton Dickinson Co., Mountain View, CA, USA) after direct immunofluorescence staining using phycoerythrin (PE)-conjugated anti-HLA-A, B, C (Class I) monoclonal antibody for 30 min. Non-specific binding was removed by washing cells twice with cold PBS. Two color flow cytometric analyses were performed. A total of 10,000 events was collected by the FACScan, and the data was analysized using the CellQuest software (Becton Dickinson Co., Mountain View, CA, USA).

H. RT-PCR

RNA was prepared by the acid-guanidium-phenol-chloroform method (27). cDNA was synthesized from total RNA with oligo (dT) primer and MMLV reverse transcriptase (Invitrogen Life Technologies, CA, USA). Samples were incubated at 42^0C for 60 minutes, and the reaction was terminated by heating at 70^0C for 15 minutes, followed by rapid chilling on ice for 2 min. PCR was carried out using human HLA-A, B, and C gene common primers (sense: 5'-GTCATGGCGCCCCGAACC-3'; antisense: 5'-GTTCTCCAGGTATCTGCG-3';

size about 580 bp) and β-actin primers (sense:5'-CCCCCATGCCATCCTGCGTCTG-3'; antisense: 5'-CATGATGGAGTTGAAGGTAGTTT-3'; size about 336 bp), respectively. The housekeeping β-actin mRNA was used as a loading control. PCR was conducted with 25 cycles for β-actin, 25 cycles for MHC class I. PCR products were separated by electrophoresis in 1-1.5% agarose gels and stained with ethidium bromide, and the bands were visualized under UV light.

I. Statistics

Results are means ± SD and were analyzed statistically by using the paired Student's *t*-test. Probability values of <0.05 were considered significant.

VI. RESULTS

A. Transfection of Keratinocytes with either Ad-GFP or Ad-GFP- IDO

Keratinocytes were susceptible to the adenoviral infection. GFP expression could be visualized by fluorescent microscopy within 12 h of the addition of recombinant adenovirus. The efficiency of transfection was determined by fluorescent microscopy and flow cytometry (Fig. 1A and Fig.1B). As shown in this figure, more than 80% of keratinocytes were transfected by a mock vector (Ad-GFP) or IDO adenoviral vector (Ad-GFP-IDO) after 72 hrs of transfection.

To determine the levels of IDO protein expression, keratinocytes were transfected with Ad-GFP-IDO at MOI of 100 for 30 hrs. Cells were incubated in a fresh medium for another 42 hrs and then harvested (i.e.72 hrs post transfection) and analyzed for IDO expression by western blot using IDO polyclonal antibody. As expected, IDO protein band with a 42 kDa molecular weight was observed in cells transfected with Ad-GFP-IDO but not in non-transfected and a AD-GFP transfected cells (Fig. 1C).

To further validate the biological activity of IDO protein, the levels of kynurenine, a metabolic product of IDO, in conditioned medium, was evaluated. As shown in Fig.1D, the levels of kynurenine were not detectable in conditioned medium of either untransfected or Ad-GFP-transfected cells. However, the levels of kynurenine in conditioned medium of cells transfected with Ad-GFP-IDO exhibited a remarkable increase. Thus, these results confirmed that a functional IDO is expressed in Ad-GFP-IDO transfected keratinocytes.

B. IDO Down-Regulates Expression of MHC Class I Antigen in Keratinocytes

The effect of IDO on the cell surface levels of MHC class I proteins was evaluated 5 days post transfection. Expression of MHC class I was monitored by staining for HLA-A, B, C with PE labeled antibody followed by flow cytometry analysis. As shown in Figure 2, expression of IDO in keratinocytes resulted in up to 4.2 fold and 3.9 fold down-regulation of surface MHC class I complexes

compared to those of non-viral infected and mock adenoviral infected cells (fluorescence intensity of 37.25 ± 3.85 vs. 157.5 ± 10.50, $p<0.001$ compared to non-viral infected control; 37.25 ± 3.85 vs. 147.25 ± 12.53, $p<0.005$ compared to mock infected control, panel B), respectively. Down-regulation of MHC class I was correlated with increased expression of IDO, which was indicated by intensity of GFP fluorescence (panel A). To show that adenovirus infection and GFP do not affect MHC class I expression, keratinocytes were transfected by mock recombinant adenovirus (Ad-GFP) and the MHC class I expression was analyzed. The result of this experiment shows no significant difference in the cell surface expression of MHC class I between Ad-GFP transfected and non-transfected keratinocytes (Figure 2, fluorescence intensity of 147.25 ± 12.53 vs. 157.5 ± 10.50, panel B). These results suggest that the IDO expression itself specifically reduces the cell surface MHC class I antigen in transfected keratinocytes.

C. Down-Regulation of MHC Class I Antigen by IDO is Tryptophan Dependent

To test the possible mechanism through which IDO mediates the down-regulation of MHC class I expression on the cell surface, we examined the effect of the degraded products of IDO and depletion of tryptophan to HLA class I expression. An addition of kynurenine at various concentrations ranging from 200 to 400 μM to the conditioned medium of keratinocytes including non-, Ad-GFP and Ad-GFP-IDO transfected cells had no significant effect on down-regulation

of MHC class I expression on day 5. An addition of L-tryptophan at a concentration of 200 μM to conditioned medium also had no effect on the expression of MHC class I on non-transfected and mock transfected keratinocytes; however, the addition of tryptophan markedly increased the expression of MHC class I (Figure 3, fluorescence intensity of 149.67 ± 7.51 vs. 44.67 ± 11.06, $p<0.001$), and nearly restored the normal level of MHC class I expression in Ad-GFP-IDO transfected keratinocytes. Furthermore, the addition of an IDO inhibitor, 1-methyl-DL-tryptophan, at a concentration of 800 μM, partially restored the expression of MHC class I on IDO transfected keratinocytes (Figure 3, fluorescence intensity of 83.0 ± 16.5 vs. 44.67 ± 11.06, $p=0.067$). This partial restoration of MHC class I expression in IDO transfected keratinocytes by IDO inhibitor was likely due to incomplete inhibition of IDO activity by the IDO inhibitor. Inhibitory activities of available IDO inhibitors are relatively low with only micromolar Ki values [29-33]. The measurement of kynurenine in conditioned medium of IDO adenovirus transfected keratinocytes exhibited about 30% enzymatic inhibition of IDO by that concentration of IDO inhibitor for 24 h. Increasing doses of IDO inhibitor did not further increase the expression of MHC class I on IDO transfected keratinocytes. Collectively, these results strongly suggest that depletion of tryptophan, but not high levels of kynurenine, is the key factor in IDO induced down-regulation of MHC class I.

D. IDO does not Alter the Level of HLA MHC Class I mRNA in Keratinocytes.

MHC class I expression at transcription levels was investigated by RT-PCR. To co-amplify class I A, B, and C gene simultaneously, the primer pairs for these genes were designed. After 25 cycles of amplification, a PCR-product with anticipated 580 bp was detected in non-, Ad-GFP-, and Ad-GFP-IDO transfected keratinocytes (Fig. 4A). Using amplified β-actin as loading controls (Fig. 4B), we found that there was no significant difference in MHC class I gene expression in Ad-GFP-IDO tranfected cells compared to either non- or Ad-GFP transfected cells (Fig. 4C). These results indicate that IDO does not affect MHC class I expression at the level of mRNA expression.

E. IDO Down-Regulates Expression of MHC Class I in HeLa Cells

The role of IDO in down-regulating MHC class I expression was further confirmed by another experimental approach. HeLa cells, which have a high efficiency rate of transfection, were selected to examine the effect of IDO on expression of MHC class I. When HeLa cells were transfected with Ad-GFP-IDO for 5 days, the fluorescence intensity of HLA-A, B, C from normal (159.67 ± 25.72) and vector transfected cells (148.67 ± 10.69) decreased to (54.67 ± 10.12, $p<0.05$) (Figure 5). Similarly, the addition of 250 μM of tryptophan completely rescues the expression of MHC class I (Figure 5, fluorescence intensity of 144.33 ± 15.31 vs. 54.67 ± 15.31, $p<0.01$). This suggests that IDO also down-regulates MHC class I expression in HeLa cells.

V. DISCUSSION

Engineered epidermal substitutes using the patient's keratinocytes have been developed and used as an adjunctive therapy for permanent skin replacement [34-35]. Though desirable, it is unlikely to have an autologous engraftment for patients who suffer from extensive skin loss from a variety of conditions including large and severe thermal injury. Another obstacle is that autologous culture method needs several weeks for cell growth and graftable epidermal substitute production. To overcome these problems, cultured keratinocyte allografts from unrelated donors have been suggested as functional skin replacement for large burn injury, as well as diabetic, elderly and immune-compromised patients who suffer from non-healing complications. Despite the absence of passenger leukocytes and langerhans cells, experimental and clinical results obtained after cultured epidermal allograft strongly suggest that allogeneic keratinocytes are rejected. Furthermore, these epidermal substitute allografts are more immunogeneic than previously believed [36-38]. Therefore, exploring an allogeneic, non-rejectable and readily available skin substitute may provide a better means of improving wound coverage.

Previous studies indicated that IDO has an immunosuppressive function on prevention of semi-allogeneic fetus from maternal immune rejection [5]. Further studies demonstrated that IDO prolongs survival of grafted allogeneic islets [39]. These studies suggest that IDO seems to be an ideal molecule to be used for non-

rejectable skin substitutes. For this purpose, two recombinant adenoviruses bearing either the GFP (Ad-GFP), a reporter gene, or the GFP plus human IDO genes (Ad-GFP-IDO) were constructed in our laboratory, and a series of experiments including IDO immunomodulatory mechanisms and its applications in non-rejectable skin substitutes were explored.

In our present work, we showed for the first time that IDO down-regulates MHC class I expression in transfected keratinocytes. Further analysis revealed that the down-regulation of MHC class I expression was due to the depletion of tryptophan. This phenomenon was also confirmed in IDO-transfected HeLa cells. Since MHC class I antigens serve as targets for allogeneic immune rejection, the findings provide a direct evidence that down-regulation of MHC class I is, at least in part, a responsible mechanism underlying IDO-mediated local immunosuppressive effect in vivo.

In fact, previous studies have strongly implied an association between IDO expression and MHC class I regulation. For instance, during pregnancy, the expression of IDO [5] and the absence of MHC class I [40-42] on trophoblasts seem to contribute to the process of maternal tolerance of the conceptus. Down-regulation of the surface expression of the class I MHC molecules may be a mechanism by which the virus can escape the host's CTL response [26]. Interestingly, some viruses such as human immunodeficiency virus (HIV), poliovirus, and cytomegalovirus (CMV) reduce MHC class I expression [24-26]

and induce IDO expression simultaneously in infected cells [20-23]. Here, our findings demonstrated that IDO induces down-regulation of MHC class I in transfected keratinocytes.

The most likely explanation for IDO induced the MHC class I down-regulation is depletion of tryptophan. Since tryptophan is the least available essential amino acid in the body, degradation of tryptophan by IDO will affect protein synthesis or transport. Indeed, the addition of tryptophan in Ad-GFP-IDO transfected keratinocyte conditioned medium completely restored down-regulation of MHC class I. Similarly, the use of IDO inhibitor partially restored the down-regulation of MHC class I induced by IDO. Furthermore, our results demonstrate that IDO seems to reduce the expression of MHC class I at the protein, but not mRNA levels, further supporting the possible role of tryptophan degradation in the regulation of MHC class I expression.

Although down-regulation of MHC expression in the donor implies a role in minimization of immune rejection against the transplanted organs or tissues, grafted tissues from knockout mice, that do not express MHC class I, are still rejected [43]. Therefore, if IDO really does prevent immune rejection, other mechanisms should also be involved. To explore this, we have studied whether IDO affects infiltrated immune cells. Interestingly, we found IDO expression on transfected fibroblasts selectively induces CD4 + Jurkat cell, THP-1 monocyte,

and PBMC apoptotic death in a co-culture system (44), suggesting that IDO is mediating multiple mechanisms to prevent immune rejection.

In conclusion, the data reported here for the first time showed that IDO down-regulates MHC class I expression. Depletion of local tryptophan seems to be one of the mechanisms through which the regulation of MHC class I by IDO is mediated. This together with our previous studies that IDO-mediated bystander immune cell damaging, strongly suggest that IDO may be an effective local immunosuppressive factor.

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Figure Legends

Figure 1

A- GFP Expression in keratinocytes after 72 Hrs Transfection with either Ad-GFP (Upper Panel) or Ad-GFP-IDO (Lower Panel). Keratinocytes were transfected with either Ad-GFP or Ad-GFP-IDO at MOI of 100. The extent of transfection was monitored by GFP expression under fluorescent microscopy. Magnification 100×.

B- Flow Cytometry Determined the Efficiency of Transfection in keratinocytes with either Ad-GFP or Ad-GFP-IDO. Keratinocytes were transfected with either Ad-GFP or Ad-GFP-IDO at MOI of 100. After 72 hrs, cells were harvested and the numbers of GFP positive cells were estimated by flow cytometry. Data are means ± SD, representative of three similar experiments.

C- Western Blot Analysis of IDO Expression. Keratinocytes were transfected with either Ad-GFP, or Ad-GFP-IDO for 72 hrs. Non-transfected and transfected cells were harvested and cell lysates were fractionated by SDS-PAGE. IDO protein was detected using a rabbit polyclonal antibody raised against a purified recombinant human IDO protein. Left arrows show the molecular weight marker, right arrow shows a 42 kDa band corresponding to the IDO protein.

D- Kynurenine Levels in IDO Transfected Keratinocyte Conditioned Medium. Keratinocytes were transfected with either Ad-GFP or Ad-IDO-GFP

using MOI of 100. Free viral particles were removed by washing the cells with PBS after 30 hrs, and fresh medium was added. Conditioned medium was collected from the same number of transfected and non-transfected cells at 72 hrs post transfection and kynurenine levels were determined as described in the Materials and Methods.

Figure 2

IDO Down-Regulates Class I MHC Proteins In Keratinocytes. Keratinocytes were transfected with either Ad-GFP or Ad-GFP-IDO for 30 hrs. Free viral particles were removed by washing the cells with PBS and fresh medium was added. At day 5 post transfection, these cells were stained with PE-conjugated anti-HLA-A, B ,C monoclonal antibody. FACS analysis was used to determine HLA-A, B, C expression on untreated, Ad-GFP, or Ad-GFP-IDO transfected keratinocytes. Panel A shows the results from two-color (PE and GFP) channel analysis; while panel B shows the quantitative analysis of MHC class I expressing levels in either non-, Ad-GFP or Ad-GFP-IDO transfected keratinocytes. Panel A is one representative of four experiments. Data are means ± SD from four separate experiments (panel B).

Figure 3

Addition of Tryptophan and IDO Inhibitor partially Restored IDO Induced Down-Regulation of MHC Class I Expression. Keratinocytes were transfected with either Ad-GFP or Ad-GFP-IDO for 30 hrs. Free viral particles were removed

and the fresh medium with either 250 μM of tryptophan or 800 μM of 1-methyl-DL-tryptophan (IDO inhibitor) was added. Cells received vehicle were also included as negative control. Cells were harvested and stained with PE-conjugated anti-HLA-A, B, C at day5 post transfection. MHC class I protein expression was determined by FACS. Panel A shows the results from two-color (PE and GFP) channel analysis; while panel B shows the quantitative analysis of HLA expressing levels determined from the PE fluorescence intensity. Data presented here are one representative of triplicate experiments (panel A). Data of panel B are means ± SD, from three separate experiments.

Figure 4

Expression of HLA–A, B, C mRNA in IDO Expressing Keratinocytes.

Keratinocytes were transfected with either Ad-GFP or Ad-GFP-IDO for 30 hrs. Medium containing free viral particles were replaced by the fresh medium. Total RNA was extracted at day 5 post transfection and cDNA was synthesized as described in the Materials and Methods. The HLA-A, B, C mRNA expression was analyzed by RT-PCR (panel A). The expression of beta-actin mRNA was also determined and used as a loading control (panel B). Panel C shows the ratio of HLA-A,B,C mRNA /beta-actin in either non-, Ad-GFP, or Ad-GFP-IDO transfected keratinocytes.

Figure 5

IDO Down-Regulates MHC Class I Expression in Hela Cells. HeLa cells were transfected with either Ad-GFP or Ad-GFP-IDO for 30 hrs. Free viral particles were removed and fresh medium was added. At day 5 post transfection, cells were stained with anti-HLA A, B, C monoclonal antibody and analyzed by FACS. Panel A shows the results from two-color (PE and GFP) channel analysis; while panel B shows the quantitative analysis of HLA expression in non-, Ad-GFP, or Ad-GFP-IDO transfected cells with or without tryptophan. Panel A is one representative experiment of three. Data of panel B are means ± SD, from three separate experiments.

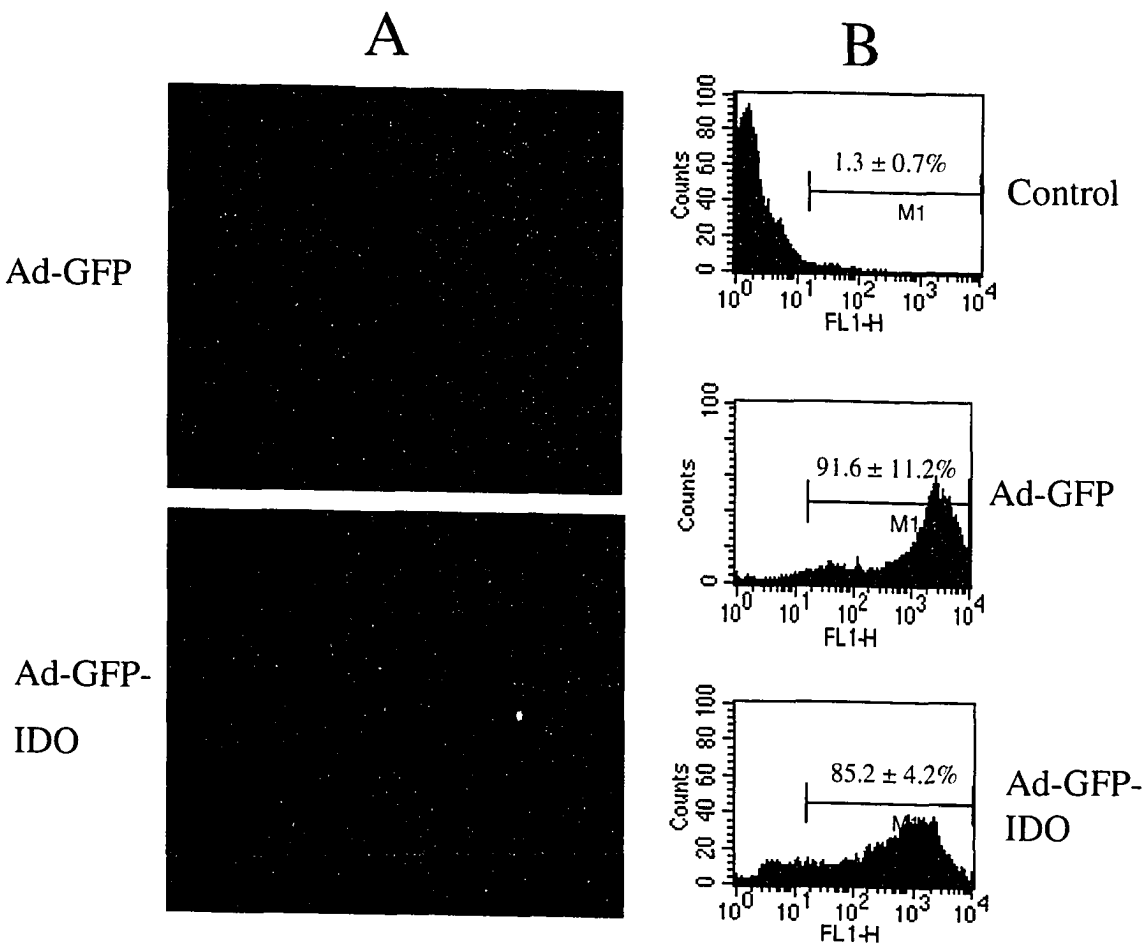

Fig. 3-1A and 3-1B

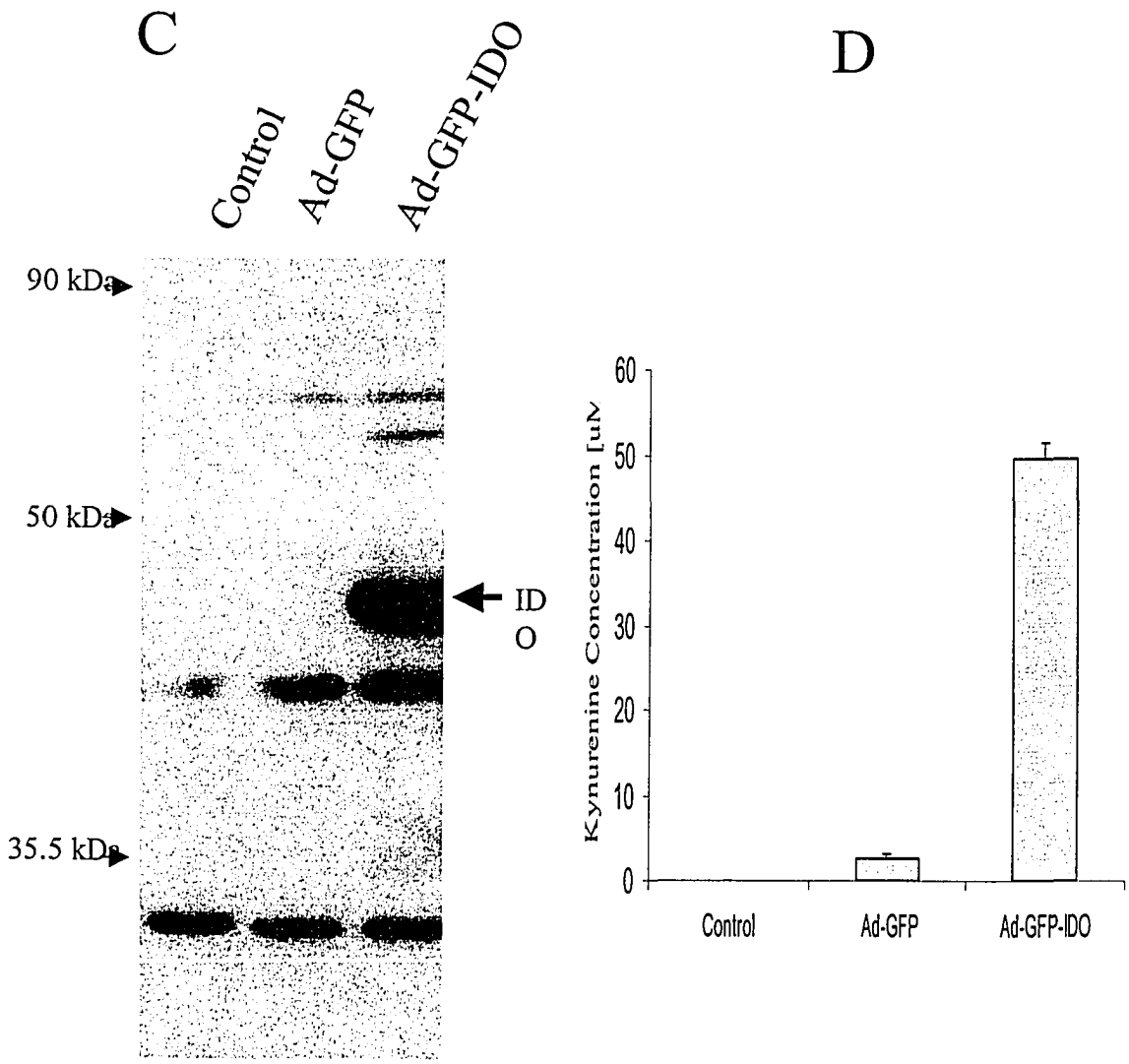

Fig. 3-1C and 3-1D

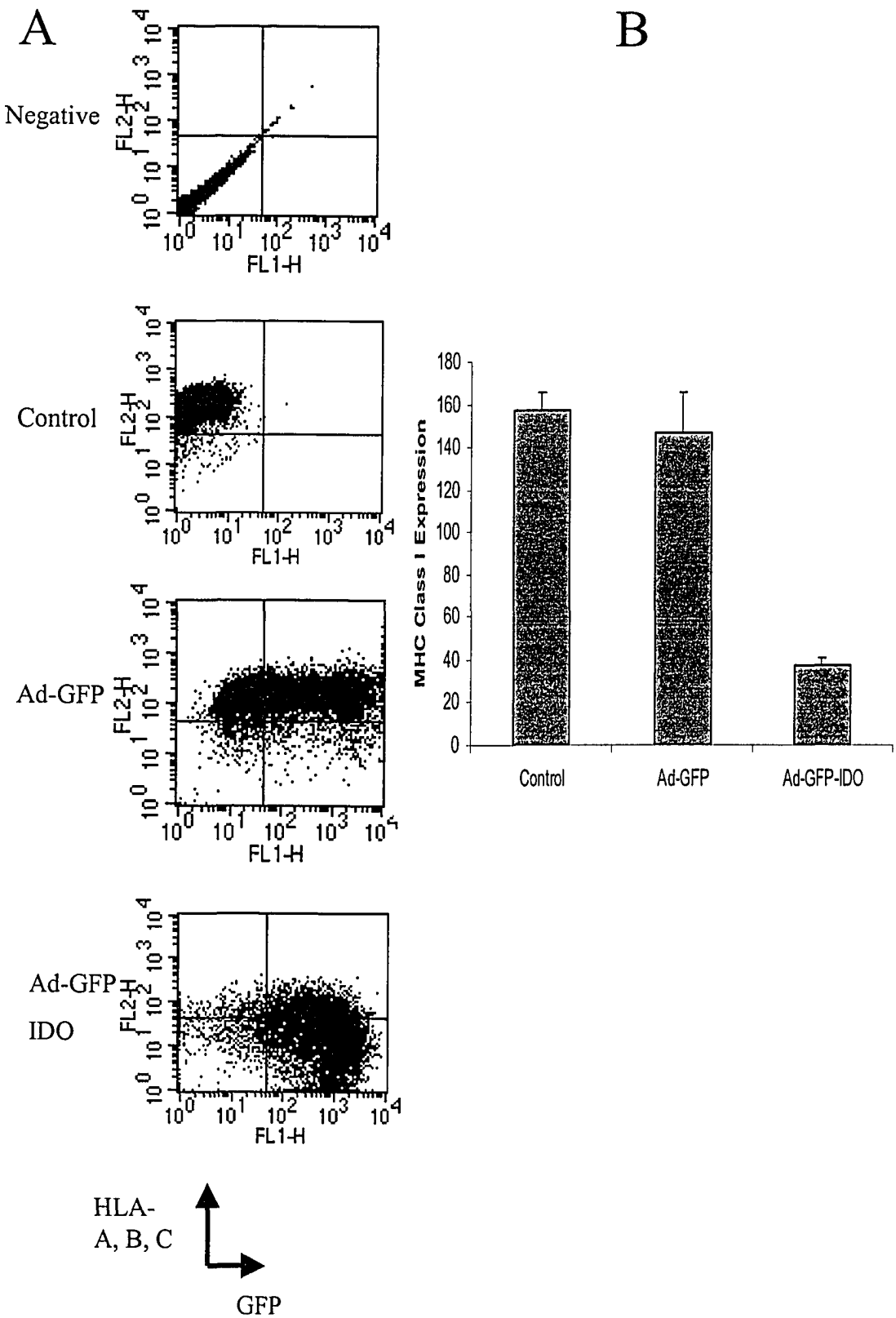

Fig. 3-2

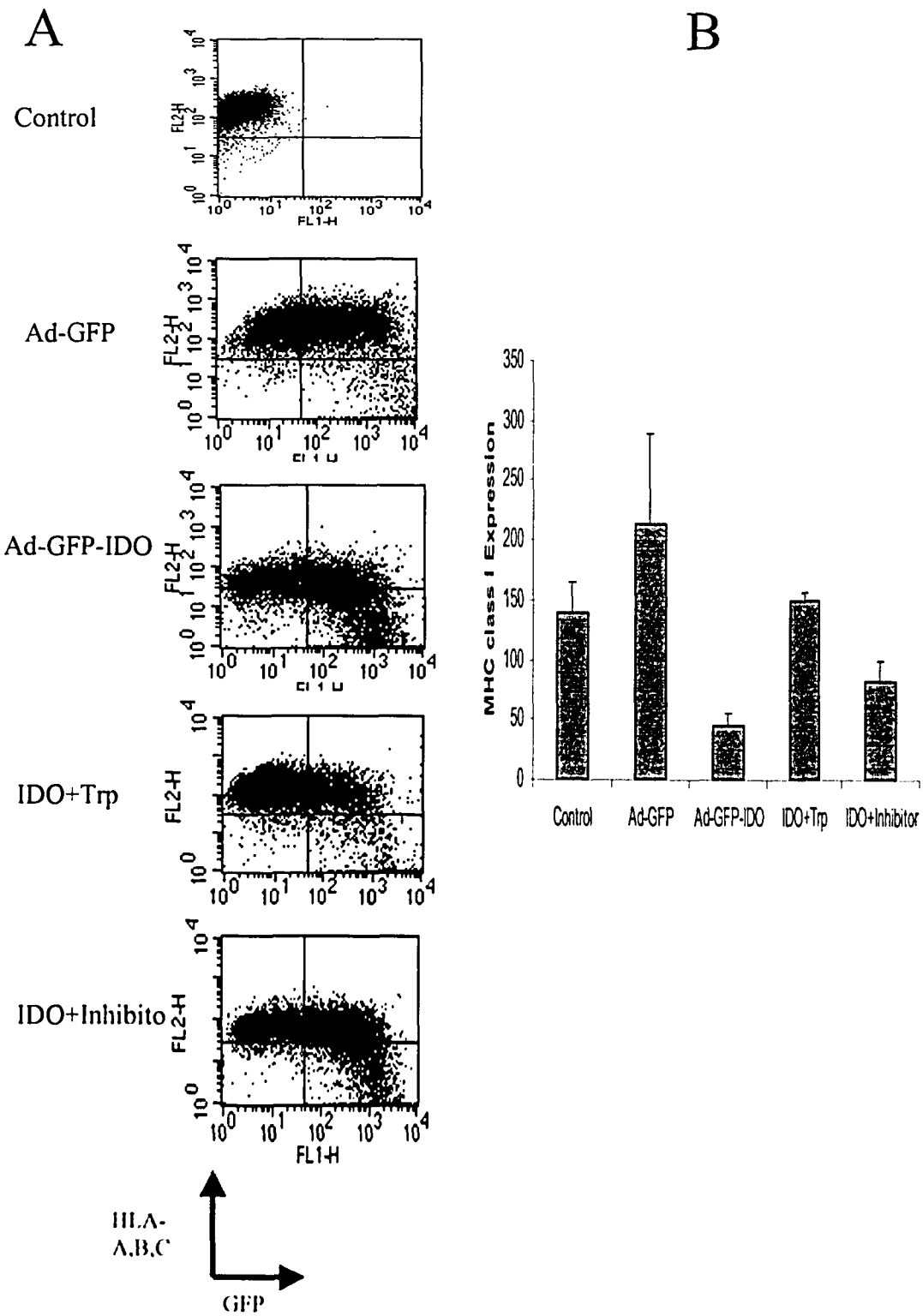

Fig. 3-3

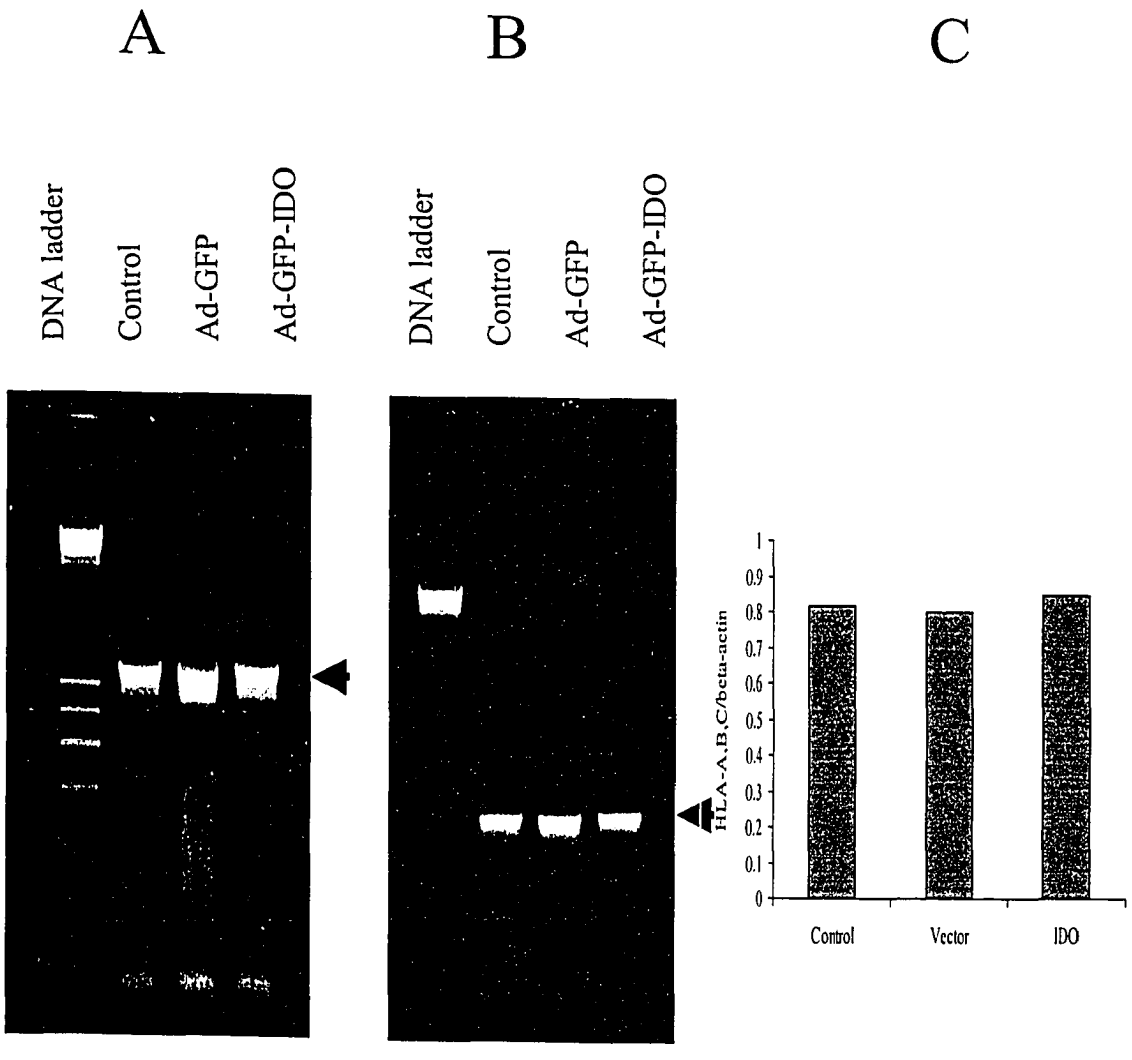

Fig. 3-4

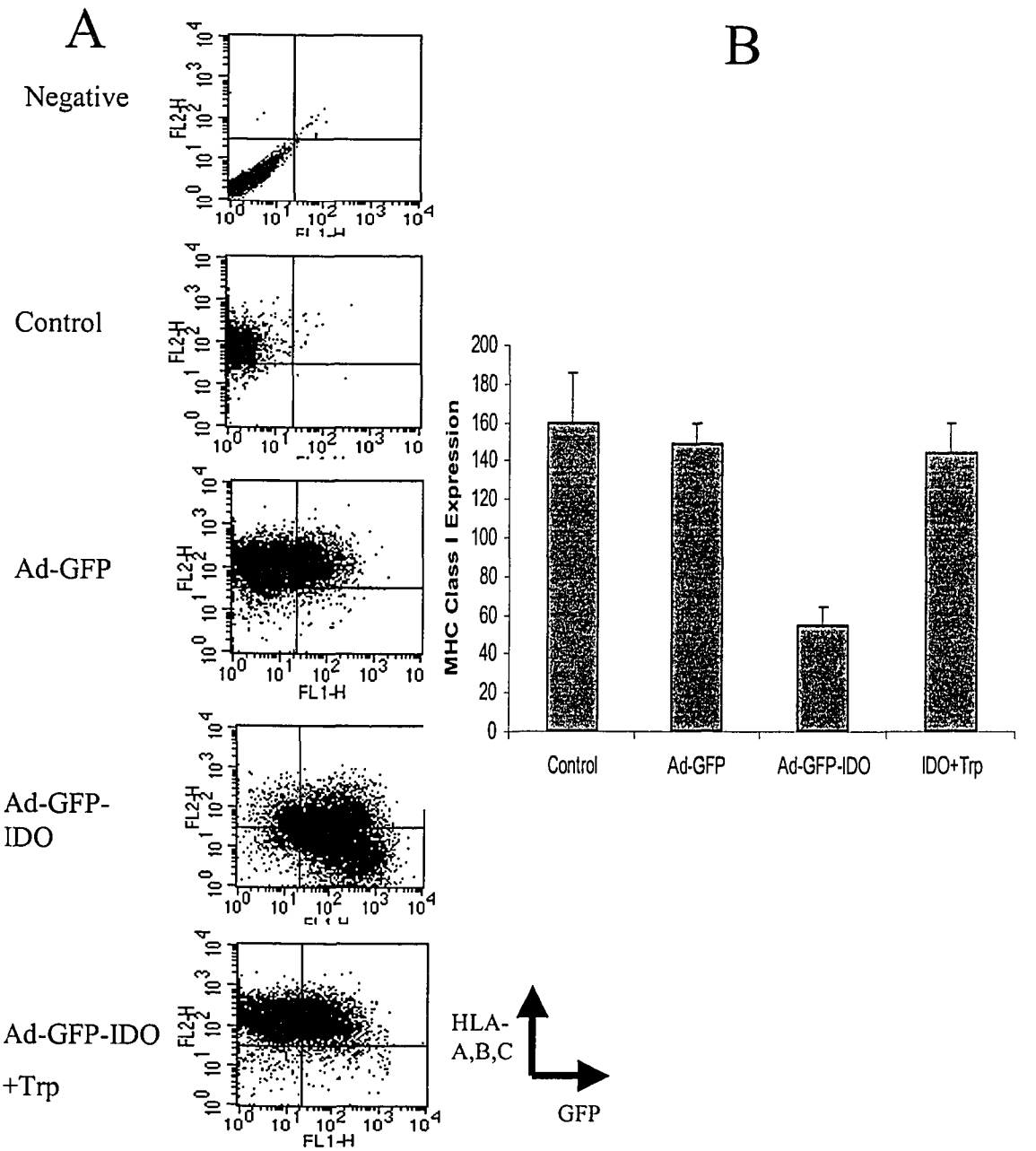

Fig. 3-5

Chapter 4

Local Expression of Indoleamine 2,3-dioxygenase (IDO) Protects Engraftment of Xenogeneic Skin Substitute

I. INTRODUCTION

Despite major improvements in tissue engineering of skin, a need remains for alternative, immediate and permanent wound closure materials for the treatment of cutaneous wounds. One potential approach is to generate a functional skin equivalent with the local immunosuppressive factor, indoleamine 2,3-dioxygenase (IDO) incorporated into genetically modified allo- or xeno-geneic skin cells (Li et al, 2004b). IDO is a rate-limiting enzyme that converts tryptophan to N-formylkynurenine (Taylor and Feng, 1991). This unstable product is further catabolized to kynurenine. It has been suggested that IDO plays a key role in the prevention of the immune rejection of the allogeneic fetus (Munn et al, 1998) and in the immune resistance of tumors (Uyttenhove et al, 2003). In a co-culture system, we have recently demonstrated that depletion of tryptophan by IDO jeopardizes the survival of CD4+ lymphocytes and THP-1 monocytes (Li et al, 2004b). We also found that IDO expression down-regulates MHC class I levels on the surface of IDO expressing keratinocytes (Li et al, 2004a). Based on this information, we hypothesize that allogenic skin cells genetically modified with an IDO gene would be develop immunologic tolerance.

In addition to host immune cell rejection as well as cytokines and growth factors released in response to engraftment, neovascularization also plays a critical role in graft survival. It is well known that for successful vascularization of the transplanted tissue to occur, several angiogenic factors important in graft

take are released (Boyce et al, 1995). Re-vascularization is characterized by the development of new capillaries from preexisting vessels and requires endothelial cell proliferation, migration and differentiation in tubular arrays (Yoshida et al, 1996). A number of growth factors (Davison and Benn, 1996), extracellular matrix molecules (Sengar, 1996), enzymes (Lorimier et al, 1996) and cell types (Schaffer and Nanney, 1996) are involved in this complex process. Revascularization is vital and highly regulated during a variety of normal physiological conditions including ovulation, embryonic development and wound healing (Rissau, 1997). Although IDO catalyzes the degradation of tryptophan which is normally required by immune cells to inflict damage to nearby cells, enhanced expression of IDO to deplete tryptophan locally may also delay re-vascularization at the transplantation site.

Thus, the aim of this study was to evaluate the effects of local IDO expression on xenogeneic graft take as well as neovacularization formation in an *in vivo* rat model. The results show that engraftment of genetically modified IDO xenogeneic fibroblasts embedded in a collagen matrix accelerates wound healing in the rat model. Furthermore, we demonstrated that IDO promotes angiogenesis *in vivo* and *in vitro*, and can protect grafted cells from host immune rejection.

II. MATERIALS AND METHODS

A. Adenoviral Vector Construction

To construct the adenovirus encoding of a protein of human IDO, we cloned the PCR product with a full-length protein into a shuttle vector, which co-expresses a green fluorescent protein (GFP) as a reporter gene following the manufacturer's instructions (Q-Biognene, Carlsbad, CA) (Li et al, 2004b). The recombinant adenoviral plasmids were generated by electroporation of BJ5183 *E. coli* using the shuttle vector either with or without IDO. Recombinant adenoviral plasmids were then purified and transfected to 293 cells using Fugene-6 transfection reagent (Roche Applied Science, Laval, QC, Canada). Adenoviral stock were prepared and titered on 293 cells as previously described (Li et al, 2004b).

B. Fibroblast Culture and Transfection

Cultures of fibroblasts were established as previously described (Ghahary et al, 2000). Fibroblasts were grown in Dulbecco's Modified Eagle Media (DMEM) with 10% fetal bovine serum (FBS) and cells from passages three to seven were used in this study.

Recombinant adenoviruses were used to infect fibroblasts at a multiplicity of infection (MOI) of 2000. Free viral particles were removed from culture medium 30 hrs after transfection. Efficiency of transfection and expression of functional IDO were assessed by counting the number of GFP positive cells, by anti-human IDO antibody in western, and measuring the levels of kynurenine in conditioned medium as previously described (Li et al, 2004a, 2004b).

C. Fibroblast-Populated Collagen Matrices

Type I collagen was isolated and purified from fetal bovine skin by repeated salt precipitation, as described by Volpin and Veis (1971). Human fibroblasts were embedded within type I collagen according to a method modified from Bell et al (1979). Briefly, either transfected or nontransfected fibroblasts were mixed with type I collagen (4.29 mg/ml in 0.1% acetic acid), 3×DMEM, chondroitin-6-sulfate (3mg/ml in 1×DMEM; Sigma, Oakville, ON, Canada), and FBS at a ratio of 1.5:3.5:3:1:1. After adjusting the pH to 7.50 with 0.4 N NaOH and the mixture was added directly to the culture plate. All dermal substitutes were maintained in DMEM with 10% FBS and used for transplantation within one week.

D. Transplantation of Xenogenic Tissue constructs *in vivo*

Procedures on all animal studies were approved by the Health Sciences Lab Animal Service Animal Welfare Committee of the University of Alberta. Ten week old Sprague Dawley rats were anesthetized by isoflurane. The dorsal surface of animal was shaved and cleaned with 70% ethanol. Six full-thickness excisional wounds were made on the dorsal surface (placing 3 wounds on each side of the midline) using a 6 mm^2 punch biopsy tool (Dormer Laboratories, Mississauga, ON, Canada). Either non-treated, mock adenovirus infected, or IDO transfected fibroblasts were embedded in a bovine collagen gel and grafted on the wounds. The wounds were then dressed with gauze impregnated with 3% Xeroform (Sherwood, St Louis, MO) and bandaged. On day 4 and 8, wounds were measured and photographed. Animals were sacrificed on day 8, 14, and 28. The entire wound, including a 2-4 mm margin of unwounded skin, was carefully excised. Each wound was divided in half. One half was fixed in 4% paraformaldehyde (Fisher, Pitsburg, PA) PBS solution and processed for paraffin embedding. The other half was snap-frozen by overlaying with Cryomatrix (Thermo Electron Corp., Pitsburgh, PA) with immediate immersion in a dry ice bath. Tissues were stored at -80 ^{0}C until analysis.

E. Histology and Immunohistochemistry

Sections were mounted on slides and stained with hematoxylin and eosin. Wounds were evaluated for the extent of re-epithelialization, and the granulation tissues was evaluated based on structure and cellularity.

Four-micron paraformaldehyde fixed and paraffin embedded sections were deparaffinized and hydrated by incubation in PBS for 10 min. To retrieve cellular antigens, sections were pretreated by heating in a microwave oven before blocking with 5% albumin in PBS solution. For antibody staining, sections were incubated with either anti-GFP horseradish peroxidase (HRP) conjugated antibody (1:40; Santa Cruz Biotechnology, Santa Cruz, CA), goat anti-PECAM-1 (CD31, 1:40; Santa Cruz), or rabbit anti-T cell CD3 peptide (1:200; Sigma), at room temperature for 1 hr. The second antibody was HRP-conjugated anti-goat IgG (Sigma) used in CD31 staining or HRP-conjugated anti rabbit IgG (Bio-Rad. Life Science, Mississauga, ON, Canada) used in CD3 staining. All second antibodies were used in a concentration of 1:400. The signal detection was carried out using DAB (3,3'-diaminobenzidine) enhanced liquid substrate system (Sigma). The slides were counterstained with hematoxylin for 5 seconds, then sections were dehydrated, mounted and examined by microscopy.

F. Endothelial Cell Culture

Human umbilical vein endothelial cells (HUVECs) were isolated by collagenase digestion (Narumiya et al, 2001). Cells were grown in a M199 medium (Invitrogen) supplied with 20% FBS and endothelial cell growth supplement (ECGS) (VWR, Mississauga, ON, Canada) at a final concentration of 10 μg per ml.

G. *In vitro* Co-culture Angiogenesis Assay

In vitro angiogenesis assay was assessed through the formation of capillary-like structures by HUVECs co-cultured with either recombinant adenoviral infected or uninfected human fibroblasts as a previously reported (Bishop et al, 1999). To distinguish the origin of the cells within tubular-like structure, HUVECs were trypsinized and labeled by incubation with 40 μg/ml 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanin (DiI, Molecular Probes, Eugene, OR) as previously described (Nehls et al, 1998). Cells were then thoroughly washed with PBS. An equal ratio of HUVECs and fibroblasts (5×10^5/ well) was mixed and seeded on a 6-well plate. Cells were incubated in M199 supplied with 20% FBS and ECGS as described above. The medium was replaced every 2 days. Images of tube structures were taken at day 20 using digital camera (Diagnostic Instruments, Sterling Heights, MI) attached to a Leica PBIRB fluorescence microscope (Opti-Tech Scientific Inc, Scarborough, ON). To distinguish endothelial cells from fibroblasts in our c0-culture system, the living HUVEC were labeled with DiI prior to co-culturing with fibroblasts. By inverted fluorescent microscopy, the DiI fluorescent label (red color) and GFP expression (green color) in recombinant adenovirus infected fibroblasts allowed us to identify different cell types.

H. Statistical Analysis

All data are given as mean ± SD. Statistical significance was performed using two-tailed Student's *t*-test for unpaired comparisons between groups. A p value less than 0.05 is considered significant.

III. RESULTS

A. Engraftment of IDO genetically Modified Human Fibroblasts Embedded in Collagen Matrix Accelerates Healing of Cutaneous Wounds

Prior to any *in vivo* experiments, the rate of IDO adenoviral infection and IDO activity were evaluated. The infection efficiency of adenovirus in fibroblasts and IDO activity in conditioned medium were then determined. As described previously (Li et al, 2004b), about 30% of fibroblasts were infected. As shown in Fig. 1A, the kynurenine levels in IDO adenoviral infected fibroblast conditioned medium was significantly increase as compared to either non-treated or empty vector infected fibroblast conditioned medium. To assess the effect of IDO on wound healing, either normal human fibroblasts, mock recombinant adenovirus infected human fibroblasts or IDO recombinant adenovirus infected human fibroblasts, embedded in a bovine collagen gels were applied onto 6 mm full-thickness wounds generated in the dorsal skin of rats and compared to control wounds which received no fibroblasts embedded collagen lattice. The size of wounds that received IDO adenovirus infected fibroblasts were indistinguishable from those that received either no lattice, lattices embedded with uninfected human fibroblasts, or mock-transfected recombinant adenovirus fibroblasts on

day 4 (33.1 ± 7.2 *vs.* 42.9 ± 12% *vs.* 35 ± 3.9% *vs.* 43.9 ± 7.7%, IDO transfected *vs.* non-treated control *vs.* non-transfected fibroblasts *vs.* mock transfected, $p>0.05$) (Fig. 1C). However, on day 8, all wounds grafted with IDO adenovirus infected fibroblasts were completely healed, while the other wounds remained open (Fig. 1B). As shown in Fig. 1C, measurement of the wounds that received engraftment of IDO adenovirus infected fibroblasts was significantly smaller compared to either non-treated, grafts with non-tranfected human fibroblasts, or grafts with transfected mock recombinant adenovirus human fibroblasts (7.0 ± 5.0% *vs.* 18.4 ± 5.2% *vs.* 19.6 ± 3.7% *vs.* 25.3 ± 4.9%, IDO transfected *vs.* non-treated control *vs.* non-transfected fibroblasts *vs.* mock transfected, $p<0.01$).

Examination of H&E stained wound sections revealed a dramatic difference in thickness of the epidermal layer between collagen gels containing IDO transfected fibroblasts and all other wounds. More rapid re-epithelialization was observed in wounds that received IDO adenovirus infected human fibroblasts (Fig. 2d) compared to other groups on day 8 post-transplantation, however, a marked delay in re-epithelialization was seen in either treated non-transfected human fibroblasts (Fig. 2b) or fibroblasts mock recombinant adenovirus transfected human fibroblasts (Fig. 2c) compared to control wounds (Fig. 2a). Granulation tissue deposition was more abundant and the number of infiltrated inflammatory cells was less in wounds that received IDO expressing fibroblasts as compared to other groups. In contrast, wounds that received either non-transfected human fibroblasts or mock adenovirus transfected human fibroblasts, showed a

significantly decrease in the amount of granulation tissue deposition and abundant inflammatory cell infiltration as compared to control wounds.

B. Host Immune Rejection of IDO genetically Modified Fibroblasts Embedded in Collagen Lattices

There have been conflicting reports of the survival and immune reaction of allografts of cultured skin cells especially fibroblasts (Erdag and Morgan, 2004; Sher et al, 1983; Hultman et al, 1996). However, like other cell types (Schmidt et al, 2003), xenogeneic fibroblasts have been shown to stimulate a high degree of host immune response (Erdag and Morgan, 2004; Isik et al, 2003; Schneider et al, 2004). To assess whether IDO plays a role in the prevention of grafted skin cells from host immune rejection, in the present study, we performed a xenograft with either non-treated, mock adenovirus infected, or IDO adenovirus infected human fibroblasts in a rat model. Cells were then traced by using GFP expression as a marker. The immune response was evaluated by infiltrated $CD3^+$ T lymphocytes. As shown in Fig 3a and 3b, there were no GFP expressing cells within the wounds that received the mock adenovirus infected fibroblasts on day 8 and 28, respectively. Similarly, we did not find GFP^+ cells in the control wounds on day 14. However, xenogeneic human fibroblasts expressing IDO survived and were easily detectable within the reticular dermis as indicated by GFP^+ staining on day 8 (Fig. 3c), 14, and 28 (Fig. 3d). These findings suggest that IDO does protect xenografts containing human fibroblasts from host immune rejection.

Furthermore, as shown in Fig.3 and Table 1, there was no significant difference in the number infiltrated $CD3^+$ T lymphocytes in wounds that received IDO expressing human fibroblasts compared to either non-treated wounds, wounds that received uninfected human fibroblasts, or mock adenovirus infected human fibroblasts on day 8, however, these differences become significant on day 14 and 28. Wounds that received either untreated human fibroblasts (Fig. 4Bb and 4Cb) or mock adenovirus infected fibroblasts (Fig. 4Bc and 4Cc) showed massive infiltrated lymphocytes and some clustered lymphocytes distributed at the reticular dermis as compared to untreated wounds on day 14 (Fig. 4Ba) and 28 (Fig. 4Ca). While wounds that received IDO adenovirus infected human fibroblasts revealed a few of infiltrated CD3 positive cells on day 14 (Fig. 4Bd) and 28 (Fig. 4Cd). This is a clear indication that the local expression of IDO by genetically modified xenogenic fibroblasts suppresses the infiltration of T lymphocytes at the wound site.

C. Increased Neo-vascularization in Wounds that Received IDO genetically Modified Fibroblasts Embedded in Collagen

Delayed or abrogated re-vascularization is thought to be a cause of rejection of allo- and xeno-genic engraftment (Boyce et al, 1995). To further evaluate the effects of IDO on neo-vascularization *in vivo*, we performed CD31 immunostaining on wound sections obtained from untreated control (Fig. 5Aa), non-transfected fibroblasts (Fig. 5Ab), mock –transfected fibroblasts(Fig. 5Ac)

and IDO adenovirus transfected fibroblasts grafted wounds (Fig. 5Ad). The results show a significant increase in the number of capillary-like vessels in wounds that received IDO-expressing fibroblasts compared to other groups on day 8. Data obtained from 6 sections randomly selected from 3 wounds of each group demonstrated a 2 to 3 fold increase in the number of vessels per mm^2 in wounds that received IDO expressing fibroblasts relative to those obtained from control wounds or those that received non-transfected fibroblasts or mock adenovirus transfected fibroblasts (Fig. 5B, $p<0.01$). We also performed CD31 staining at later time points on another set of samples obtained from animals that received similar treatment on day 14 and 28. The findings showed no significant difference in vascular density between wounds that received IDO expressing human fibroblasts and controls.

D. IDO Expression Promotes Formation of Vessel like Structures *in vitro*

To further investigate the impact of IDO expression on re-vascularization, in a co-culture system, HUVECs and human dermal fibroblasts with or without recombinant adenovirus infection were mixed in a ratio of 1:1 in 6-well plates.

DiI labeled endothelial cell cords were extended and elongated to form multicellular capillaric-like structure when co-cultured with IDO expressing fibroblasts (Fig. 6c) for a period of 20 days. However, these structures were not seen when HUVECs were grown in the presence of non-transfected fibroblasts (Fig. 6a) or mock adenovirus transfected fibroblasts (Fig. 6b) for the same period.

These findings strongly support the results obtained from the *in vivo* experiment described above, suggesting that enhanced IDO expression can initiate angiogenesis.

E. Depletion of Tryptophan by IDO Results in Formation of Capillary-like Structures *in vitro*

To determine how vessel-like structur es are formed in response to the local expression of IDO, the formation of these structures in response to tryptophan degraded products such as kynurenine and the depletion of tryptophan in a co-culture system was evaluated. As shown in Fig 7, the addition of kynurenine at a concentration of 50 μg (Fig. 7b) and 100 μg (Fig. 7c) to the conditioned medium of co-culture HUVECs with non-transfected human fibroblasts had no effect on the formation of capillary-like structure compared to control (Fig. 7a). However, under similar experimental conditions, the addition of L-tryptophan at a concentration of 50 μg (Fig. 7e) and 100 μg (Fig. 7f) to the conditioned medium of co-culture HUVECs and IDO-expressing fibroblasts, markedly decreased DiI labeled endothelial cell extension, elongation and branching as compared to those fibroblasts without the supplemental tryptophan (Fig. 7d). This data suggests that the depletion of local available tryptophan in response to IDO expression leads to the enhanced formation of capillary-like structures in vitro and may be an explantation for the enhanced angiogenesis seen in our in vivo model.

V. DISCUSSION

A number of recent studies suggest that IDO may play a key role in local immunosuppression (Munn et al, 1998, 1999, 2004; Uyttenhove et al 2003; Hayashi et al, 2004; Friberg et al, 2002). In an attempt to develop a non-rejectable skin substitute containing either allo- or xeno-geneic skin cells, a series of experiments were conducted to test whether IDO can function as a local immuno-suppressive factor to protect allo- or xeno- geneic skin cell engraftment. In previous studies, we have demonstrated that immune cells, but not primarily fibroblasts, keratinocytes and endothelial cells, are susceptible to low tryptophan levels in conditioned medium (Li et al, 2004b). These findings, therefore, lead us to hypothesize that IDO may function as a local immunosuppressive factor that protects allo-geneic or xenogeneic engraftment in an animal model. In this study, wounds that received IDO expressing human fibroblasts healed faster compared to controls (untreated wounds, wounds that received non-transfected human fibroblasts or mock adenovirus transfected fibroblasts in an animal model. Further studies found that at least two mechanisms might be accountable for promotion of healing in wounds treated with IDO expressing fibroblasts. First, the logical explanation would simply be engrafted skin substitute remained intact due to protective role of IDO and that resulted in earlier epithelialization. and this would shorten the wound closure time. Another mechanistic reason might be the one that we have shown in this manuscript indicating that IDO induces angiogenesis and that, at least in part, would promotes healing process. The graft take that results from IDO expression was supported by experiments showing the presence

of GFP positive cells within the wound sites at all time points. This finding was further confirmed by demonstrating a significant decrease in the number of infiltrated $CD3^+$ T lymphocytes 2 and 4 weeks post-transplantation. Additionally, IDO directly promotes neo-vascularization as demonstrated *in vivo* and *in vitro*, which may in part be accounted for the more rapid healing. Depletion of tryptophan by IDO in the local environment seems to contribute to the formation of capillary-like structures which were not seen when supplemental tryptophan was provided to the endothelial cells that are contained within these structures.

It has been well established that IDO expression by a variety of cell types can suppress the immune system by multiple mechanisms. It has been shown that IDO depletes tryptophan availability in the microenvironment (Munn et al, 1999), which depletes nearby activated lymphocytes (Li et al, 2004b). In other studies, evidence shows that IDO promotes the generation of toxic tryptophan metabolites such as 3-hydroxyanthranilic acid (3-HAA) and quinolinic acid (QA), which induce apoptosis in activated lymphocyte (Lee et al, 2002) and inhibit lymphocyte proliferation (Frumento et al, 2002; Terness et al, 2002). It has also been demonstrated that IDO scavenges O_2^- (Sun, 1989) which in turn decreases local inflammatory damage (Daley-Yates et al, 1988). These unique and synergistic activities of IDO likely collaborate to inhibit the immune response by the host. The molecular mechanism of T cell suppression induced by IDO was further explained in a recent study by Munn et al. who demonstrated that activation of GCN2 kinase in T cells by uncharged tRNA mediates T cell proliferative arrest

and anergy induction (Munn et al, 2005). They found that T cells with a targeted disruption of GCN2 were not susceptible to IDO-mediated suppression of proliferation *in vitro*. *In vivo*, proliferation of GCN2-knockout T cells was not inhibited by IDO expressing dendritic cells. GCN2 was originally identified as a regulator of translation in response to starvation for one of many different amino acids (Wek et al, 1989). Uncharged tRNA that accumulates during amino acid depletion binds to a GCN2 regulatory domain homologous to histidyl-tRNA synthetase enzyme, triggering enhanced eukaryotic initiation factor-2α (eIF2α) kinase activity (Sood et al, 2000). The activation of eIF2α kinase can provide a signal transduction pathway linking eukaryotic cellular stress in response to alterations in the control of gene expression at the translational level (Clemens, 2001), which in turn results in cell cycle arrest, lineage-specific differentiation, or apoptosis (Crosby et al, 2000; Zhang et al, 2002; Rao et al, 2004). This also is consisted with our previous study showed that immune cell damage induced by IDO can be restored with the addition of tryptophan (Li et al, 2004b). These findings collectively explain why fewer infiltrated $CD3^+$ T lymphocytes were seen in wounds that received IDO genetically modified fibroblasts on day 14 and 28.

One of the main limitations in using skin substitutes for as wound coverage is delayed re-vascularization (Sahota et al, 2004). Young and colleagues (Young et al, 1996) have suggested that split-thickness skin grafts survive first by diffusion of nutrients through the graft (imbibition), then initial vascularization by inosculation, and finally by neovascularization. Since composite skin substitutes

lack a capillary network, they cannot easily re-vascularize. Nutrients for these grafts are only supplied by imbibition and neovascularization. As such, imbibition alone is unlikely to be sufficient to support the permanent implantation of skin substitutes until neovascularization is established (Young et al, 1996). Improvements in skin substitutes by establishing a capillary-like network either with the addition of endothelial cells (Supp et al, 2002; Sahota et al, 2003; Black et al, 1998), or by genetically modifing skin cells with vascular endothelial growth factor (Supp et al, 2000, 2002), have demonstrated that initiating early neovascularization significantly increases graft take. In addition to protecting xenografted skin cells from immune rejection, IDO seems also to initiate neovascularization in the early stages of wound healing in our model. Although evidence that enhanced IDO expression promotes angiogenesis was obtained using our co-culture experiment, the mechanism of how enhanced IDO expression locally in healing wounds initiates angiogenesis *in vivo* and *in vitro* appears to be related to the depletion of tryptophan and warrants further study.

In summary, our findings suggest that IDO expression may function as a local immuno-suppressive factor and stimulates neovascularization that protects allo- or xeno- geneic human dermal grafts which may significantly increase the feasibility and variety of allograft skin constructs available for transplantation in wound healing disorders.

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VII. TABLES

Table 1. Infiltrated CD3 positive cell number per high power field at various times postgrafting

	Day 8	Day 14	Day 28
Untreated wounds	23.2 ± 12.2	14.5 ± 5.3	11.9 ± 7.5
Untreated fibroblasts	19.8 ± 7.8	39.5 ± 27.7[a]	25.8 ± 15.1[a]
Mock Ad fibroblasts	18.3 ± 7.1	45.6 ± 15.6[b]	23.4 ± 21.2
IDO Ad fibroblasts	17.5 ± 11.2	1.6 ± 1.6[c]	5.7 ± 5.1[c]

[a]$P<0.05$ and [b]$P<0.01$ vs. untreated wounds, respectively; [c]$P<0.01$ vs. wounds receiving either untreated fibroblasts or mock adenovirus infected fibroblasts. n= 10 high power fields. Data were expressed as the mean ± SD

VIII. FIGURE LEGENDS

Figure 1. Grafting IDO genetically modified human fibroblasts populated collagen gels accelerates wound healing in rats. **Panel A.** Kynurenine levels in IDO transfected fibroblast conditioned medium. Fibroblasts were infected by either Ad-GFP or Ad-GFP-IDO. Free viral particles were removed by washing the cells with PBS after 30 hrs, and fresh medium was added. Conditioned medium was collected from the same number of infected and non-infected cells at 72 hrs post transfection. Kynurenine levels were determined. **Panel B.** Photographic findings of wounds on day 8 post transplantation. Wound closure analysis on day 4 and 8 (n = 6). **Panel C.** The effect of IDO expressing fibroblasts on wound healing was comparable to that of other groups on day 4 and 8, respectively.

Figure 2. H&E staining of wound sections on day 8 for untreated (a) uninfected fibroblasts (b) mock adenovirus infected fibroblasts (c) and IDO adenovirus infected fibroblasts (d) treated wounds. The green arrows represent the wound edge; the black arrows represent re-epithelialization areas.

Figure 3. Detection of grafted fibroblasts by GFP immunohistochemistry. Sections of wounds that received either mock adenovirus infected fibroblasts (a) at day 8 post-transplantation and (b) at day 28 post-transplantation, IDO adenovirus infected fibroblasts (c) at day 8 and 28 (d) post-transplantation were

prepared and GFP expression was detected by immunostaining. Arrows show GFP positive cells.

Figure 4. Detection of CD3+ infiltrated lymphocytes within xenografts. Immunohistochemical staining of CD3+ lymphocytes in the sections of untreated control wounds on day7 (Aa), day14 (Ba) and 28 (Ca) as well as those wounds that received non-transfected fibroblasts on day7 (Ab), 14 (Bb) and28 (Cb) are shown. Similarly. immuno-staining for CD31 expressing cells infiltrated within those wounds that received mock adenovirus transfted fibroblasts on day7(Ac), 14 (Bc) and 28 (Cc) as well as wounds that received IDO adenovirus transfted fibroblasts on day 7(Ad), 14 (Bd), day 28 (Cd) are shown. The arrows show cluster distributions of infiltrated $CD3^+$ lymphocytes at the wound site.

Figure 5. Detection and quantitative analysis of capillary-like structures in wound sections on day 8 post-transplantation. **Panel A.** Either untreated (Aa), treated with uninfected fibroblasts (Ab), mock adenovirus infected fibroblasts (Ac) and IDO adenovirus infected fibroblasts (Ad) wounds were excised and sections were strained with CD31 antibody. **Panel B** shows the quantitative analysis of capillary-like structures shown in **Panel A.** Data are expressed as the mean ± SD (n=6).

Figure 6. IDO induces capillary-like structure formation in HUVECs co-cultured with IDO expressing fibroblasts. DiI-labeled HUVECs were co-cultured with

either untreated fibroblast (a and d), mock adenovirus infected fibroblasts (b and e) or IDO adenovirus infected fibroblasts (c and f) and DiI fluorescence (a-c) and GFP (d-f) fluorescence staining cells were then detected. Magnification: 400 ×.

Figure 7. Depletion of tryptophan promotes the formation of capillary-like structures in HUVECs. DiI-labeled HUVECs were co-cultured with untreated fibroblasts in the absence (a) or in the presence of 50 μg/ml (b) and 100 μg/ml of kynurenine for 20 days. Similarly, DiI-labeled HUVECs were co-cultured with IDO adenovirus infected fibroblasts in the absence (d) or in the presence of 50 μg/ml (e) and 100 μg/ml (f) of L-tryptophan for 20 days. The images show DiI fluorescent labeling cells. Magnification: 400 ×

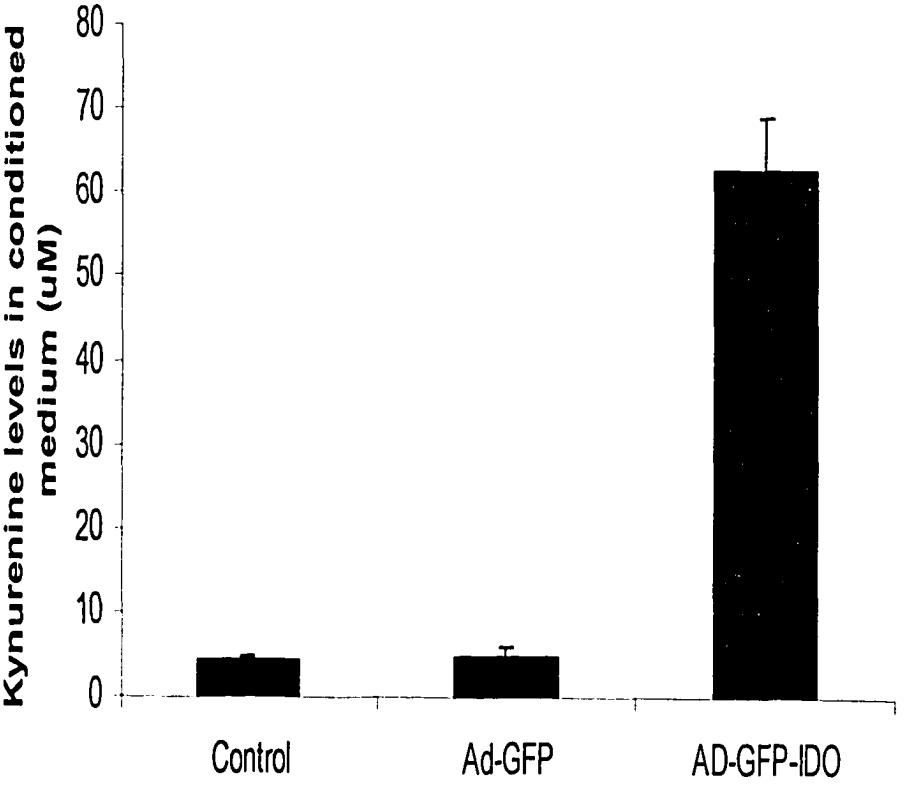


Fig. 4-1A

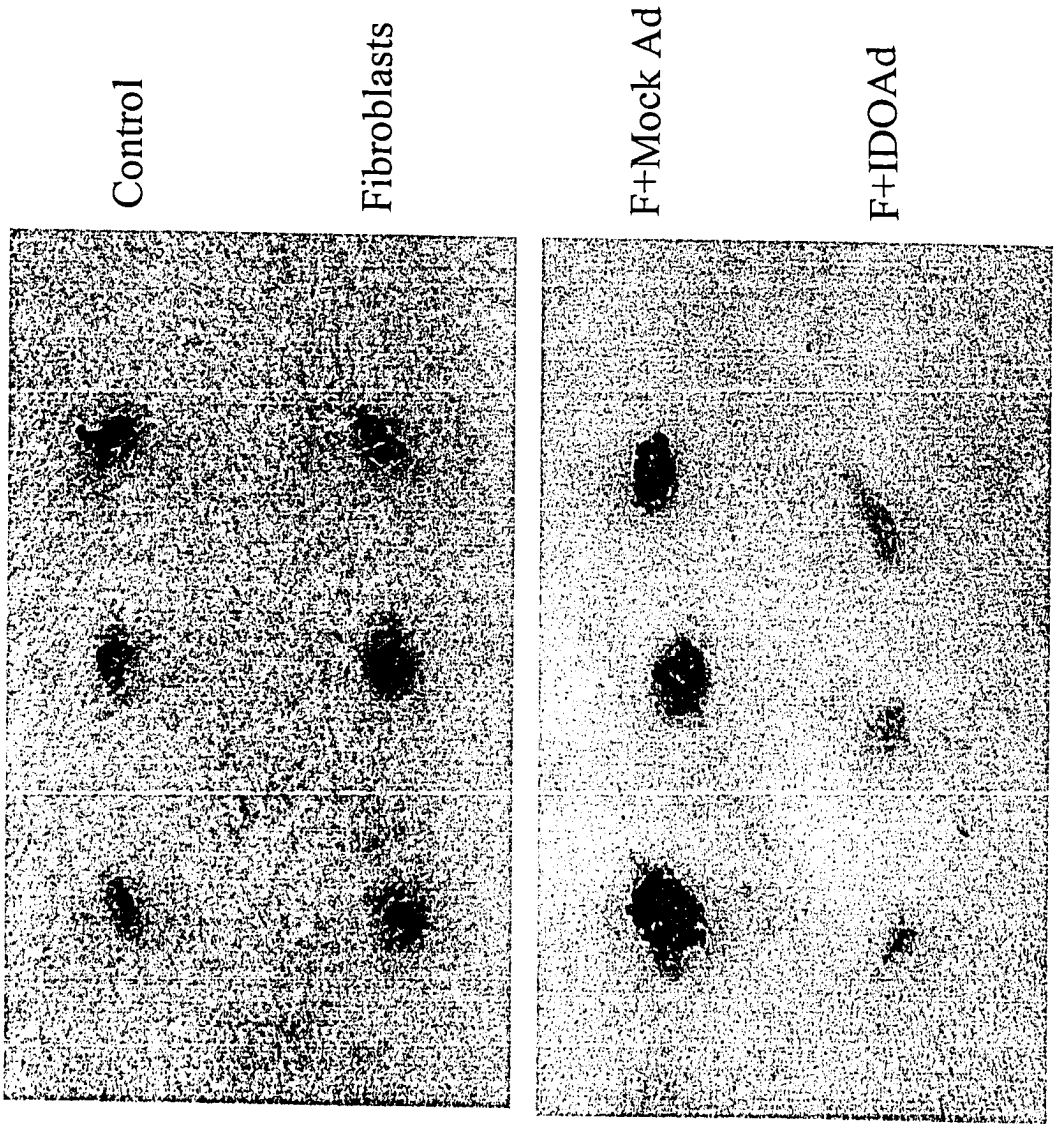
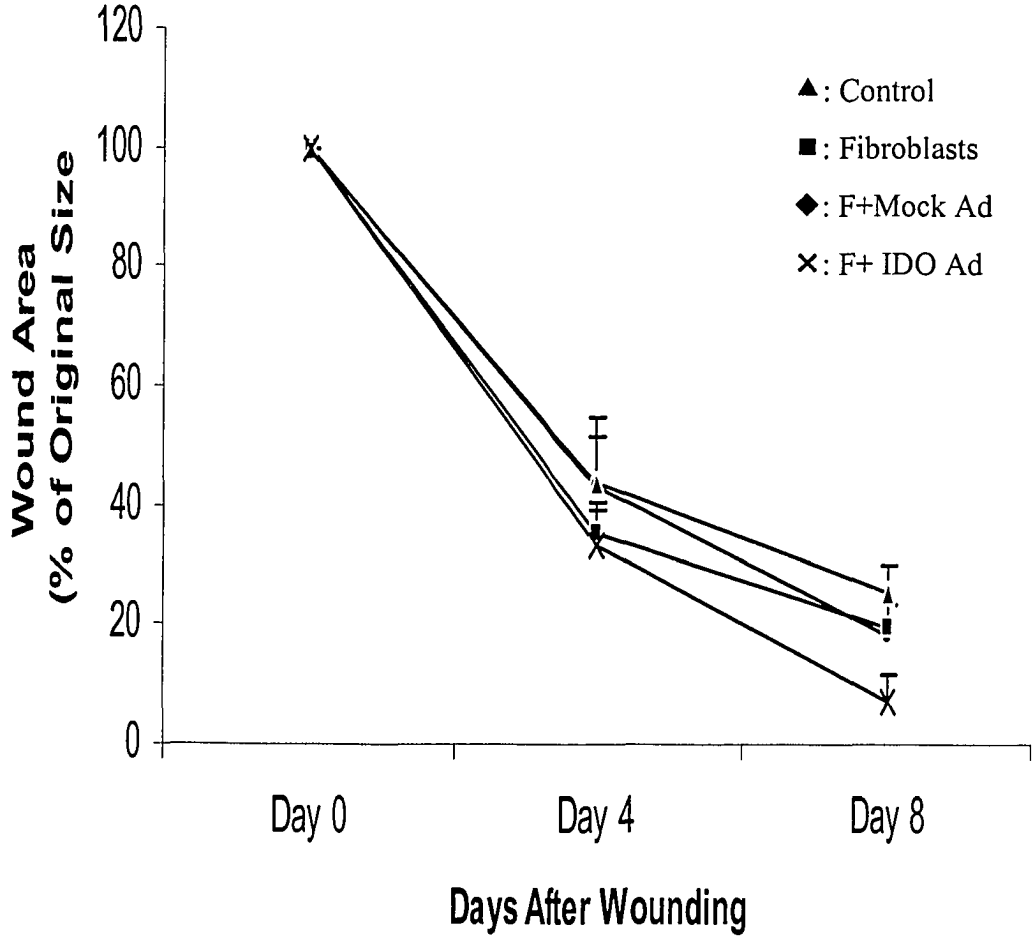


Fig. 4-1B



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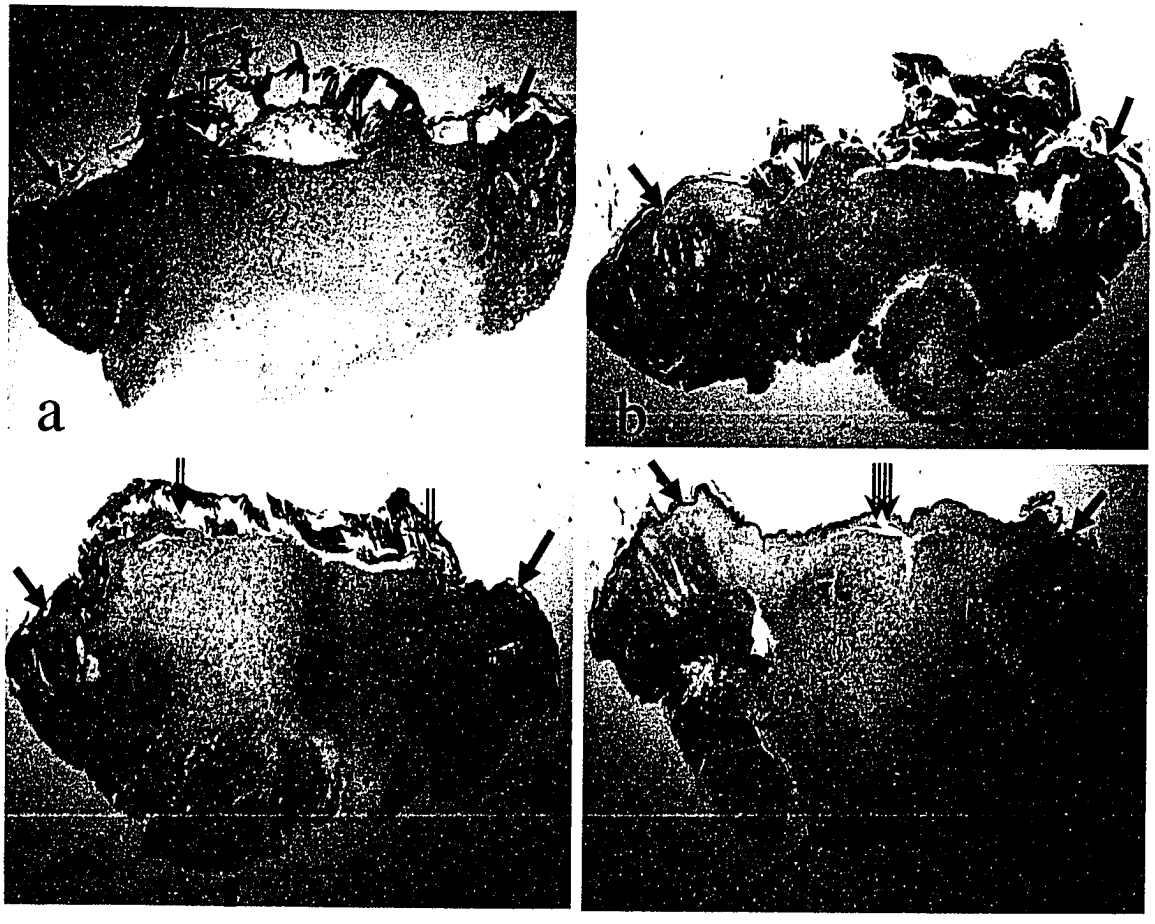


Fig. 4-2

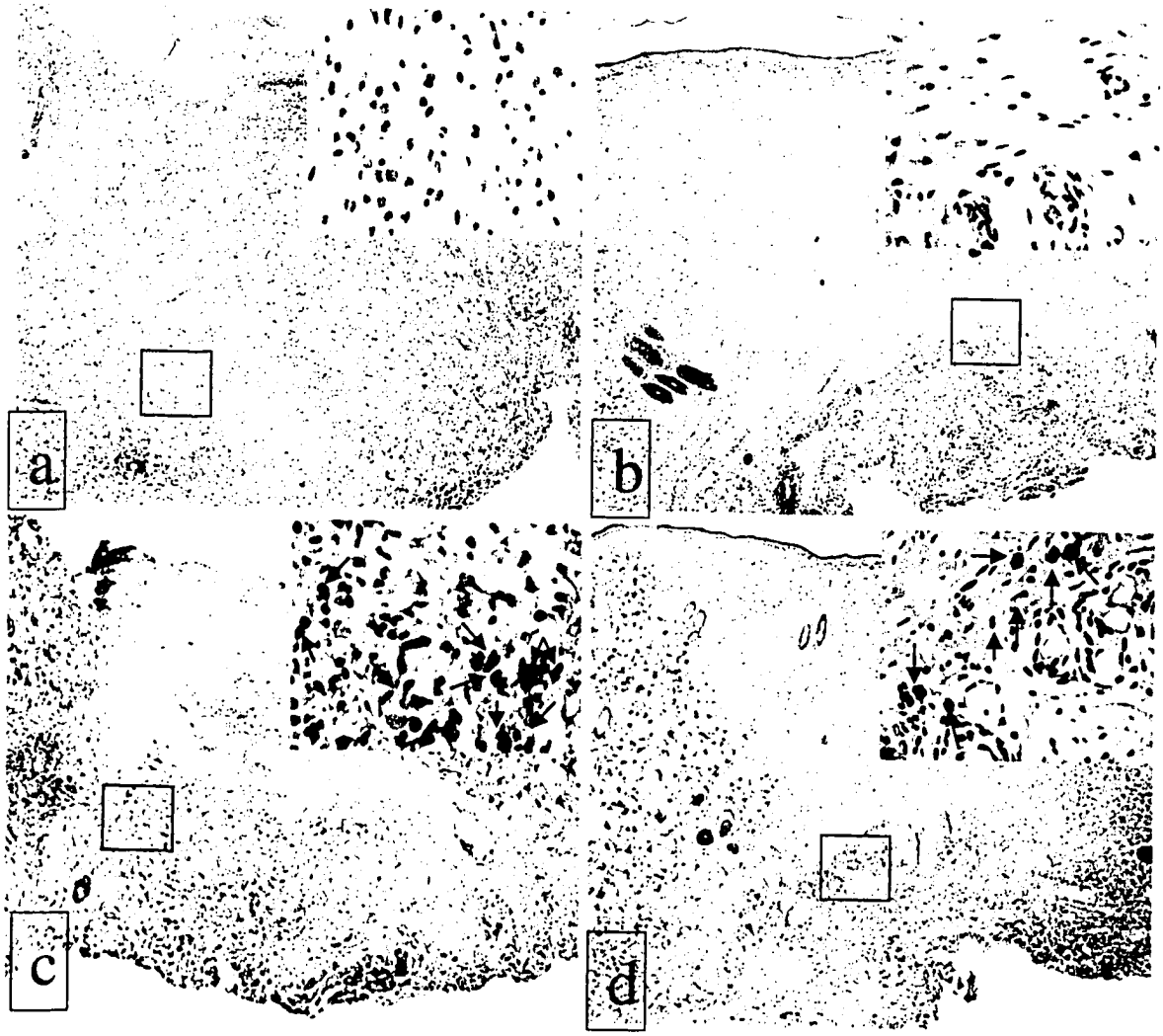
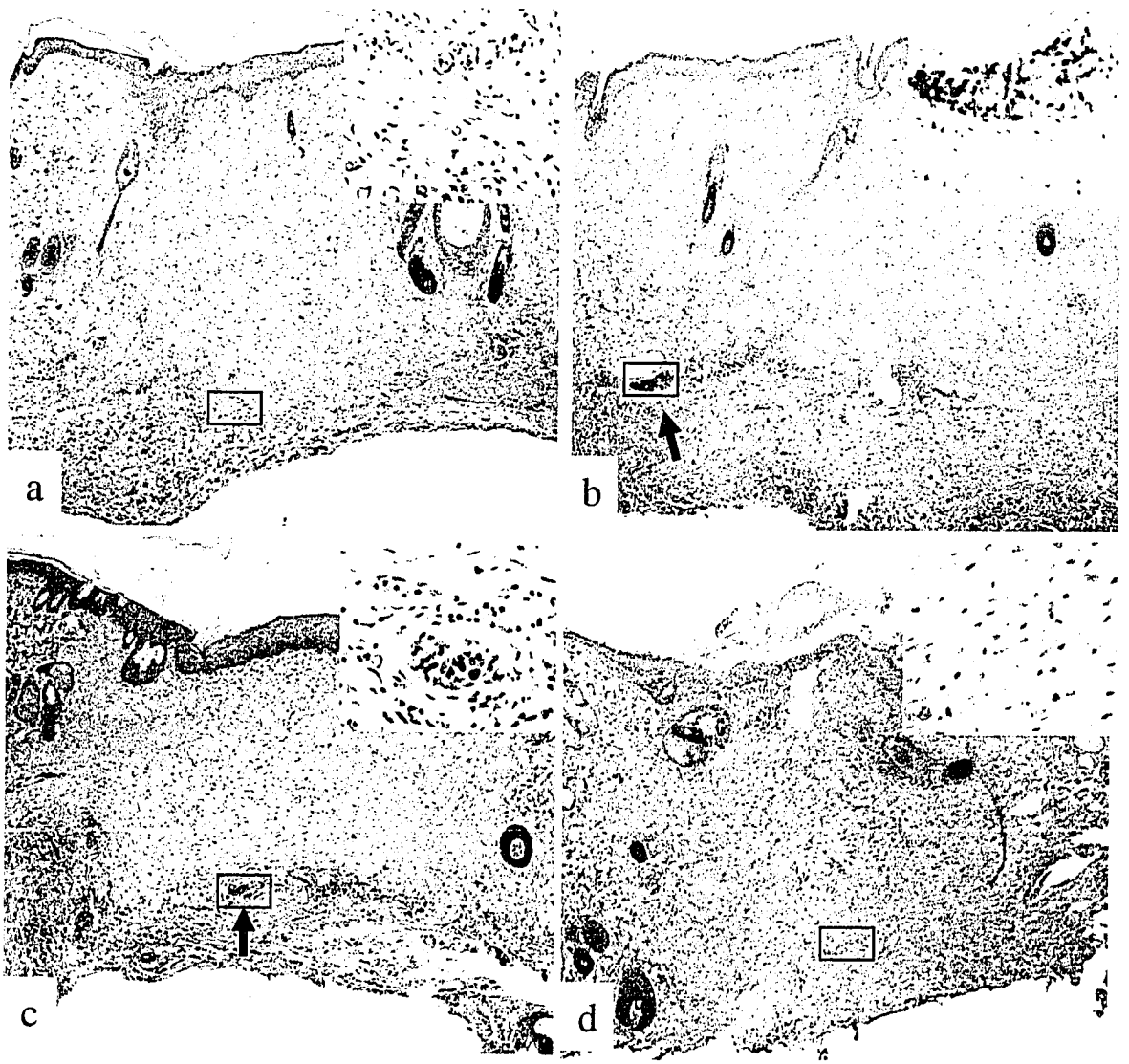


Fig. 4-3

Fig. 4-4A



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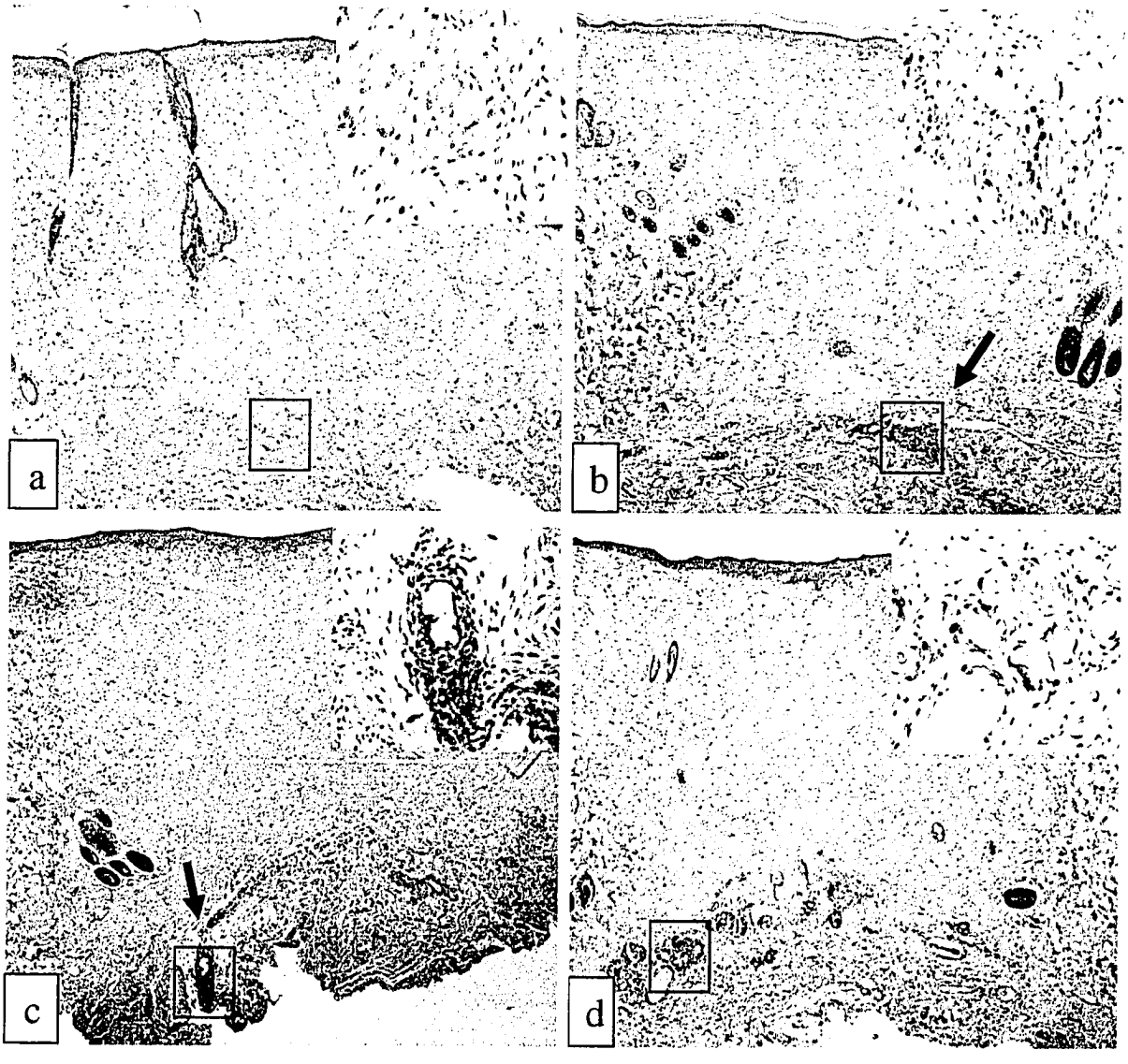

Fig.4-4C

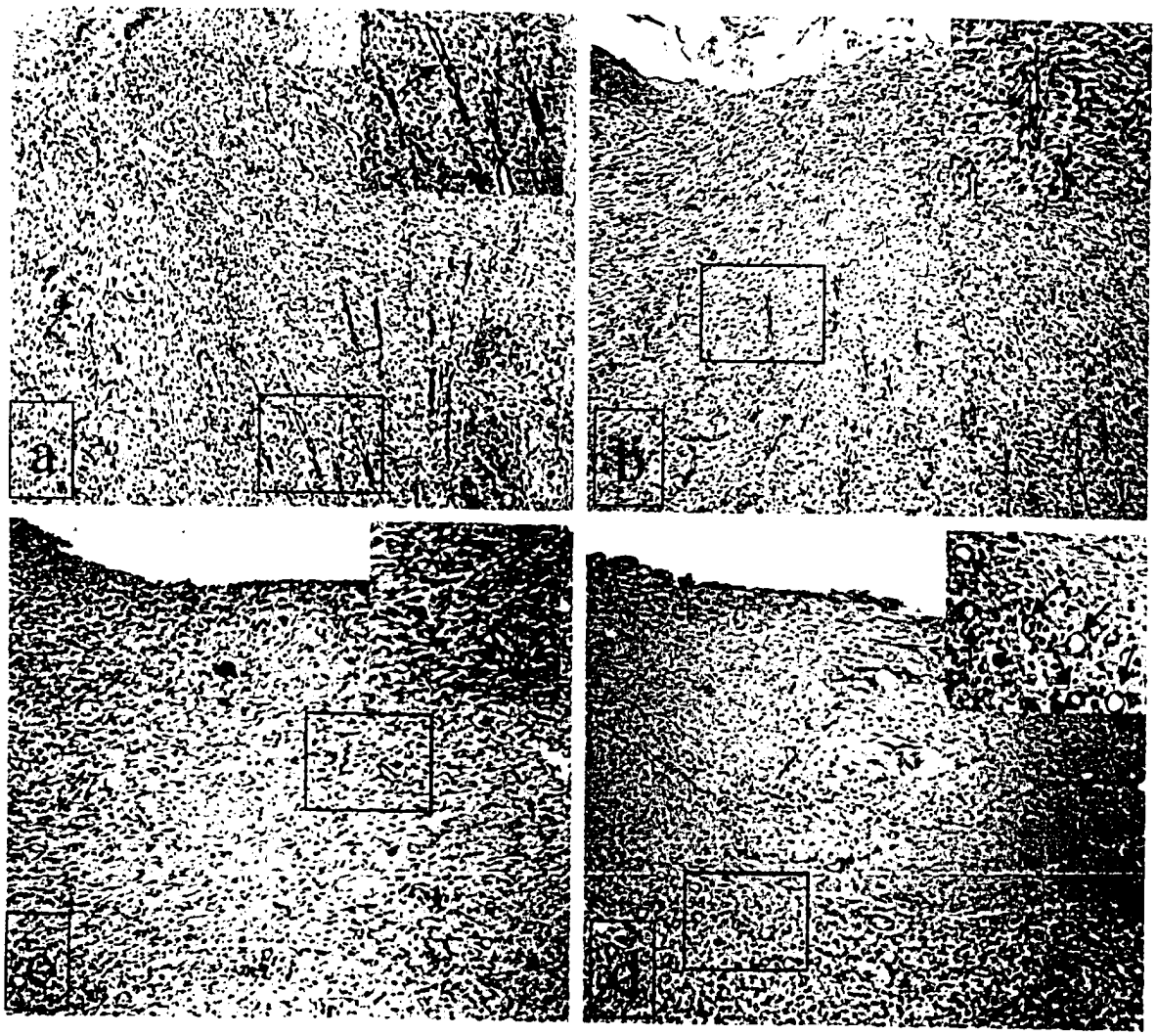

Fig. 4-5A

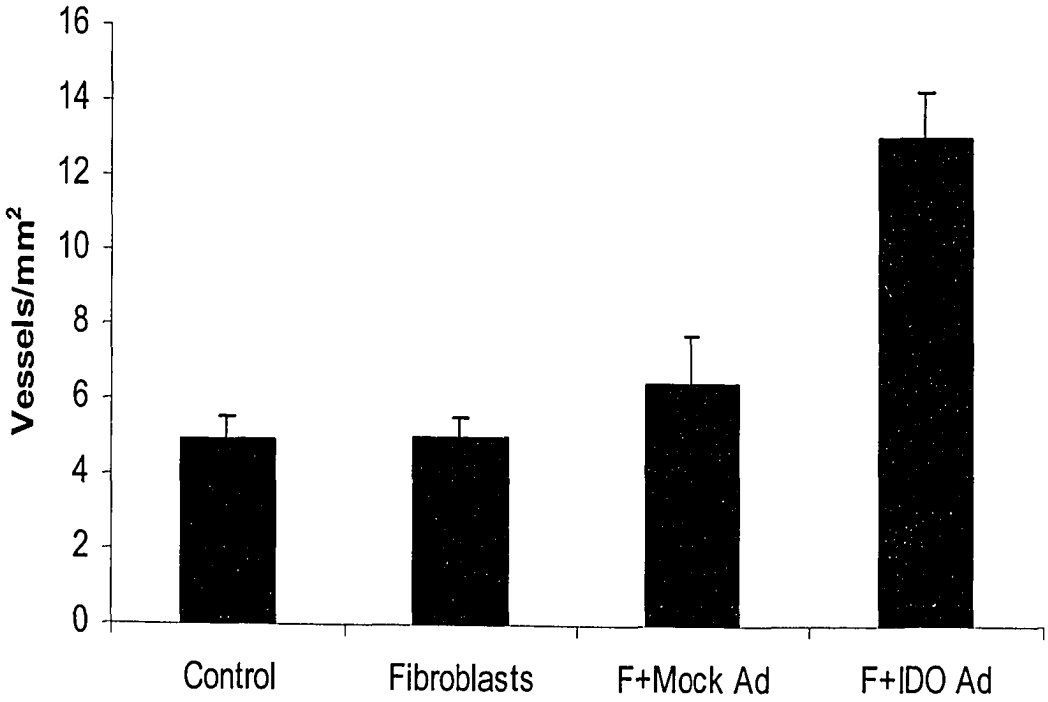

Fig. 4-5B

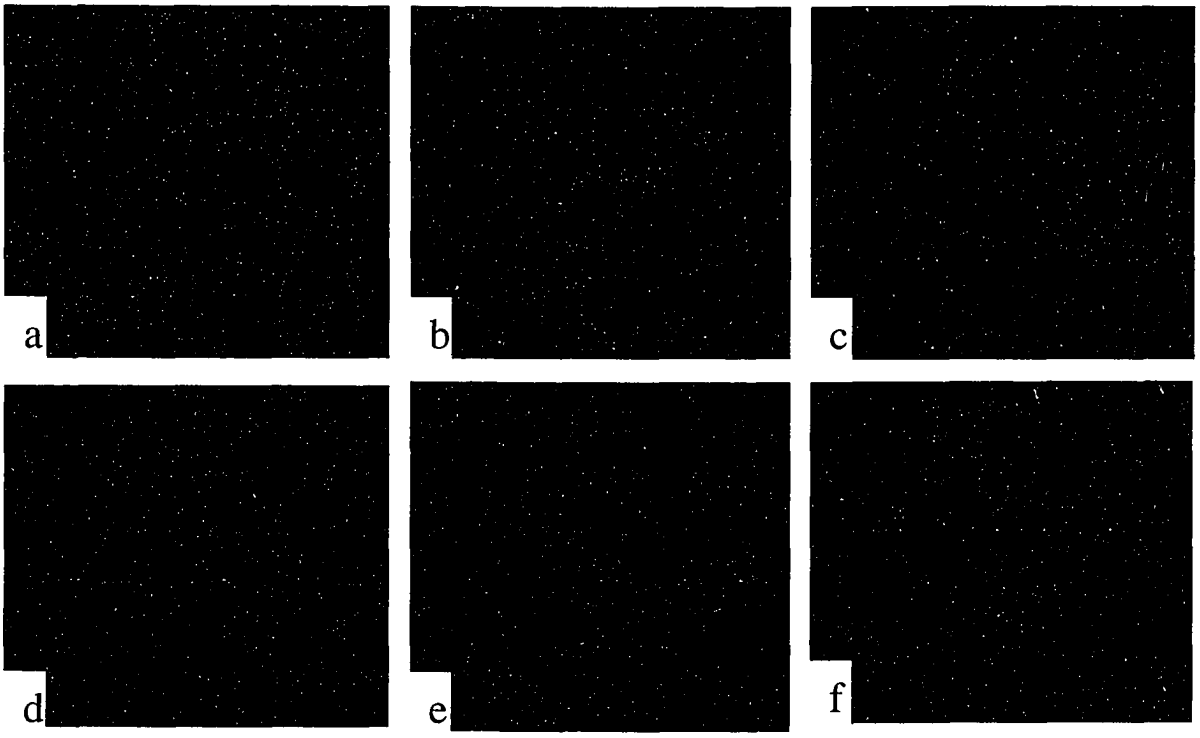

Fig. 4-6

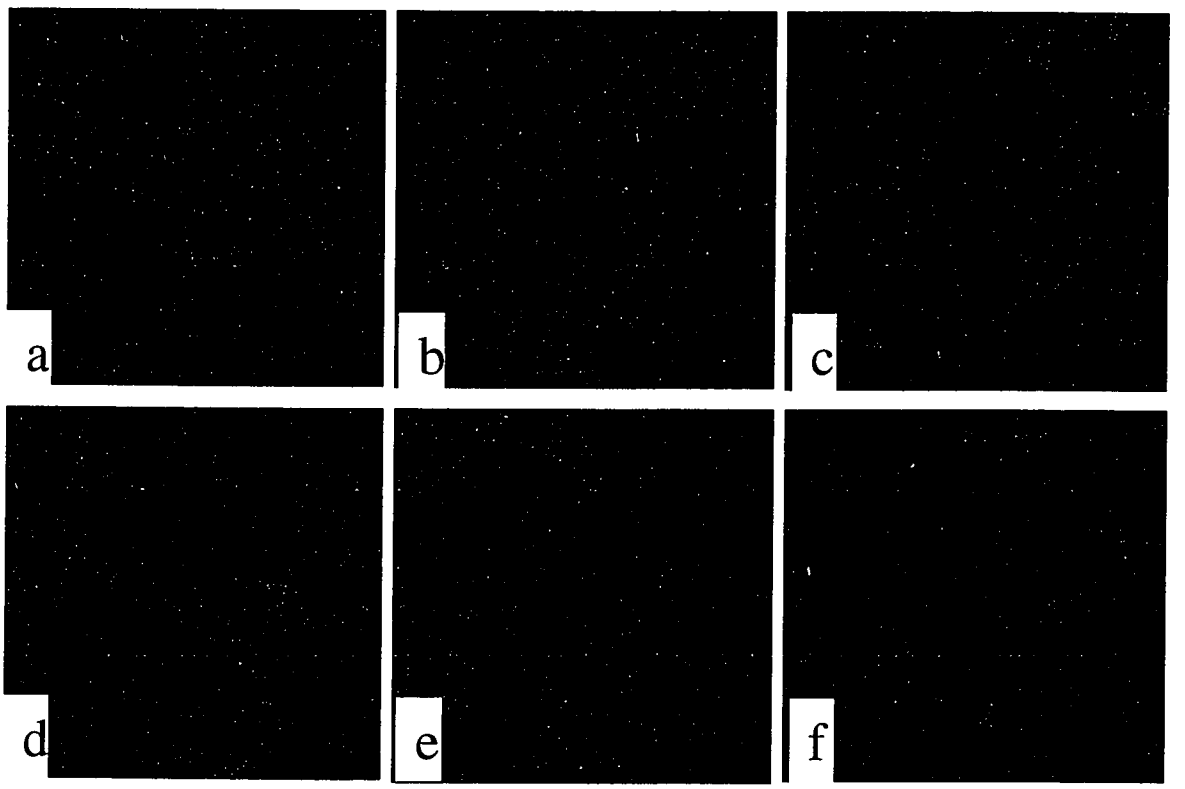

Fig. 4-7

Chapter 5

General Discussion and Conclusions

I. General Discussion and Conclusions

In an effort to generate a non-rejectable skin substitute, the current study was undertaken through a series of experiments. We have: 1) generated a recombinant control adenovirus containing GFP gene only and a recombinant IDO adenovirus containing human IDO plus GFP genes; 2) successfully transfected IDO into human dermal keratinocytes, fibroblasts as well as HUVECs with these recombinant adenoviruses; 3) demonstrated that IDO expressed in fibroblasts significantly induces bystander Jurkat cell, THP-1 monocyte and PBMC apoptosis via depletion of tryptophan in a co-culture system; 4) demonstrated that normal primary skin cells and HUVECs are not sensitive (including the effects of proliferation and survival) to low levels of tryptophan in conditioned medium; 5) demonstrated that IDO expressed by keratinocytes down-regulates MHC class I expression on the cell surface, which is also tryptophan dependent; 6) demonstrated that engraftment of IDO into genetically human fibroblasts populated in collagen gel accelerates wound healing in a rat model; 7) demonstrated that IDO could improve engraftment of human xenogeneic fibroblasts; and 8) found that IDO promotes angiogenesis *in vitro* and *in vivo* via degradating tryptophan in the local microenvironment. In addition, we have also produced a recombinant human IDO protein in *E. Coli.* and generated a polyclonal antibody.

Our first specific aim for this study was to generate a recombinant adenovirus containing the human IDO gene and to use this virus to transfect skin fibroblasts and keratinocytes. It is well known that one of the major advantages of using adenoviral vectors for delivery of therapeutic DNA is that both dividing and non-dividing cells can be infected readily and most cells are susceptible to infection (1). Meanwhile, adenoviral DNA is not incorporated into the target cell genome, thereby avoiding any potential untoward effects of gene insertion (2). Therefore, it was anticipated that this approach would generate a high efficiency of transfection and avoid severe side effects on virus infected skin cells. Indeed, in the present study, more than 90% of keratinocytes were shown positive for GFP expression. This was consistent with previous studies in which keratinocytes were shown to be susceptible to infection by adenoviral vectors (3-5). Although it has been indicated that adenovirus receptor is low or absent in human fibroblasts (6), and they are difficult to be infected with adenovirus type 5 vectors (7), in our study, through the use of a higher MOI and extension of virus exposure time, 30-50% of GFP expressing cells shown to be positive as evaluated by FACS analysis. Therefore, adenoviral vectors are suitable to transduce primary keratinocytes and fibroblasts.

We next analyzed the expression of IDO protein in infected cells. As expected, by western blot using a polyconal antibody, we detected a 42 kDa protein in IDO recombinant adenovirus infected cells, which was

consistent with the molecular weight of human IDO (8). Importantly, the levels of kynurenine used an index for IDO induced tryptophan degradation increased significantly in conditioned medium collected from cells infected with IDO adenovirus. These findings collectively suggest that IDO expressed in these cells is also active.

Despite the fact that endogenous IDO contributes to the maternal tolerance towards the allogeneic fetus (9), host tolerance toward tumor (10), self-tolerance in NOD mice (11), inducing experimental asthma (12) and autoimmune diseases (13), the mechanisms by which IDO mediates its immune suppressive effect are not completely understood. In term of its wide expression throughout the immune system, and more specifically in monocyte-derived macrophage and dendritic cells (14-17), it was previously hypothesized that immune suppression by IDO in a local environment might be attribute to IDO expressing antigen presenting cells which transfer into local lymph nodes and can inhibit immune cell proliferation in the local lymph nodes (18, 19). Indeed, recent studies demonstrated that IDO expressed in macrophages and dendritic cells could suppress lymphocyte proliferation in vitro (17, 20). IDO expression in antigen presenting cells might be involved in the mechanism of its in vivo immune regulation. However, it is not clear whether other cells that express IDO and also participate in this process in a local environment possess the same effects. For example, what is the function of IDO that is expressed by trophoblasts of the placenta and tumor cells? Will

IDO expressed in skin cells inhibit an immune rejection? Obviously, these IDO expressing cells cannot be transferred into the local lymph nodes to suppress lymphocyte proliferation. To clarify these questions and to provide theoretic evidence to determine if the engraftment of IDO genetically modified skin substitutes would be non-rejectable, we performed a co-culture experiment in which immune cells were co-cultured with IDO expressing fibroblasts. We found that IDO not only suppresses lymphocyte proliferation but also induces CD4+ Jurkat cell, THP-1 lymphocyte, CD4 +, CD8+ and B cell apoptosis. The effect of IDO on bystander immune cells could be abrogated by the addition of tryptophan. This finding strongly suggest that IDO, in fact, can directly damage surrounding immune cells via the depletion of tryptophan. According to our current findings, for the first time, we postulated that IDO functions as a local immunosuppressive factor via the depletion of local tryptophan which in turn induces surrounding activated/ resting T lymphocyte apoptosis. Thus the role of IDO in the local environment might not be related to the immune response in the local lymph nodes.

We also tested whether IDO expressed in skin cells will affect normal skin cell proliferation and induces skin cell and blood vessel endothelial cell damage. Results showed that IDO expressed in keratinocytes, fibroblasts and endothelial cells, does not significantly inhibit their proliferation nor damage these normal primary cells as compared to that co-culture with mock adenovirus infected cells. These results were further

confirmed by *in vivo* experiments in a rat model. In this animal model, engraftment of IDO expressed human fibroblasts into excised wounds did not delay the healing. In contrary, it accelerated wound healing as compared to either non-treated wounds or wounds receiving either non-treated fibroblasts or mock adenovirus infected fibroblasts. The mechanism in which wound healing has been promoted by IDO needs to be further explored.

It is well established that trophoblasts and most tumor cells are deficient in MHC class I on their cell surface (21, 22). On the other hand, these cells express IDO that likely to be responsible for MHC class 1 deficiency seen in these cells. The best example of the relationship between MHC class I and IDO expression is the fact that the anterior chamber of the eye, an immune privileged site, has high IDO activity and simultaneously lacks MHC class I expression (23, 24). It was known that infection by viruses such as HIV, CMV and poliovirus induces cells to express IDO and down-regulate MHC class I molecules. According to this information, we hypothesized that IDO expressed in some cells or tissues might affect MHC class I expression. In the present study, we found that IDO expressed in skin keratinocytes significantly down-regulates MHC class I molecules on the cell surface. Suppression of MHC class I proved to be tryptophan dependent as an addition of tryptophan restored the low levels of MHC class I. Our findings are, therefore, considered to be the first indication that IDO down-regulates MHC class I expression. MHC class I molecules are necessary for the

presentation of peptide antigens to CTLs (25). Total or partial loss of MHC class I is considered to be one of the main mechanism through which tumor and viral infected cells are not attacked by the immune cells (26-28). Therefore, this finding further indicates that skin cells, genetically modified with IDO, will be non-rejectable.

We also determined whether the immunosuppressive function of IDO seen in vitro could be used to protect skin cells genetically modified with IDO from host immune rejection. By engraftment of either non-treated, a mock adenovirus or IDO adenovirus infected human fibroblasts populated in collagen gels into a rat model, we found that engraftment of fibroblasts with or without a mock adenovirus infection could initiate an immune response. This is mainly because we have found a significant increase in the number of infiltrated CD3 positive T lymphocytes on day 14 and 28, and disappearance of grafted cells. However, wounds receiving IDO adenovirus infected human fibroblasts showed a significant decrease of infiltrated CD3 positive T lymphocytes and survival of grafted fibroblasts. The data presented in our study seems to support the fact that xenogeneic fibroblasts are recognized by lymphocytes and provoke an immune rejection, and IDO could protect xenografted fibroblasts from host immune rejection.

There were clear differences in the number of blood vessel-like structure by H &E staining in wounds receiving IDO genetically modified

fibroblasts compared to other controls on day 8 post-transplantation. To compare the number of blood vessel like structures in these wounds treated with different regiments, we further stained with endothelial cell marker, CD31. We found that there was at least a 2 to 3 fold increase in the number of capillary-like structures in wounds receiving IDO genetically modified fibroblasts as compared to other controls on day 8. However, the difference in blood vessel density was no longer significant on day 14 and day 28, indicating that IDO would not constitutively promote angiogenesis so that cause abnormal wound healing once a capillary network is built and the depletion of tryptophan is improved. The result showing the promotion of IDO induced angiogenesis *in vivo* was further confirmed by a co-culture experiment performed in vitro. HUVECs co-cultured with IDO expressing fibroblasts showed to be extended, elongated and branched out to form a capillary-like structure for a period of 20 days. Furthermore, we found that formation of these structures is induced by the depletion of tryptophan. As the lack of vascularization after grafting has been suggested to be one of the major reasons for poor graft take and survival of tissue-engineered reconstructed skin (29), we speculated that the initiation of revascularization by IDO genetically modified fibroblasts at the early stage of post-transplantation will drastically improve the outcomes of tissue engineered organs.

To conclude, in this thesis, I propose that IDO plays an important role in the modulation of local immune response through at least two novel

mechanisms of IDO inducing bystander immune cell death and down-regulation of MHC class I molecules on the cell surface. In addition to its suppressive function, IDO also promotes neovascularization, an important factor for engraftment take. Therefore, these results confirm that a skin substitute containing IDO genetically modified allo- or xeno- geneic skin cells would be protect from host immune rejection when grafted. As such, the findings of this study collectively suggest the potential use of IDO expressing cells for development and application of a non-rejectable skin substitute to be used as both a wound coverage as well as a rich source of wound healing promoting factors.

II. Suggestions for Future Studies

A. Increase of Adenoviral Vector Transduction Efficiency in Skin Fibroblasts

Introducing a functional gene of interest into cultured cells offers a powerful mean to study the possible biological role of this gene in a given biological system. Adenoviral vectors have efficiently been used to deliver these genes of interest into target cells. However, several cell types and tissues are refractory to recombinant adenovirus infection, mainly because of low level expression of coxackievirus-adenovirus receptor (CAR). These include endothelium, smooth and adult skeletal muscle, brain tissue, primary tumors, hematopoietic cells, and skin fibroblasts (6, 30-34). Although we have achieved about 30% efficiency of infection in fibroblasts with recombinant adenoviral vectors by a high MOI and extension of virus exposure time, there is a need to

improve both the rate of infection as well as reproducibility of IDO gene in target cells such as fibroblasts.

Several strategies have been tested to increase the efficiency of adenovirus infection. Kim et al. used bifunctional molecules with soluble extracellular domain of CAR fused to peptide-targeting ligands and showed a significant improvement in transduction efficiency of adenoviral vectors from 8.3% to 80% (35). These peptide ligands include a cyclic RDG peptide and the receptor-binding domain of apolipoprotein E. Oriliky and Schaack reported that either Superfect, Polylysine or Cholesterol mixed with adenovirus before exposure to cells would increase the transduction efficiency from 0.1% to 30-45% (36). It is also reported that the use of human albumin solder could increase the transduction of adenovirus into targeted cells (37).

According to these pioneer works, we would propose to test whether these approaches would improve the transduction efficiency of our recombinant adenovirus. The efficiency of transduction would be evaluated by flow cytometry for GFP expression. Cell proliferation and cytotoxicity would be tested as described in this thesis.

B. To Explore the Molecular Mechanism by which Immune, but not Primary Skin Become Sensitive to Depletion of Tryptophan

One of the remarkable findings in our studies was to show that there is a significant difference in cell survival between immune cells and normal skin cells as well as blood vessel endothelial cells to the depletion of tryptophan. As discussed previously, two mechanisms have been postulated to account for the inhibition of lymphocyte proliferation by IDO: the mammalian target of rapamycin (mTOR) signal pathway and the GCN2 stress-kinase pathway.

To clarify whether these molecular mechanisms are accountable for the different sensitivity between T cells and other primary cells to the depletion of tryptophan. We would propose to perform several sets of experiments.

Firstly, we would test whether mTOR, GCN2 kinase and eIF2α are expressed differently in T cells, fibroblasts, keratinocytes, and blood vessel endothelial cells. Secondly, in a co-culture system, CD4 + Jurkat cells would be co-cultured with either fibroblasts, keratinocytes or endothelial cells with or without rapamycin, and cell proliferation and survival would be evaluated. This approach would address whether mTOR activation only affects immune cells but not normal skin cells and endothelial cells. Thirdly, siRNA would be used to knockout either mTOR or GCN2 expression in Jurkat cells, and Jurkat cells would then be co-cultured with or without IDO expressed fibroblasts. The proliferation and cell survival of Jurkat cells would further be evaluated as described in this thesis.

C. Further Evaluation of IDO genetically Modified Skin Substitutes Containing Fibroblasts and Keratinocytes in Xenotransplantion

It became clear that a bilayered skin substitute with dermis and epidermis was a desirable goal for a more definitive treatment of burn patients (38). Cultured allo- or xeno-keratinocytes has been proved to have high immunogeneicity and are rejected when they are grafted on normal immunocompetent mice (39, 40). For the ultimate goal of making a clinically applicable and non-rejectable skin substitute, we propose to determine whether IDO can improve and /or prevent engraftment of allo or xeno-grafting bilayered skin substitutes rejection. To perform this experiment, skin cells including fibroblasts and keratinocytes would be infected by IDO recombinant adenovirus and a bilayered skin substitute would be prepared by using fibroblasts populated collagen gel on which keratinocytes are seeded. The immune response and the fate of skin substitute applied on the healing wounds would be evaluated using the methods as described in this study.

III. References

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