Local Expression of Indoleamine 2,3-Dioxygenase Protects Engraftment of Xenogeneic Skin Substitute

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The expression of indoleamine 2,3-dioxygenase (IDO), which metabolizes tryptophan, an essential amino acid, into kynurenine, has been identified as having a key role in the prevention of the immune rejection of the semi-allogeneic fetus during pregnancy. We have previously demonstrated that IDO expressed in fibroblasts causes bystander $CD4^+$ T cell damage as well as THP-1 cell damage by apoptosis. As T cells are primarily responsible for graft rejection, here, we asked the question of whether engraftment of IDO-expressing xenogeneic fibroblasts populated in a collagen matrix can be immuno-protected in an animal model. The results show a significant reduction in the number of infiltrated $CD3^+$ T lymphocytes on days 14 and 28 post-transplantation in the wounds receiving IDO-expressing fibroblasts relative to controls. IDO-expressing human fibroblasts embedded in bovine collagen on wounds in a rat model accelerates wound healing by promoting neovascularization during the early stages and providing protection of the xenograft fibroblasts. Using a co-culture system, we further confirm that IDO can induce angiogenesis through the depletion of tryptophan. These findings suggest that IDO may have an application in promoting the engraftment of skin substitutes and other transplanted organs.

Journal of Investigative Dermatology (2006) **126,** 128–136. doi:10.1038/sj.jid.5700022

INTRODUCTION

The need for alternative, immediate, and permanent wound closure materials has promised potential applications for the treatment of cutaneous wounds. One approach postulated by our research group is to generate a functional skin equivalent with a local immunosuppressive factor such as indoleamine 2,3-dioxygenase (IDO) incorporated to genetically modify either allo- or xenogeneic skin cells (Li *et al.*, 2004b). IDO is a rate-limiting enzyme that converts tryptophan to *N*-formyl-kynurenine (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 semi-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 downregulates major histocompatibility complex (MHC) class I levels on the surface of IDO-expressing keratinocytes (Li *et al.*, 2004a). Based on this information, we hypothesize that skin cells genetically modified with an IDO gene would be non-rejectable.

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 when vascularization at the transplantation site is formed, several trophic factors important in graft take are released, and grafted cells survive (Boyce *et al.*, 1995). Re-vascularization is characterized by the development of new capillaries from pre-existing 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. Re-vascularization is vital and highly regulated during a variety of normal physiological conditions, including ovulation, embryonic development, and wound healing (Rissau, 1997). As IDO catalyzes the degradation of tryptophan that causes bystander immune cell damage, we then asked the question of whether the expression of IDO would damage or 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 showed 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*

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Abbreviations: DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanin; GFP, green fluorescent protein; HUVEC, human umbilical vein endothelial cells; IDO, indoleamine 2,3-dioxygenase; mTOR, mammalian target of rapamycin

Received 16 June 2005; revised 8 August 2005; accepted 29 August 2005

and *in vitro,* and IDO can protect grafted cells from host immune rejection.

RESULTS

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 the conditioned medium were then determined. As described in the previous study (Li *et al.*, 2004b), about 30% of fibroblasts were infected (data not shown). As shown in Figure 1a, the kynurenine levels in IDO adenoviral-infected fibroblast conditioned medium were significantly increased as compared to either non-treated or empty vector-infected fibroblast conditioned medium. To assess the effect of IDO on wound healing, normal human fibroblasts, mock recombinant adenovirus-infected human fibroblasts, or IDO recombinant adenovirus-infected human fibroblasts, embedded in bovine collagen gels, were applied onto 6 mm full-thickness wounds generated in the dorsal skin of rats. The sizes of wounds that received IDO adenovirus-infected fibroblasts were indistinguishable from those that received no treatment, uninfected human fibroblasts, or mock recombinant adenovirus-infected fibroblasts on day 4 (33.1 ± 7.2 *vs* 42.9 ± 12 *vs* 35 ± 3.9 *vs* $43.9 \pm 7.7\%$, IDO *vs* non-treated versus fibroblasts versus mock, $P > 0.05$) (Figure 1c). However, on day 8, all wounds grafted with IDO adenovirus-infected fibroblasts were completely healed, while the other wounds remained open (Figure 1b). As shown in Figure 1c, measurement of the wounds that received engraftment of IDO adenovirus-infected fibroblasts was significantly smaller compared to either non-treated grafts with non-treated human fibroblasts or grafts with mock recombinant adenovirus-infected human fibroblasts (7.0 ± 5.0 *vs* 18.4 ± 5.2 *vs* 19.6 ± 3.7 *vs* $25.3 \pm 4.9\%$, IDO *vs* non-treated versus fibroblasts versus mock, $P < 0.01$).

Examination of hematoxylin and eosin-stained wound sections revealed a dramatic difference in thickness of the epidermal layer between IDO collagen gels and all other control wounds. More rapid re-epithelialization was observed in wounds that received IDO adenovirus-infected human fibroblasts (Figure 2d) compared to other groups on day 8 post-transplantation; however, a marked delay in re-epithelialization was seen in either engraftment of non-treated human fibroblasts (Figure 2b) or engraftment of mock recombinant adenovirus-infected human fibroblasts (Figure 2c) compared to non-treated wounds (Figure 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 (data not shown). In contrast, wounds that received

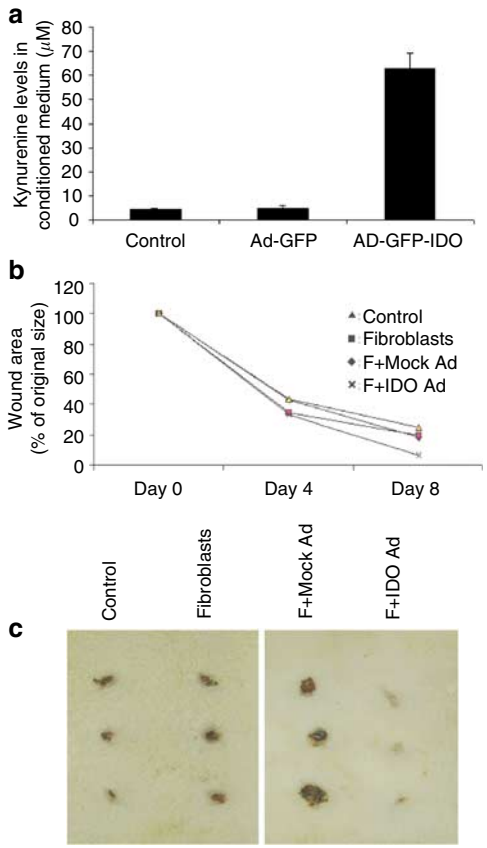

Figure 1. Grafting IDO genetically modified human fibroblasts populated collagen gels accelerates wound healing in rats. (**a**) Kynurenine levels in IDO-transfected fibroblast conditioned medium. Fibroblasts were infected by either Ad-GFP or Ad-GFP-IDO. Free viral particles were removed by washing the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

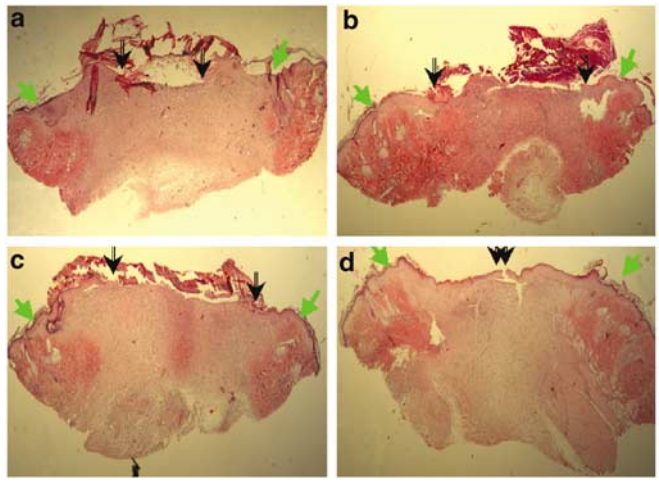

Figure 2. Hematoxylin and eosin staining of wound sections on day 8 for untreated (a), uninfected fibroblasts (b), mock adenovirus-infected fibroblasts (c), and IDO adenovirus-infected fibroblasts (d) treated wounds. The green arrows represent the wound edge, and the black arrows represent re-epithelialization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

either non-treated human fibroblasts or mock adenovirus-infected human fibroblasts showed a significant decrease in the amount of granulation tissue deposition and abundant inflammatory cell infiltration as compared to non-treated wounds (data not shown).

Efficacy of engrafting IDO genetically modified fibroblasts embedded in collagen gels in preventing acute host rejection

There have been conflicting reports of the survival and immune reaction of allografts of cultured skin cells, especially fibroblasts (Sher *et al.*, 1983; Hultman *et al.*, 1996; Erdag and Morgan, 2004). However, like other cell types (Schmidt *et al.*, 2003), xenogeneic fibroblasts have been shown to stimulate a high degree of host immune response (Isik *et al.*, 2003; Erdag and Morgan, 2004; 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 Figure 3a and b, there were no GFP-expressing cells within the wounds that received the mock adenovirus-infected fibroblasts on days 8 and 28, respectively. Similarly, we did not find GFP-positive cells in the control wounds on day 14 (data not shown). However, xenogeneic human fibroblasts expressing IDO survived and were easily detectable within the reticular dermis as indicated by GFP-positive staining on days 8 (Figure 3c), 14 (data not shown), and 28 (Figure 3d). These findings suggest that IDO does protect xenografted human fibroblasts from host immune rejection. Furthermore, as shown in Figure 3 and Table 1, there was no significant difference in the number of 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 days 14 and 28. Wounds that received either untreated human fibroblasts (Figure 4b, f) or mock adenovirus-infected fibroblasts (Figure 4c, g) showed massive infiltrated lymphocytes and some clustered lymphocytes distributed at the reticular dermis as compared to untreated wounds on days 14 (Figure 4a) and 28 (Figure 4e). However, wounds that received IDO adenovirus-infected human fibroblasts revealed a few infiltrated $CD3^+$ cells on days 14 (Figure 4d) and 28 (Figure 4d). This is a clear indication that the local expression of IDO by genetically modified xenogeneic fibroblasts suppresses the infiltration of T lymphocytes at the wound site.

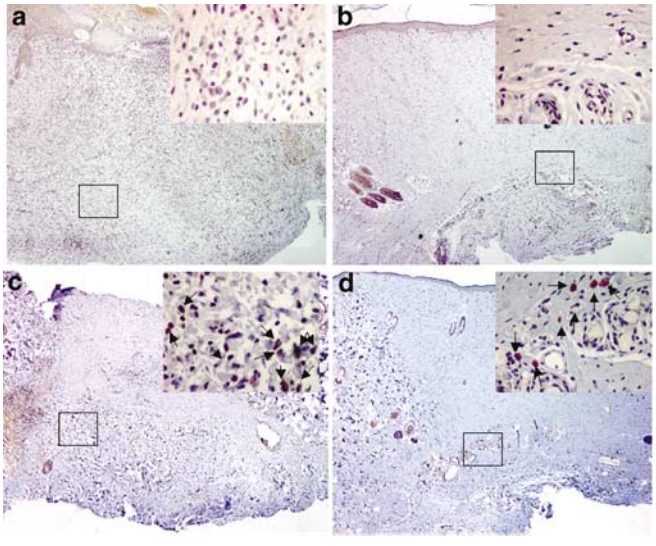

Figure 3. Detection of grafted fibroblasts by GFP immunohistochemistry staining. Sections of wounds that received either mock adenovirus-infected fibroblasts (**a**) at day 8 post-transplantation and (**b**) at day 28 post-transplantation, IDO adenovirus-infected fibroblasts (**c**) at days 8 and 28 (**d**) post-transplantation were prepared and GFP expression was detected by immunostaining. Arrows show GFP-positive cells.

Increased neovascularization in wounds that received IDO genetically modified fibroblasts embedded in collagen

Slow re-vascularization is thought to be a cause for the rejection of allo- and xenogeneic engraftment (Boyce *et al.*, 1995). To further evaluate the effects of IDO on neovascularization *in vivo*, we performed CD31 immunostaining on wound sections obtained from untreated control (Figure 5a), fibroblasts (Figure 5b), mock-infected (Figure 5c), and IDO adenovirus-infected fibroblasts grafted wounds (Figure 5d). The results showed 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 six sections randomly selected from three wounds of each group demonstrated a two- to three-fold increase in the number of vessels per mm^2 in wounds that received IDO-expressing fibroblasts relative to those obtained from wounds of either non-treated, those that received fibroblasts, or mock adenovirus-infected fibroblasts (Figure 5e, $P<0.01$). We also performed CD31 staining at later time points on another set of

Table 1. Infiltrated $CD3^+$ 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[1]	25.8±15.1[1]
Mock adenovirus fibroblasts	18.3±7.1	45.6±15.6[2]	23.4±21.2
IDO adenovirus fibroblasts	17.5±11.2	1.6±1.6[3]	5.7±5.1[3]

[1] $P<0.05$.
[2] $P<0.01$ versus untreated wounds, respectively.
[3] $P<0.01$ versus wounds receiving either untreated fibroblasts or mock adenovirus-infected fibroblasts.
n=10 high-power fields.
Data were expressed as the mean±SD.

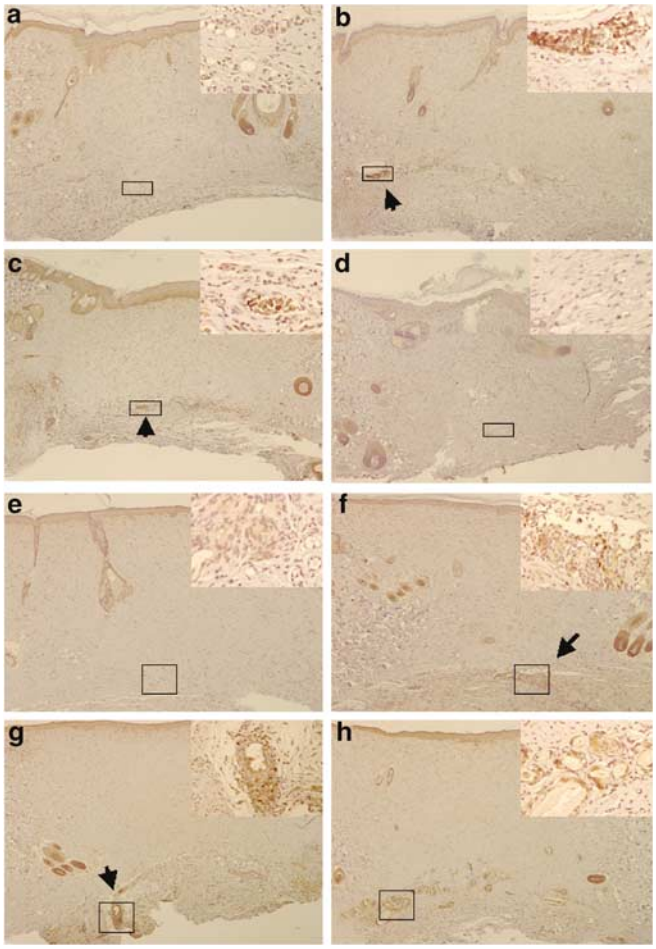

Figure 4. Detection of CD3$^+$-infiltrated lymphocytes within xenografts. Immunohistochemical staining of CD3$^+$ lymphocytes in sections of untreated wounds on days 14 (**a**) and 28 (**e**) as well as those wounds that received untreated fibroblasts on days 14 (**b**) and 28 (**f**) are shown. Similarly, immunostaining for CD31-expressing cells infiltrated within those wounds that received mock adenovirus-infected fibroblasts on days 14 (**c**) and 28 (**g**) as well as wounds that received IDO adenovirus-infected fibroblasts on days 14 (**d**) and 28 (**h**) are shown. The arrows show cluster distributions of infiltrated CD3$^+$ lymphocytes at the wound site.

samples obtained from animals that received similar treatment on days 14 and 28. The findings showed no significant difference in microvascular density between wounds that received IDO-expressing human fibroblasts and controls (data not shown).

IDO expression promotes the formation of vessel-like structures *in vitro*

To further investigate the impact of IDO expression on revascularization, 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 six-well plates. To distinguish endothelial cells from fibroblasts in our co-culture system, the living HUVECs 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. In this system, the DiI signal was clearly detectable for up to 30 days.

The results showed that the DiI-labeled endothelial cell cords were extended and elongated to form multicellular capillaries when co-cultured with IDO-expressing fibroblasts (Figure 6c) for a period of 20 days. However, these structures were not seen when HUVECs were grown in the presence of untreated fibroblasts (Figure 6a) or mock adenovirus-infected fibroblasts (Figure 6b) for the same period. These findings strongly support the results obtained from the *in vivo* experiment described above, indicating that IDO can directly initiate angiogenesis.

Depletion of tryptophan by IDO results in the formation of capillary-like structures *in vitro*

To determine how vessel-like structures 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 were evaluated. As shown in Figure 7, the addition of kynurenine at a concentration of 50 μg (Figure 7b) and 100 μg (Figure 7c) to the conditioned medium of co-culture HUVECs with non-treated human fibroblasts had no effect on the formation of capillary-like structure relative to that of control (Figure 7a). However, under similar experimental conditions, we found that the addition of L-tryptophan at a concentration of 50 μg (Figure 7e) and 100 μg (Figure 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 without the addition of tryptophan (Figure 7d). These data suggest that the depletion of tryptophan is probably the main reason by which capillary-like structures are formed in response to IDO expression.

DISCUSSION

A number of recent studies suggest that IDO may play a key role in local immunosuppression (Munn *et al.*, 1998, 1999, 2004; Friberg *et al.*, 2002; Uyttenhove *et al.* 2003; Hayashi *et al.*, 2004). In an attempt to develop a non-rejectable skin substitute containing either allo- or xenogeneic skin cells, we have conducted a series of experiments to test whether IDO can function as a local immunosuppressive factor to protect allo- or xenogeneic 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 the 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- or xenogeneic engraftment in an animal model. In this study, we found that wounds that received IDO-expressing human fibroblasts healed faster compared to controls (untreated wounds, wounds that received untreated human fibroblasts, or mock adenovirus-infected fibroblasts in an animal model). Further studies found that at least two mechanisms might be accountable for the promotion of healing in wounds treated with IDO-expressing fibroblasts. First, the logical explanation would

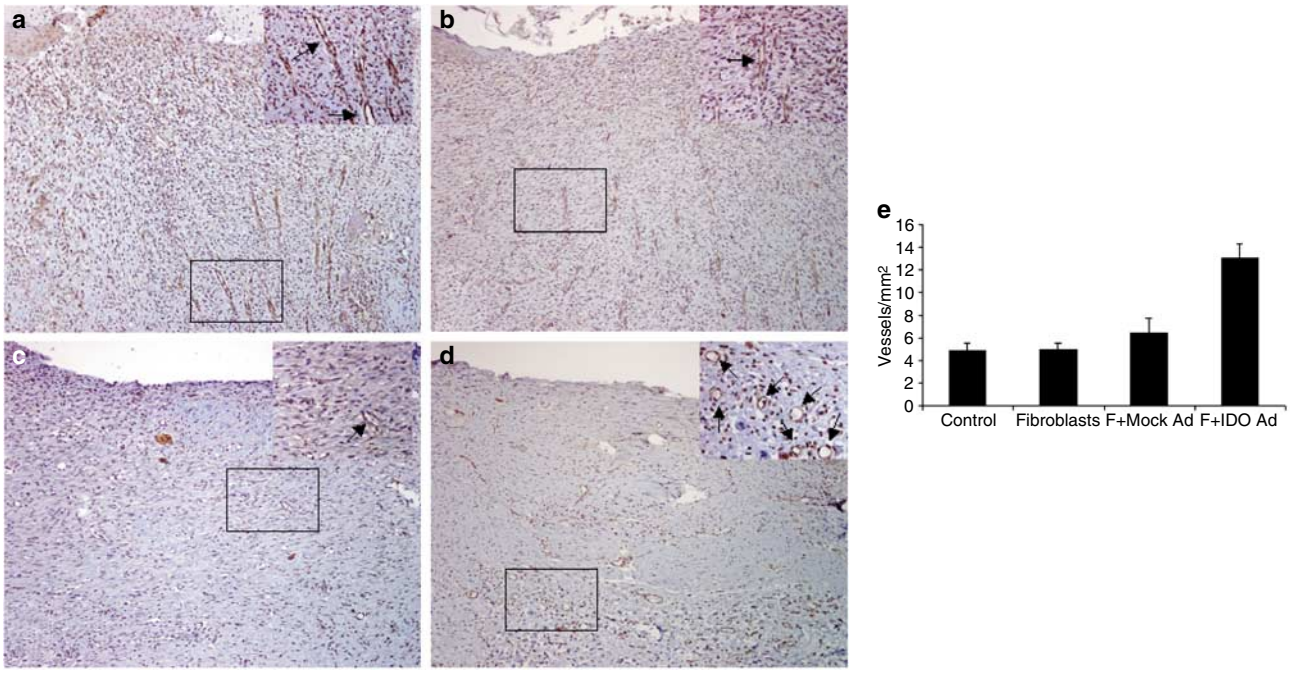

Figure 5. Detection and quantitative analysis of capillary-like structures in wound sections on day 8 post-transplantation. Either untreated (**a**), treated with uninfected fibroblasts (**b**), mock adenovirus-infected fibroblasts (**c**), and IDO adenovirus-infected fibroblasts (**d**) wounds were excised and sections were strained with CD31 antibody. (**e**) Quantitative analysis of capillary-like structures shown in (**a**). Data are expressed as the mean ± SD ($n = 6$).

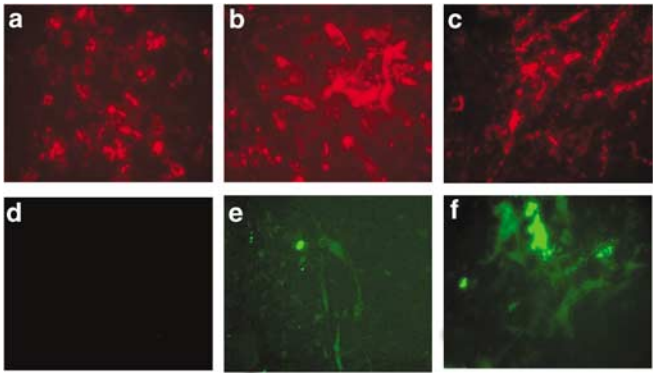

Figure 6. IDO induces tube formation in HUVECs co-cultured with IDO-expressing fibroblasts. DiI-labeled HUVECs were co-cultured with either untreated fibroblast (**a** and **d**), mock adenovirus-infected fibroblasts (**b** and **e**) or IDO adenovirus-infected fibroblasts (**c** and **f**), and DiI fluorescence (**a–c**) and GFP (**d–f**) fluorescence staining cells were then detected. Original magnification: × 400.

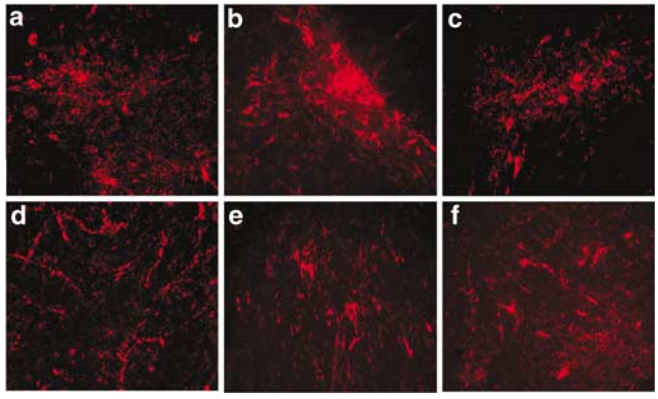

Figure 7. Depletion of tryptophan promotes the formation of capillary-like structures in HUVECs. DiI-labeled HUVECs were co-cultured with untreated fibroblasts in the absence (**a**) or in the presence of 50 μg/ml (**b**) and 100 μg/ml (**c**) of kynurenine for 20 days. Similarly, DiI-labeled HUVECs 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. Original magnification: × 400.

simply be that engrafted skin substitute remained intact due to the protective role of IDO and this resulted in earlier epithelialization, thus shortening the wound closure time. Another mechanistic reason might be the one that we have shown in this paper, indicating that IDO induces angiogenesis and this, at least in part, would promote the 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, we found that IDO directly promotes neovascularization as demonstrated *in vivo* and *in vitro*, which may in part be accounted for by the more rapid healing. Depletion of tryptophan by IDO in the local environment seems to contribute to the formation of capillary-like structures, mainly because an addition of tryptophan prevented endothelial cells forming these structures.

It has been well established that IDO expression by a variety of cell types can suppress the immune system in multiple ways. It has been shown that IDO depletes tryptophan availability in the microenvironment (Munn *et al.*, 1999), which damages surrounding activated lymphocytes (Li *et al.*, 2004b). In other studies, evidence shows that IDO promotes the generation of various toxic tryptophan metabolites such as 3-hydroxyanthranilic acid and quinolinic acid, which induce activated lymphocyte death (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.* (2005), demonstrating that activation of GCN2 kinase in T cells by uncharged tRNA mediates T cell proliferative arrest and anergy induction. They found that T cells with a targeted disruption of GCN2 were not susceptible to IDO-mediated suppression of *in vitro* proliferation. *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 control 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 kinase activity (Sood *et al.*, 2000). The activation of eukaryotic initiation factor-2α 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 idea is also supported by our previous study showing 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 days 14 and 28.

One of the main limitations in using skin substitutes for a wound coverage is delayed re-vascularization (Sahota *et al.*, 2004). 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 (Black *et al.*, 1998; Supp *et al.*, 2002; Sahota *et al.*, 2003) or by genetically modifying skin cells with vascular endothelial growth factor (Supp *et al.*, 2000, Supp and Boyce, 2002) have been studied. The results of these studies demonstrated that initiating early neovascularization significantly increases graft take. In addition to protecting xenografted skin cells from immune rejection, IDO also seems to initiate neovascularization in the early stages as shown in Figure 5. Direct evidence that IDO promotes angiogenesis was obtained using our co-culture experiment (Figure 6). Although understanding the mechanism of how IDO initiates angiogenesis *in vivo* and *in vitro* requires further study, the fact that IDO-mediated angiogenesis was reversed with the addition of tryptophan may indicate that the depletion of tryptophan by an unknown mechanism stimulates angiogenesis.

In summary, our findings suggest that IDO expression may function as a local immunosuppressive and neovascularization stimulatory factor that protects allo- or xenogeneic dermal grafts. If true, this will be the first report on the possibility of developing a non-rejectable skin substitute to be used for a variety of dermal wound healing disorders.

MATERIALS AND METHODS

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-Biogene, 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 described previously (Li *et al.*, 2004b).

Fibroblast culture and transfection

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

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

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, fibroblasts either with or without transfection in a density of 6×10^5/ml were mixed with type I collagen (4.29 mg/ml in 0.1% acetic acid), 3 × Dulbecco's modified Eagle's media, chondroitin-6-sulfate (3 mg/ml in 1 × Dulbecco's modified Eagle's media; Sigma, Oakville, ON, Canada), and fetal bovine serum at a ratio of 1.5:3.5:3:1:1. After adjusting the pH to 7.50 with 0.4 N NaOH, the mixture was added

directly to the culture plate. All dermal substitutes were maintained in Dulbecco's modified Eagle's media with 10% fetal bovine serum and used for transplantation within 1 week.

Grafting to Sprague–Dawley rats

Procedures on all animal studies were approved by the Health Sciences Lab Animal Service Animal Welfare Committee of the University of Alberta, Edmonton, Alberta, Canada. Rats (10 weeks old) 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 (three 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 transfection 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, USA), and the grafted areas were bandaged. On days 4 and 8, wound closure was measured and photographed. Animals were killed on days 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) phosphate-buffered saline solution and processed for paraffin embedding. The other half was snap-frozen by overlaying with cryomatrix (Thermo Electron Corp., Pittsburgh, PA) with immediate immersion in a dry ice bath. Tissues were stored at −80°C until analysis.

Histology and immunohistochemistry

Sections were mounted on slides and stained with hematoxylin and eosin using standard techniques. Wounds were evaluated for the extent of re-epithelialization, and the granulation tissues were evaluated based on formation, architecture, and cellularity.

Paraformaldehyde-fixed and paraffin-embedded sections (4 μm) were deparaffinized and hydrated by incubation in phosphate-buffered saline for 10 minutes. To retrieve cellular antigens, a microwave oven heating pretreatment was performed before blocking with 5% albumin in phosphate-buffered saline solution. For antibody staining, sections were incubated with either anti-GFP horseradish peroxidase-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 hour. The second antibody was horseradish peroxidase-conjugated anti-goat IgG (Sigma) used in CD31 staining or horseradish peroxidase-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 3,3′-diaminobenzidine-enhanced liquid substrate system (Sigma). The slides were counterstained with hematoxylin for 5 seconds, and then sections were dehydrated, mounted, and examined under microscopy.

Endothelial cell culture

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

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 according to a previously reported method (Bishop *et al.*, 1999). To distinguish the origin of the cells in the tube 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 described previously (Nehls *et al.*, 1998). Cells were then thoroughly washed with phosphate-buffered saline. An equal ratio of HUVECs and fibroblasts (5×10^5/well) was mixed and seeded onto a six-well plate. Cells were incubated in M199 supplied with 20% fetal bovine serum and endothelial cell growth supplement as described above. The medium was replaced every 2 days. Images of tube structures were taken at day 20 using a spot Jr digital camera (Diagnostic Instruments, Sterling Heights, MI) attached to a Leica PBIRB fluorescence microscope (Opti-Tech Scientific Inc., Scarborough, ON, Canada).

Kynurenine measurement

The biological activity of IDO was evaluated by measuring the levels of tryptophan-degraded product, L-kynurenine, present in the conditioned medium derived from IDO and control vector-transfected cells. The amount of L-kynurenine was measured by a previously established method (Takikawa *et al.*, 1988). Briefly, proteins in the conditioned medium were precipitated by trichloroacetic acid, and after centrifugation, 0.5 ml of supernatant was incubated with an equal volume of Ehrich's reagent for 10 minutes at room temperature. Absorption of the resultant solution was measured at 490 nm by a spectrophotometer within 2 hours. The values of kynurenine in the conditioned medium were calculated by a standard curve with the defined kynurenine (0–100 μM) concentration.

Statistical analysis

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

CONFLICT OF INTEREST

The author states no conflict of interest.

ACKNOWLEDGMENTS

This study was supported by the Toronto Hospital for Sick Children Foundation (A Ghahary, EE Tredget) and CIHR grants (A Ghahary, EE Tredget). We thank Dr JM Carlin (Department of Microbiology, Miami University, Oxford, OH) for his gift of human IDO cDNA and Dr ST Davidge (Department of Physiology, University of Alberta, Edmonton, Alberta, Canada) for her assistance in the preparation of primary endothelial cell cultures.

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