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Immune Cell Proliferation Is Suppressed by the Interferon-γ-Induced Indoleamine 2,3-Dioxygenase Expression of Fibroblasts Populated in Collagen Gel (FPCG)

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Abstract Indoleamine 2,3-dioxygenase (IDO), a tryptophan-catabolizing enzyme, is an intracellular enzyme possessing various immunosuppressive properties. Here, we report the possible use of this enzyme to suppress proliferation of immune cells cocultured with IDO-expressing fibroblasts of an allogenic skin substitute. Fetal skin fibroblasts embedded within bovine collagen were treated with cytokine interferon-γ (IFN-γ) to induce expression of IDO mRNA and protein. Expression of IDO mRNA was evaluated by Northern analysis. IDO enzyme activity was evaluated by measurement of kynurenine and tryptophan levels in the IFN-γ untreated and treated fibroblasts. The results of Northern analysis showed a dose-dependent increase in expression of IDO mRNA in response to various concentrations of IFN-γ used. The levels of kynurenine and tryptophan measured, as the bioactivity of IDO, were significantly different in the IFN-γ treated fibroblasts, compared to those of controls ($P < 0.001$). In a lasting effect experiment, the expression of IDO mRNA was gradually reduced to an undetectable level within 32 h of IFN-γ removal. The results of Western blot analysis, however, revealed a significantly longer (192 h) lasting effect of IFN-γ on IDO protein level, relative to that of mRNA expression. To demonstrate immunosuppressive effects of IDO on proliferation of immune cells, IDO-expressing fibroblasts were cocultured with peripheral blood mononuclear cells (PBMC) for a period of 5 days. The results of ^{3}H-thymidine incorporation showed a significant reduction in proliferation of PBMC when cocultured with IDO-expressing fibroblasts, compared to those cocultured with non-IDO-expressing fibroblasts ($P < 0.001$). Furthermore, addition of IDO-inhibitor (1-methyl-D-tryptophan) reversed the suppressive effects of IDO on PBMC proliferation in a dose-dependant fashion. To test the viability of immune cells cocultured with IDO-expressing fibroblasts, FACS analysis of the PI stained PBMC was conducted and no significant difference was found between these cells and the controls. In another set of experiments, we showed that migration rate and subsequent proliferation of IDO-expressing fibroblasts are also the same as those of control cells. In conclusion, IDO-expressing allogenic fibroblasts embedded within collagen gel suppress the proliferation of allogenic immune cells, while they still remain viable in this IDO-induced tryptophan-deficient culture environment. J. Cell. Biochem. 90: 206–217, 2003.

Key words: indoleamine 2,3-dioxygenase (IDO); interferon-γ; fibroblasts; skin substitute

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

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layer of lethally irradiated 3T3-cells was initially introduced by Rheinwald and Green [1975]. These investigators were later able to grow keratinocytes to confluency, 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 [Morhenn et al., 1982; Thivolet et al., 1986], this has not been a routine procedure due to: (1) sheets of keratinocytes prepared from layers of cultured keratinocytes without matrix are very fragile and difficult to cultivate, (2) the rate of graft-take is relatively low (50–60%), (3) patients with large injuries do not have enough of uninjured skin to be used for cell culture, (4) leaving the burn wound open for 3–6 weeks until cells become available, increases the risk of infection, heat, and fluid loss, and finally (5) generating another wound would further increase the risk of 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 allogenic and readily available skin substitute seems logical. Therefore, we have designed and recently conducted a series of experiments to examine the benefit of using allogenic fibroblasts expressing indoleamine 2,3-dioxygenase (IDO) as a local immunosuppressive factor.

IDO is an intracellular-catalyzing enzyme that converts tryptophan, the least available essential amino acid, into *N*-formylkynurenine, which is then converted to L-kynurenine by the enzyme formamidase [Takikawa et al., 1986]. IDO-induced tryptophan depletion has been illustrated to play important roles in physiological condition such as suppression of T cell responses in vivo and in vitro [Mellor et al., 2002]. It also prevents maternal T-cell mediated rejection of allogenic fetuses [Munn et al., 1998; Mellor et al., 2001] and prolongs graft survival of IDO-expressing pancreatic islet cells [Alexander et al., 2002]. Expression of IDO by human trophoblasts in placenta, in the contact zone between fetus and mother immune cells, protects the fetus from mother's immune cell rejection [Kamimura et al., 1991]. The immunosuppressive role of IDO has been demonstrated by showing suppression of T cell proliferation present in the vicinity of the IDO-expressing macrophages. It has been suggested that local tryptophan deprivation might play a key role in suppression of the immune cell proliferation [Munn et al., 1999]. Induction of IDO is specific to IFN-γ, but not to interferon α or β [Takikawa et al., 1988] and this cytokine, therefore, has been used to induce IDO expression in dermal fibroblasts.

Interferon-gamma (IFN-γ), a pleotropic cytokine secreted by the natural killer (NK) and activated T cells, is an important component of our immune system [Billiau and Dijkamans, 1990; Boehm et al., 1997]. IFN-γ exerts its effects by binding to the specific receptors on human fibroblasts [Anderson et al., 1982], which will then activate the Jak-STAT signal transduction pathway [Sadir et al., 2000]. Activated STAT1 will be translocated to the nucleus and bind to response elements at the promoter region of the target gene [Heim, 1999]. IFN-γ receptor complex is then internalized and subsequently degraded [Anderson et al., 1982]. IDO expression is shown to be halted in IFN-γ resistant mutant cell lines [Feng and Taylor, 1989], which may confirm the crucial role of this cytokine in stimulation of IDO expression and production. IFN-γ-induced IDO expression has been previously shown to possess anti-parasitic [Dai et al., 1994], anti-viral [Cheney et al., 2002], anti-tumor [Takikawa et al., 1990; Burke et al., 1995], and anti-proliferative [Maza and Peterson, 1988] activities. The anti-proliferative activity of IFN-γ depends on tryptophan concentration and this can be reversed by addition of tryptophan in a dose- and time-dependant manner [Ozaki et al., 1988; Leung et al., 1992].

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 survival of pancreatic islet cells [Alexander et al., 2002], we hypothesize that IDO expression may function as a local immunosuppressive factor to protect allogenic skin substitutes. In this study, therefore, we evaluated the proliferation of human PBMC cocultured with IFN-γ-induced IDO-expressing dermal fibroblasts. The finding of the present study reveals that IFN-γ induces the expression of IDO protein and its enzyme activity, thus generating a tryptophan-deficient environment in which human PBMC are unable to proliferate. In contrast to immune cells, however, IFN-γ-induced IDO-expressing dermal fibroblasts are able to migrate out of the collagen gel and proliferate at the same rate as the untreated control cells.

METHODS AND MATERIALS

Fibroblast Cell Culture

Cultures of human foreskin fibroblasts were established as described previously by Ghahary et al. [1997]. In brief, punch biopsy samples were prepared from human foreskin. The tissue was collected in DMEM with 10% FBS (GIBCO, Grand Island, NY), minced into small pieces of less than 0.5 mm in any dimension, washed with sterile medium six times, and distributed into 60×15 mm Petri culture dishes (Corning Inc., Corning, NY), four pieces per dish. A sterile glass cover-slip was attached to the dish with a drop of sterile silicone grease to immobilize the tissue fragment. DMEM + Ab (penicillin G sodium 100 U/ml, streptomycin sulfate 100 μg/ml, and amphotericin B 0.25 μg/ml) (3 ml) with 10% FBS was added to each dish and incubated at 37°C in a water-jacked humidified incubator in an atmosphere of 5% CO_2. The medium was replaced twice weekly. After 4 weeks of incubation, cells were released from dishes by brief (5 min) treatment with 0.1% trypsin (Life technologies Inc., Gaithersburg, MD) and 0.02% ethylenediaminetetraacetic acid (EDTA) (Sigma, St. Louis, MO) in PBS (pH 7.4) and transferred to 75 cm^2 culture flasks (Corning Inc.). Thereafter, once visual confluence was reached, the cells were subcultured 1:6 by trypsinization. Fibroblasts from passages 3 to 7 were used for this study.

Fibroblasts Populated in Collagen Gel

Fibroblasts populated in collagen gel (FPCG) were prepared, using bovine type I procollagen extracted in our laboratory by a procedure described by Volpin and Veis [1971], utilizing a modification procedure of Bell et al. [1979]. Experiments were performed using 6-well plates (ICN Biomedicals Inc., Aurora, OH) with 35 mm diameter. Each well contained 350 μl $3 \times$ DMEM + Ab, 26 μl 0.4 N NaOH, 440 μl cell suspension (2×10^6 cells/ml) in $1 \times$ DMEM + 10% FBS, 125 μl FBS (for a final concentration of 10%), and 870 μl of acid-extracted fetal bovine type I procollagen (2.14 mg/ml). Each treatment group was prepared in triplicates and was immediately transferred to a humidified incubator at 37°C in an atmosphere of 5% CO_2.

IFN-γ Treatment

FPCG were placed in the incubator for 3 h for the collagen gel lattices to be formed. After this period, collagen gels were released from the plates, using surgical blade (Fisher Scientific, Osaka, Japan) and fibroblasts were treated with 2,000 U of human recombinant IFN-γ (Sigma) for a period of 48 h. To find the lasting effect of IDO expression after IFN-γ removal, after this period, conditioned medium was replaced with a fresh medium with no IFN-γ at the indicated time-points. Collected conditioned media were used to determine the levels of kynurenine and tryptophan.

Northern Blot Analysis

FPCG were harvested and the collagen lattice was digested with bacterial collagenase (Sigma) in PBS (4 mg/10 ml) at 37°C for 30 min. Isolated cells were pelleted by centrifugation at 1,100 rpm for 10 min. Pellets were then lysed with 500 μl of 4 M guanidium isothiocyanate (GITC) solution and total RNA from each group was isolated by the guanidium isothiocyanate/CsCl procedure of Chirgwin et al. [1979] using phenol:chloroform (1:1), followed by chloroform:isoamyl alcohol (49:1). Total RNA from each individual fibroblast culture was then separated by electrophoresis (10 μg per lane) on a 1% agarose gel containing 2.2 M formaldehyde and was blotted onto a nitrocellulose paper. To control the loading, quantities of 18S ribosomal RNA were compared visually by ethidium bromide fluorescence. The blots were baked for 2 h at 80°C under vacuum and prehybridized for 4 h at 45°C in a prehybridization solution. Hybridization was performed at 45°C in the same solution, using IDO, 18S, or type I procollagen cDNA. The cDNA probes were labeled with P-α^{32}-dCTP by nick translation. Filters were washed initially at room temperature with $2 \times$ sodium citrate/sodium chloride buffers and 0.1% sodium dodecylsulfate for 1 h and finally washed for 20 min at 65°C in $0.1 \times$ sodium citrate/sodium chloride buffer and 0.1% sodium dodecylsulfate. Autoradiography was performed by exposing Kodak X-Omat film to nitrocellulose filters at −80°C, in presence of an intensifying screen. Quantitative analysis of autoradiographs was accomplished by densitometry. The cDNA probe for 18S was obtained from the American Type Culture Collection (Rockville, MD). The other cDNA probes were gifts: IDO (Dr. J.M. Carlin, Department of Microbiology, Miami University, Oxford, OH) and type I procollagen (Pro α_1 (I)) (Dr. G. Tromp, Dr. H. Kuivaneimi, and Dr. L. Ala-Kokko,

Department of Biochemistry and Molecular Biology, Jefferson Institute of Molecular Medicine, Philadelphia, PA).

Determination of Total Kynurenine and Tryptophan in the Conditioned Medium

The biological activity of IDO was evaluated by measuring the level of tryptophan or its degradative product, kynurenine, present in the conditioned medium derived from IFN-γ treated fibroblasts. Conditioned medium related to the same number of cells was first deproteinized by adding 100 μl of acetone to 50 μl of the cell culture medium. Sample was vortexed, cooled on ice, and centrifuged at 13,000 rpm for 15 min at 4°C. The supernatant of each sample received 10 μl of 0.25 M $HClO_4$ and IS (4-amino-hippuric acid) solution and the mixture was incubated at 25°C for 25 min. The mixture was then evaporated to dryness under vacuum. One hundred microlitres of 14% BF_3–propanol was added to the residue in a screw-capped glass tube and was heated at 90°C for 2 h. The solution was again evaporated to dryness under vacuum. The residue was dissolved in 40 μl of 20% MeOH in water and was analyzed by liquid chromatography with electrospray mass spectrometry (LC/EMS), using a Hewlett Packard series 1100 mass selective detector controlled by 1100-MED Chem Station. The mass spectrometer was operated in the positive ion mode. Tryptophan and kynurenine quantitative analyses were performed in the selected ion-monitoring mode.

Western Blot Analysis

To measure the lasting effect of IDO expression in dermal fibroblasts following the interferon-γ removal, Western blot analysis was performed on the harvested fibroblasts every 48 h post interferon-γ removal for a total period of 240 h. Briefly, cell extracts were prepared by adding 2×10^6 fibroblasts to the lysate buffer (50 mM Tris-HCl [pH = 7.40], 150 mM NaCl, 1 mM EDTA (Sigma), 1 mM EGTA (Sigma-Aldrich Canada, Ltd.), 0.025% NaN_3, 1% TritonX-100, 0.5% Igepal CA-630 (Sigma), and protease inhibitor cocktail (Sigma)). Extracts were centrifuged at 13,000 rpm for 10 min at 4°C and 50 μg (BSA protein assay [Pierce]) of the total protein was run on a 10% SDS–PAGE. Proteins were then transferred to a PVDF membrane (Millipore Corp., Bedford, MA) by Mini Trans-Blot Cell (Bio-Rad Laboratories Ltd., Mississauga, Ont.). Blots were incubated with either IDO (raised in our laboratory) or β-actin (Santa Cruz Biotechnology, Santa Cruz, CA) monoclonal primary antibodies. Horseradish peroxidase (HRP)-conjugated secondary antibody (Sigma) was used and visualized using ECL + Plus™ western blotting detection system (Amersham Biosciences).

Peripheral Blood Mononuclear Cell (PBMC) Isolation and Mixed Lymphocyte Reaction

Samples of 25–30 ml of human blood were drawn into heparinized tubes from normal individuals. Total PBMC was isolated by density gradient sedimentation on Histopaque 1077 (Sigma) following the manufacturer's protocol. Briefly, whole blood was layered onto an equal volume of Histopaque and centrifuged at 2,000 rpm for 20 min at room temperature. Mononuclear blood cells were isolated and added to RPMI 1640 + 10% FBS (GIBCO) and pelleted by centrifugation at 2,000 rpm for 10 min and were further washed twice in RPMI 1640. For the mixed lymphocyte reaction experiment, fibroblasts were treated with 2,000 U of IFN-γ for a period of 48 h, after which IFN-γ was removed, cells were washed three times with PBS, and fresh medium (with no IFN-γ) was replaced. Subsequently, 5×10^4 fibroblasts were plated into 12-well plates (Costar, Cambridge, MA), irradiated with 2,000 U of gamma rays, and cocultured with 2×10^5 of PBMC in RPMI 1640 + 10% FBS for a period of 5 days. To show the specificity of IDO action on proliferation of PBMC, IDO inhibitor, 1-methyl-D-tryptophan (Aldrich Chem. Co., Milwaukee, WI), was added to coculture samples in different concentrations of 50, 100, 200, 400, and 800 μM.

^{3}H-thymidine Incorporation Assay

To measure the rate of peripheral blood mononuclear cell (PBMC) proliferation in response to exposure to allogenic fibroblasts, ^{3}H-thymidine proliferation assay was performed on the PBMC cocultured with fibroblasts. In brief, ^{3}H-thymidine (PerkinElmer Life Sciences Inc., Boston, MA) was added to conditioned medium of each sample for a final concentration of 2 μCi/ml and was incubated for 16 h. After this period, PBMC were harvested, washed three times with PBS, dissolved in GITC, and added to the scintillation fluid (Aldrich Chem. Co.). Radioactive counting was performed using the scintillation counter (Beckman) and measured in counts per min

(cpm). PBMC proliferation rate was then calculated by dividing obtained radioactive counts by the highest radioactive count, for each set of experiment.

Fluorescence-Activated Cell Sorting (FACS) Analysis

To determine whether IDO expression in fibroblasts has any effects on the cocultured PBMC viability, propydium iodide (PI) staining followed by flow cytometry was performed to measure the ratio of dead (stained) to live cells. Harvested blood cells were stained with 10 μg/ml of PI (Sigma) for 10 min and washed twice with PBS. Flow cytometry was used to quantify the relative number of cells stained for the PI.

Fibroblast Gel Migration and Proliferation Assay

To investigate the effects of IDO expression on the rate of fibroblasts migration from the skin substitute and on their subsequent proliferation, fibroblast migration and proliferation assays were performed. Briefly, 6 mm punch biopsies were taken from the IFN-γ treated and untreated FPCG. Punch biopsies were placed in triplicates onto 6-well plates. The number of fibroblasts migrating out of the gel was measured every 2 days for a period of 8 days by removing the punch biopsies and counting the number of attached cells, using a hemocytometer.

Statistical Analysis

Student unpaired two-tailed *t*-test was used to compare the levels of tryptophan and kynurenine, the rate of fibroblast migration and proliferation, PBMC proliferation, and densitometry results of Western and Northern analyses between IFN-γ treated and untreated fibroblasts.

RESULTS

IFN-γ Induces IDO Expression in Fibroblasts in a Dose-Dependant Manner

Northern blot analysis of the total RNA extracted from IFN-γ treated FPCG revealed that IDO expression is markedly induced at the IFN-γ concentration of 1,000 U/ml and this expression is further increased with increasing concentrations of IFN-γ. Fibroblasts normally do not express detectable levels of IDO mRNA; however, upon treatment with IFN-γ, IDO expression was markedly induced in the skin fibroblasts (Fig. 1A). Combined densitometry results from three separate experiments revealed that IFN-γ reduces type I procollagen mRNA/18S and increases IDO mRNA/18S ratios in a dose-dependant fashion in the FPCG (Fig. 1B).

Different strains of FPCG were also either untreated (control) or treated with 2,000 U of IFN-γ for a period of 48 h. Northern blot analysis of the total RNA extracted from four different strains of human fibroblasts revealed that IDO expression is inducible at the mRNA level in all fibroblast strains upon IFN-γ treatment (Fig. 2A). Combined densitometry results from four separate experiments showed that IFN-γ markedly increases the expression of IDO mRNA/18S (Fig. 2B) and significantly decreases

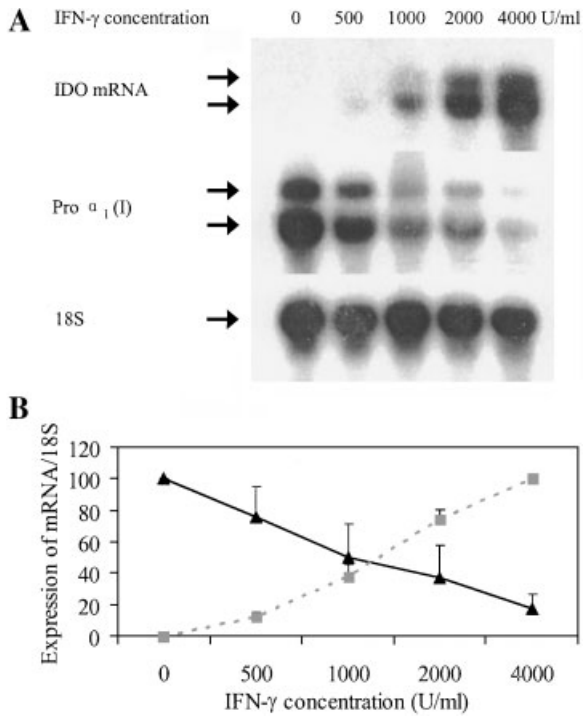

Fig. 1. IFN-γ-induced expression of IDO mRNA in fibroblasts populated within collagen gel (FPCG) is dose-dependent. Fibroblasts were treated with either 0 (control), 500, 1,000, 2,000, or 4,000 U of IFN-γ for a period of 48 h. The total RNA was extracted and Northern blot analysis was performed to determine the expression of mRNA for IDO and type I procollagen. The same blot was then re-hybridized with cDNA specific for 18S ribosomal RNA, which was used as a RNA loading control. **Panel A**, depicts the pattern of IDO and type I procollagen mRNA expression in fibroblasts treated with various concentrations of IFN-γ. **Panel B**, depicts the densitometry data on the ratio of either IDO mRNA/18S (squares) or type I procollagen mRNA/18S (triangles) as a function of various concentrations of IFN-γ used.

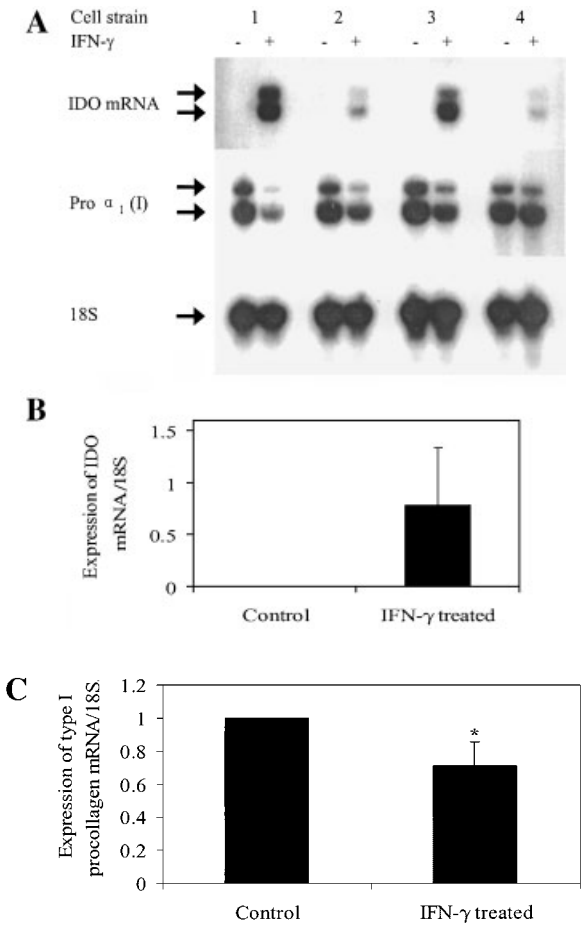

Fig. 2. IDO mRNA expression is inducible in different fibroblast strains upon IFN-γ treatment. Four different strains of human fibroblasts were either untreated (control) or treated with 2,000 U of IFN-γ for 48 h and Northern analysis was performed to evaluate the expression of IDO and type I procollagen mRNA using 18S ribosomal RNA as a loading control (**panel A**). The ratio of either IDO mRNA/18S rRNA (squares) or type I procollagen mRNA/18S rRNA (triangles) of each autoradiogram was then determined and the mean ± SD was calculated and presented in **panels B** and **C**, respectively. The *P*-value (*$P < 0.001$) for collagen mRNA expression between treated and untreated fibroblasts is considered to be extremely significant. Statistical evaluation on IDO mRNA expression, however, was not performed because the level of IDO mRNA expression was not detectable in all four different strains of untreated fibroblasts.

the expression of type I procollagen mRNA/18S ratios ($P < 0.001$, n = 4) (Fig. 2C).

IDO Expression Reduces in Fibroblasts Upon IFN-γ Removal

Collagen embedded fibroblasts, treated with 2,000 U of IFN-γ for 48 h, showed a high level of IDO mRNA expression and that gradually reduced upon IFN-γ removal up to the 32 h timepoint examined. Representative Northern blot analysis of the total RNA extracted from fibroblasts at 8, 16, 24, and 32 h post IFN-γ removal is depicted (Fig. 3A). Densitometry results from three separate experiments revealed a reduction in IDO mRNA expression in fibroblasts after the interferon-γ removal (Fig. 3B). Western blot analysis of IDO in fibroblasts treated with 2,000 U of IFN-γ illustrated that collagen embedded fibroblasts express IDO even at 192 h post interferon-γ removal (Fig. 3C). Combined densitometry results from three separate experiments also showed that IDO protein level was reduced in the collagen embedded fibroblasts after the IFN-γ removal (Fig. 3D).

IFN-γ-Induced IDO Expression Increases Kynurenine and Decreases Tryptophan Levels

To measure the enzyme activity of IDO in the collagen populated fibroblasts, tryptophan, and kynurenine levels were measured in the collected conditioned media after treatment with different doses of IFN-γ for 48 h. Fibroblasts treated with 500, 1,000, 2,000, and 4,000 U of IFN-γ had higher levels of kynurenine (10.01 ± 0.94, 10.72 ± 1.46, 10.68 ± 1.43, and 11.05 ± 0.25 μg/ml, respectively), compared to the untreated (control) fibroblasts (0.93 ± 0.58 μg/ml) (*$P < 0.001$, n = 3) (Fig. 4A). Data from four separate experiments showed that fibroblast treatment with 2,000 U of IFN-γ for 48 h decreases tryptophan levels (4.96 ± 1.74 vs. 17.56 ± 1.23 μg/ml) (*$P < 0.001$, n = 4) (Fig. 4B) and increases kynurenine levels (14.68 ± 2.14 vs. 1.29 ± 0.16 μg/ml) in fibroblast conditioned medium (*$P < 0.001$, n = 4) (Fig. 4C). The relative production level of kynurenine in fibroblasts remained high at 32 h post IFN-γ removal ($94 \pm 6\%$), which was significantly higher than that in control samples ($9 \pm 4\%$) (*$P < 0.001$, n = 3) (Fig. 4D).

IDO-Expressing Fibroblasts Suppress PBMC Proliferation in a Coculture System

^{3}H-thymidine incorporation assay on PBMC proliferation revealed that PBMC proliferation is significantly reduced when cocultured with IDO-expressing fibroblasts. Furthermore, addition of IDO inhibitor (1-methyl-D-tryptophan) reversed the inhibitory effects of IDO on lymphocyte proliferation in a dose-dependant manner, up to a point where PBMC proliferation rate was not significantly different between IDO-expressing and non-IDO-expressing (control) fibroblasts at the concentration of 800 μM

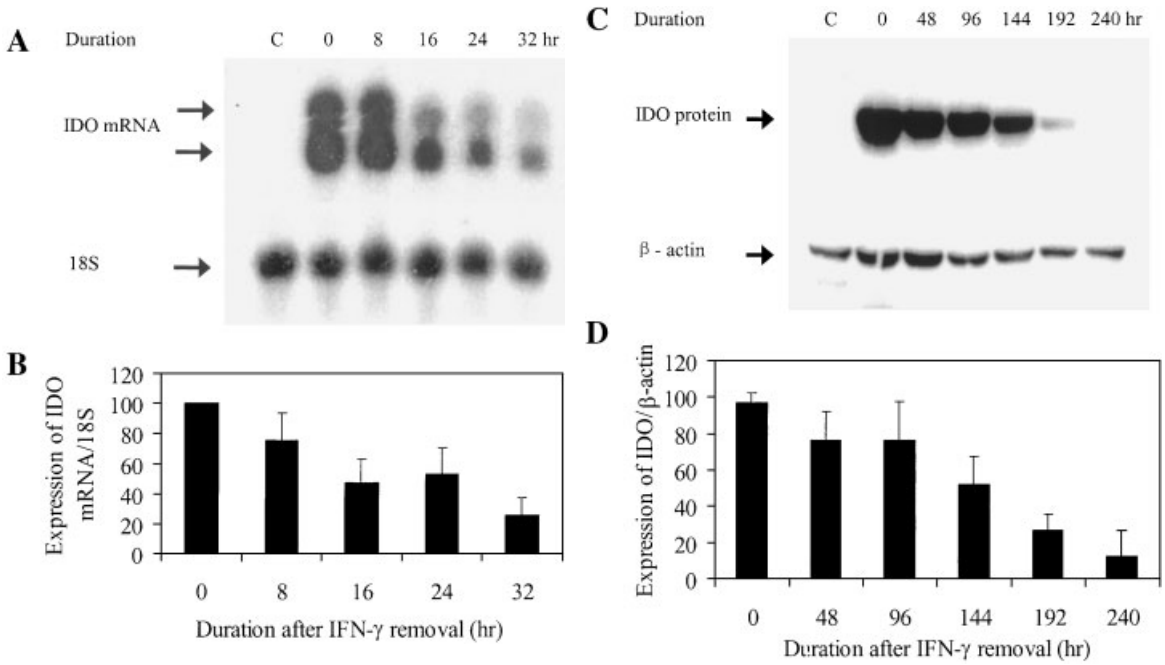

Fig. 3. IDO expression in fibroblasts embedded in a collagen is reduced after IFN-γ removal. Fibroblasts populated in the collagen gel (FPCG) were treated with 2,000 U of IFN-γ for 48 h. The conditioned medium was then replaced with fresh medium with no IFN-γ. Cells were harvested at 8, 16, 24, and 32 h post IFN-γ removal and total RNA was extracted and subjected to Northern analysis. **Panel A** represents the pattern of IDO mRNA expression and 18S ribosomal RNA in fibroblasts untreated (**C**), treated with IFN-γ for 48 h (0), or treated with IFN-γ for 48 h, and harvested at the indicated time-points post IFN-γ removal. Densitometry of autoradiograms related to three separate experiments was used to determine the ratio of IDO mRNA expression/18S ribosomal RNA. Data is expressed as the mean ± SD for each time point (**panel B**). **Panel C**, represents the pattern of IDO protein detected by Western blot analysis of collagen embedded fibroblasts harvested from either IFN-γ untreated (C), or treated for 48 h and harvested at the indicated time-points post IFN-γ removal. The presence of β-actin in the same samples was also evaluated as a protein loading control. The intensity of each IDO protein band of Western blot was then determined by densitometry and data is expressed as the ratio of IDO/β-actin protein (**panel D**). Data represents the mean ± SD for three separate experiments.

1-methyl-D-tryptophan (*, $^{**}P < 0.05$ and <0.001, respectively, $n = 3$) (Fig. 5A). The result of FACS analysis on viability of PBMC cultured alone, or in the vicinity of either IDO-expressing or non-expressing fibroblasts showed that PBMC viability is not significantly different amongst these three cell populations examined (Fig. 5B).

IDO-Expressing Collagen Embedded Fibroblasts can Migrate and Proliferate Despite the Tryptophan Depletion

The results of fibroblast migration assay revealed that IDO-expressing fibroblasts are able to migrate out of the collagen gel lattices (Fig. 6A). Fibroblast proliferation assays of IDO-expressing and non-expressing fibroblasts also showed that the rate of fibroblast proliferation was not significantly different between IDO-expressing and control cells (Fig. 6B).

DISCUSSION

It is now recognized that improved quality of wound healing with an acceptable biologic function is achievable only if the skin substitute has both dermal and epidermal layers. Considering the fact that 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 early wound coverage to prevent or reduce heat and fluid loss, and to prevent wound infection. The skin substitute should also establish wound coverage through which dermal–epidermal wound healing modulating factors are released locally to facilitate granulation tissue formation, re-epithelialization, and improve

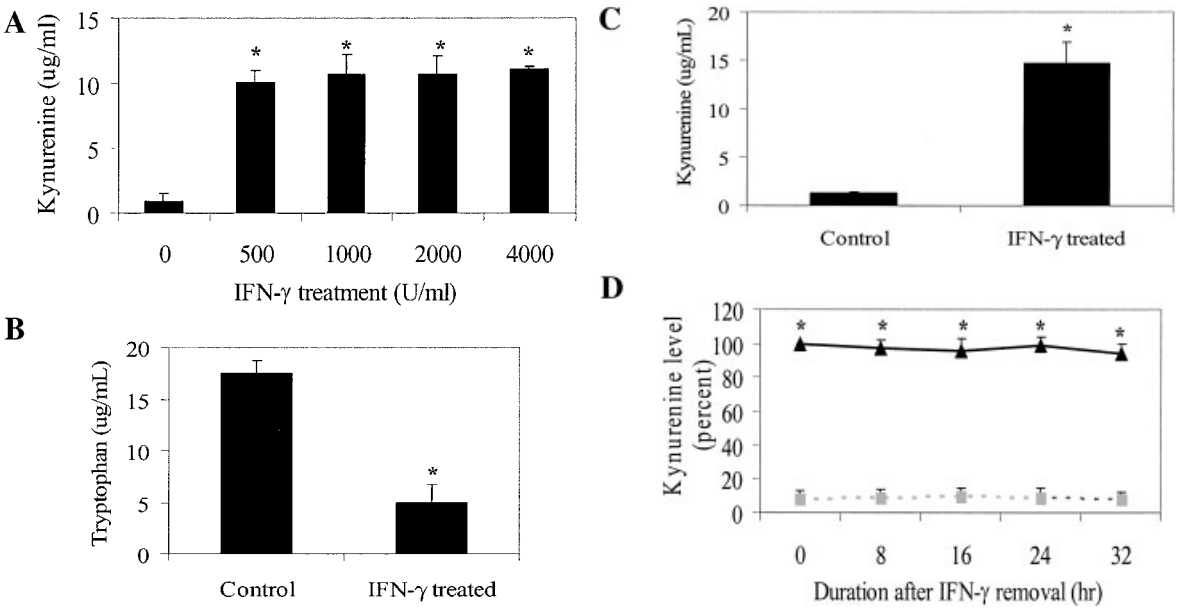

Fig. 4. Kynurenine and tryptophan levels measured in the conditioned medium of FPCG. A decreased level of tryptophan in the conditioned medium collected from IFN-γ-induced IDO-expressing fibroblasts was inverse to the levels of kynurenine. In **panel A**, collagen-populated fibroblasts were treated with either nothing (control), 500, 1,000, 2,000, or 4,000 U of IFN-γ and the conditioned medium was collected and evaluated for the kynurenine content. For statistical analysis, in a similar experimental setting, four different cell strains of fibroblasts were treated with 2,000 U of IFN-γ for 48 h and the conditioned medium was collected and evaluated for either tryptophan (**panel B**) or kynurenine (**panel C**) contents. Data is expressed as the mean ± SD for four separate experiments. In another experiment, the relative levels of kynurenine in the conditioned medium collected from fibroblasts either untreated (squares) or treated (triangles) with 2,000 U of IFN-γ for 48 h and harvested at the indicated time-points post IFN-γ removal, were determined (**panel D**). Data is shown as the mean ± SD for three separate experiments. The asterisks denote a significant difference in either tryptophan or kynurenine levels between IFN-γ untreated (control) and treated fibroblasts (*$P < 0.001$).

closure of non-healing wounds, such as those seen in the diabetic patients.

Though desirable, it is unlikely to have 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 immuno-compromised patients who suffer from non-healing complications. Furthermore, preparation of an autologous skin substitute is time-consuming, reducing the benefit for patients whose survival depends on an early application of wound coverage. As an alternative, the use of a non-living extracellular matrix substitute could be examined, but these are not capable of releasing wound healing promoting factors. Therefore, exploring an allogenic, 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 IDO, a tryptophan catabolizing enzyme, generates a tryptophan-deficient wound microenvironment in which infiltrated cells are unable to proliferate and to destroy the grafted skin substitute.

As a part of a series of experiments, here we have established that dermal fibroblasts grown in a three-dimensional collagen gel are highly responsive to IFN-γ-induced IDO mRNA expression. As IDO catalyzes an essential amino acid, tryptophan, to *N*-formylkynurenine, which is then converted to kynurenine [Higuchi and Hayaishi, 1967; Hayaishi, 1996; Munn et al., 1998], and as the presence of tryptophan is required for protein synthesis, stimulated immune cells would not be able to proliferate in such a low tryptophan level environment.

Previous studies have shown that IDO expression can inhibit immune cell proliferation in different cell types such as dendritic [Hwu et al., 2000] and HeLa cells [Logan et al., 2002]; however, immunosuppressive properties of IDO in primary cell cultures of dermal fibroblasts in a three-dimensional system, utilizing a collagen gel (which is more similar to an in vivo system),

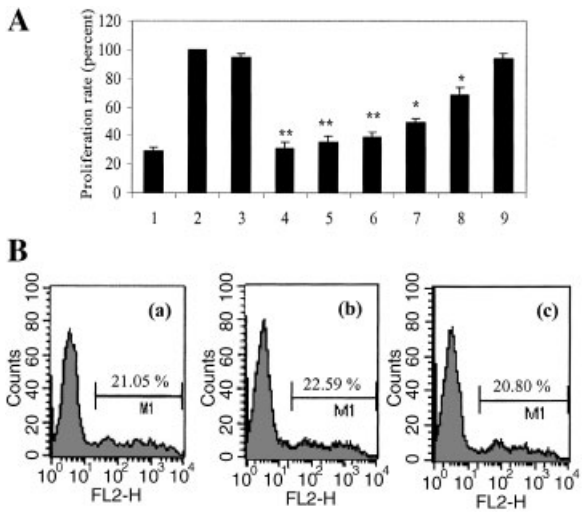

Fig. 5. The proliferation of PBMC cocultured with IDO-expressing dermal fibroblasts was suppressed. Fibroblasts were treated with 2,000 U of IFN-γ for 48 h. To eliminate the effects of IFN-γ, after this period, conditioned medium was replaced with fresh medium with no IFN-γ. IFN-γ treated or untreated fibroblasts were then cocultured with isolated human PBMC for a period of 5 days. ^{3}H-thymidime was then added to each coculture sample at a final concentration of 2 μCi/ml and 16 h later, the floating PBMC were then harvested, washed twice with PBS, and their radioactive count was measured. In **panel A**, PBMC were cultured either alone (**lane 1**), with non-IDO-expressing fibroblasts (**lane 2**), with non-IDO-expressing cells plus 200 μM of IDO inhibitor (1-methyl-D-tryptophan) (**lane 3**), or with IDO-expressing fibroblasts in the absence of IDO inhibitor (**lane 4**), or in presence of various concentrations of IDO inhibitor at final concentrations of 50, 100, 200, 400, or 800 μM (**lanes 5–9**). Data represents the mean ± SD for three separate experiments. The asterisks (*, **$P < 0.05$ and <0.001, respectively) denote a significant difference in proliferation of PMBC in control samples (cocultured with non-IDO-expressing fibroblasts) and those either cocultured with IDO-expressing fibroblasts (lane 2 vs. 4) or IDO-expressing cells in the presence of various concentration of IDO inhibitor (lane 2 vs. 5, 6, 7, and 8). PBMC proliferation was restored at 800 μM concentration of 1-methyl tryptophan (lane 9), where no significant difference was observed in rate of cocultured PBMC proliferation between control and IDO producing fibroblasts (lane 2 vs. 9). **Panel B**: To evaluate the viability of PBMC, cells were stained with PI and the number of positive PBMC was measured in the samples cultured either alone (**a**), cocultured with no IDO-expressing fibroblasts (**b**), or cocultured with IDO-expressing fibroblasts (**c**) for 5 days.

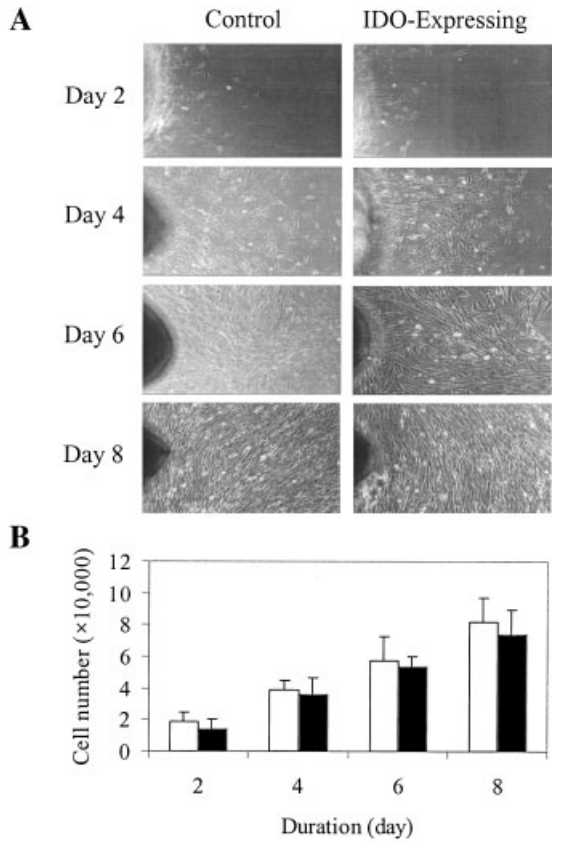

Fig. 6. IDO-expressing and non-expressing fibroblasts have a similar pattern of cell migration and proliferation. Fibroblasts embedded in the collagen gel were treated with either nothing (control) or 2,000 U of IFN-γ (IDO-expressing) for 2 days. Conditioned medium was then replaced with fresh medium (with no IFN-γ) and three 6 mm punch biopsies were taken from each gel and placed onto 6-well plates (three biopsies per each well) and the pattern of migrating cells were microscopically photographed at days 2, 4, 6, and 8 (**panel A**). At the end of each time-point, punch biopsies were removed, cells were harvested and proliferation assay was performed (**panel B**). Solid and open bars represent IDO-expressing and non-expressing fibroblasts, respectively.

has not yet been elucidated. The results of mixed lymphocyte reaction assay showed that IFN-γ-induced IDO in dermal fibroblasts suppresses the proliferation of stimulated PBMC. This suppression is completely restored by addition of an IDO inhibitor (1-methyl-D-tryptophan) at 800 μM concentration. The results of FACS analysis indicated that IFN-γ induced IDO expression does not reduce the viability of PBMC and this finding revealed that IDO expression generates a tryptophan depleted environment in which immune cells are unable to proliferate without compromising the skin cell viability.

Although our findings collectively suggest that immune cells cocultured with IDO-expressing fibroblasts are unable to proliferate, the main concern is whether IFN-γ-induced IDO-expressing cells would be able to proliferate in the same environment. In an IDO adenoviral infected cells, we have recently demonstrated that there is a marked difference in responsiveness to tryptophan depletion amongst the three different immune cell types examined (unpublished data). In fact, CD4+ cells, which play a

major role in graft rejection, are very sensitive to low tryptophan levels as more than 82% of these cells died in low tryptophan culture conditions; while, THP-1 (monocytes) were significantly less sensitive to the same environment. In the same study, we also showed that IDO adenoviral infected 293 cells with a high levels of IDO expression are attached to the bottom of the culture dish and survive better than immune cells as evaluated by FACS analysis of PI staining cells. These findings indicate that cells with different degree of proliferating abilities might respond differently to a tryptophan-deficient environment. In fact, the result of the cell migration from collagen gel and subsequent cell proliferation indicates that primary cultured fibroblasts appear to be less sensitive to IDO-induced low tryptophan environment. This is because the pattern of cell migration and proliferation of the collagen-populated fibroblasts was comparable between IFN-γ treated and untreated cells. A better survival of IDO-expressing cells relative to that of high proliferative immune cells may not be surprising because IDO-expressing cells such as trophoblasts of placenta survive better than infiltrated immune cells during pregnancy. Munn et al. [1999] recently reported that antigen-presenting cells can regulate T cell activation through tryptophan catabolism and speculated that the expression of IDO by certain antigen presenting cells in vivo allows them to suppress unwanted T cell responses. These investigators demonstrated that the expression of IDO during murine pregnancy is required to prevent rejection of allogenic fetus by maternal T cells.

The result of lasting effect of IFN-γ on expression of IDO mRNA in dermal fibroblasts revealed that the level of IFN-γ-induced IDO expression in treated cells seems to be transient. This is because the level of IDO mRNA expression in treated cells returned to its undetectable level within almost 2 days upon IFN-γ removal. However, the lasting effect of IFN-γ treatment on production of IDO protein seems to be significantly longer (more than 6 days) than that of IDO mRNA expression. This finding indicates that IDO protein is stable and can be stored within cells for a longer time-period. To further prolong the IFN-γ-induced IDO expression, we are currently examining the use of IFN-γ protein conjugated polymers based on the fact that a slow release of IFN-γ may be necessary to sustain the level of IDO expression to be protective for engraftment. In fact, the slow release of IFN-γ may also be beneficiary for those patients who suffer from severe thermal injury. This is because we have recently found (data not shown) that the level of IFN-γ in patients with severe burn injury is significantly lower relative to that of normal controls. The lower level of anti-fibrogenic factor such as IFN-γ may contribute to the development of hypertrophic scarring frequently seen upon severe thermal injury. We have also previously demonstrated that IFN-γ reduces fibroblast proliferation and synthesis of type I and III collagen, and for this reason the authors suggested that IFN-γ could be used as a therapeutic agent to treat dermal fibrotic condition [Harrop et al., 1995]. Further, according to the recent article by Halloran et al. [2001], engraftment of the kidney in IFN-γ knockout mouse appears to be manifested by an increase in vascular injury, congestion of pretubular capillaries, and massive necrosis of parenchymal cells. Thus, the use of IFN-γ may in fact be beneficial in preventing thrombosis, congestion, and necrosis of grafted tissues and more likely, less scar formation.

In conclusion, we demonstrated that IFN-γ treatment induces the expression of IDO mRNA and protein in dermal fibroblasts grown in a three-dimensional collagen environment. The tryptophan deficiency created an environment in which stimulated immune cells are unable to proliferate. We are aware of the fact that further studies are required to address many issues which have not been raised in this study and 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 a non-rejectable skin substitute is evaluated.

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