Inhibition of Type I Collagen mRNA Expression Independent of Tryptophan Depletion in Interferon-γ–Treated Human Dermal Fibroblasts

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Interferon-γ (IFN-γ) is a pleiotropic cytokine that modulates type I collagen synthesis. In addition, IFN-γ also exerts potent effects on cellular tryptophan levels by inducing the expression of indoleamine 2,3-dioxygenase (IDO) and tryptophanyl-tRNA synthetase. Because recent evidence indicates that IDO-mediated oxidative tryptophan catabolism is important in cellular responses to IFN-γ, we investigated the role of IDO in the IFN-γ–induced modulation of type I collagen gene expression. IFN-γ (≥50 U/ml) stimulated IDO expression in human dermal fibroblasts *in vitro*, resulting in a >90% depletion of tryptophan in the culture media following incubation for 48 h. Higher concentrations of IFN-γ (≥500 U/ml) caused a marked decrease in type I collagen mRNA levels. Time-course studies indicated that maximal induction of IDO mRNA expression in IFN-γ–treated fibroblast cultures (24 h) preceded the maximal decrease in collagen mRNA (96 h). Type I collagen mRNA levels were also markedly and selectively decreased in fibroblasts maintained in tryptophan-depleted cultures. Addition of exogenous tryptophan (up to 2500 μM) to IFN-γ–treated fibroblasts restored "normal" concentrations of tryptophan in the culture media, but did not abrogate the IFN-γ–induced decrease in collagen mRNA. Addition of the tryptophan metabolite kynurenine, in concentrations similar to those generated in fibroblast cultures following IFN-γ treatment for 48 h, had no significant effect on type I collagen mRNA levels. These results indicate that although IFN-γ causes activation of IDO and enhanced tryptophan catabolism in fibroblast cultures, neither the ensuing tryptophan starvation nor the accumulation of kynurenine in the culture media can fully account for the inhibitory effects of IFN-γ on type I collagen mRNA expression. ***Key words: extracellular matrix/fibrosis/cytokines/gene regulation/indoleamine 2,3-dioxygenase. J Invest Dermatol 105: 388–393, 1995***

Interferons are multifunctional cytokines that induce an antiviral state, inhibit the growth of certain tumor cells and intracellular pathogens, and activate the expression of a set of cellular genes in many cell types both *in vitro* and *in vivo* [1]. Interferon-γ (IFN-γ), a product of activated T lymphocytes, macrophages, and natural killer cells, stimulates the production of indoleamine 2,3-dioxygenase (IDO), tryptophanyl-tRNA synthetase, nitric oxide synthetase, and other proteins of unknown function [2–4]. These gene products mediate many of the important biologic activities associated with IFN-γ [5]. Recent studies indicate that induction of cellular IDO, the enzyme that catalyzes the oxidation of tryptophan to *N*-formylkynurenine [6], represents an important mechanism contributing to the inhibitory effects of IFN-γ on the growth of some tumor cells [7,8]. Similarly, induction of IDO by IFN-γ in a variety of host cells has been implicated in the inhibition of the intracellular replication of parasites such as *Toxoplasma gondii* and *Chlamydia psittaci* [9–11]. We have recently shown that abrogation of interleukin-1β (IL-1β)–induced expression of collagenase and stromelysin by IFN-γ in cultured dermal fibroblasts was mediated through depletion of tryptophan, and could be overcome by the addition of exogenous tryptophan to the cultures [12]. These studies suggest that modulation of cellular tryptophan metabolism plays a crucial role in mediating certain cellular responses to IFN-γ.

In addition to the roles in immune responses, IFN-γ exerts potent effects on collagen production. *In vitro*, IFN-γ inhibits the synthesis of types I and II collagens [13–16] and abrogates the stimulatory effects of transforming growth factor-β on collagen synthesis in fibroblasts [17,18]. Therefore, IFN-γ may have a physiologic role as well as important therapeutic potential in preventing excessive collagen accumulation during tissue repair in the skin. The mechanisms whereby IFN-γ causes inhibition of collagen gene expression require elucidation. In the present study, we examined whether induction of IDO expression and tryptophan catabolism in cultured dermal fibroblasts played a role in the modulation of type I collagen mRNA expression by IFN-γ *in vitro*. The results indicate that IFN-γ caused a decrease in type I collagen mRNA levels and a marked increase in IDO mRNA levels. Increased IDO expression was associated with enhanced tryptophan degradation, resulting in depletion of this amino acid from the culture media and a corresponding rise in concentrations of kynure-

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Abbreviations: IDO, indoleamine 2,3-dioxygenase.

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nine. The levels of type I collagen mRNA were markedly reduced in fibroblasts cultured in tryptophan-depleted media. However, repletion of tryptophan in IFN-γ-treated cultures failed to prevent the decrease in type I collagen mRNA. Therefore, the inhibitory effects of IFN-γ on type I collagen mRNA expression in fibroblasts appear to be mediated primarily by mechanisms independent from induction of IDO gene expression and consequent depletion of tryptophan and accumulation of kynurenine.

MATERIALS AND METHODS

Materials All tissue-culture reagents were obtained from Gibco (Grand Island, NY). L-tryptophan and kynurenine (purity approximately 99%), and cycloheximide were obtained from Sigma Chemical Co. (St. Louis, MO). Different batches of L-tryptophan used in these experiments gave consistent and reproducible results. Recombinant human IFN-γ was from Boehringer-Mannheim (Indianapolis, IN).

Fibroblast Cultures Cell lines used in the present experiments were established from forearm skin biopsies of six healthy adults by explant techniques previously described [19]. Cells were seeded into 75-cm^2 tissue-culture flasks containing modified Eagle's medium (MEM), 1% vitamins and 2 mM L-glutamine, supplemented with 5% fetal bovine serum (FBS). When the cells reached visual confluence, fresh medium with or without ascorbic acid (50 μg/ml), and 5% FBS (extensively dialyzed against 0.05 M Tris, pH 7.5, 0.15 M NaCl to remove free amino acids) were added to the cultures. In selected experiments, tryptophan-free MEM (Select-Amine, Gibco) was used. Cellular toxicity was evaluated at the end of the incubation period by trypan blue staining, and release of LDH into the media, as described previously [20].

Analysis of RNA Total RNA was extracted from confluent fibroblasts using the CsCl centrifugation method, and subjected to Northern analysis [21]. Hybridizations were performed with cDNAs previously labeled with [α-^{32}P]dCTP to a specific activity at least 1×10^8 cpm/μg by nick translation. Autoradiograms were analyzed by scanning at the linear range of the absorbance curve with a laser densitometer (LKB, Bromma, Sweden). The following cDNAs were used: human type I collagen [22], HLA-DR α chain [23], fibronectin [24], and rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [25], all obtained from the American Type Culture Collection (Rockville, MD); and human IDO, a gift of Dr S. Gupta (Hipple Cancer Center, Dayton, OH) [26].

Determination of Tryptophan and Kynurenine Concentrations Total free tryptophan and kynurenine were assayed by high-performance liquid chromatography on a C-18 column eluted isocratically using a solvent of phosphate buffer (0.1 M), adjusted to pH 3.6 with sodium hydroxide, and containing 0.001 M disodium–ethylenediaminetetraacetic acid and 5% acetonitrile. Samples of culture media were deproteinized with perchloric acid (0.6 M). Tryptophan was detected by its native fluorescence (activation at 290 nm and emission at 360 nm) using a Hewlett-Packard model HP1046A programmable fluorimeter. Kynurenine was detected in the same chromatograms by ultraviolet absorbtion at 360 nm using a Rainin UV-1 absorbance detector in series with the fluorimeter. Chromatogram recordings and calculations were done using the Rainin Dynamax program (Rainin Instrument Co., Woburn MA).

RESULTS

IFN-γ Modulates Type I Collagen mRNA Expression and Tryptophan Metabolism in Fibroblast Cultures To examine the effect of IFN-γ on collagen mRNA levels, IFN-γ (500 U/ml) was added to confluent fibroblasts. After various lengths of incubation, cultures were harvested and mRNA were examined by Northern analysis. Exposure of the cultures to IFN-γ for up to 8 h was not associated with detectable changes in type I collagen mRNA (data not shown). However, incubation with IFN-γ for 24 h caused a greater than 50% decrease in type I collagen mRNA levels, with maximal (>90%) inhibition at 96 h **(Fig 1*A*)**.

To determine whether inhibition of collagen mRNA expression by IFN-γ was related to modulation of tryptophan metabolism, we measured IDO mRNA levels in untreated and in IFN-γ–treated fibroblasts. IDO mRNA was undetectable in untreated fibroblasts **(Fig 1*B*)**. Exposure to IFN-γ (500 U/ml) for as little as 3 h caused a small but detectable increase in IDO mRNA that peaked at 24 h. A single transcript with an apparent molecular size of 2.2 kb was observed. The stimulation of IDO mRNA by IFN-γ was dose dependent, with maximal effect at at least 50 U/ml IFN-γ; in contrast, maximal inhibition of collagen mRNA required at least 500 U/ml (data not shown).

The product of tryptophan oxidation by IDO is formylkynurenine, which in most cells is further catabolized by kynurenine formidase to produce kynurenine [27]. To examine the effects of IFN-γ on tryptophan metabolism in dermal fibroblasts, culture supernatants from fibroblasts exposed to IFN-γ for various periods were analyzed for tryptophan and kynurenine concentrations. Tryptophan concentrations in media of fibroblasts not exposed to IFN-γ remained unchanged following a 96-h incubation, and the cells produced no kynurenine (data not shown). Exposure of fibroblasts to IFN-γ (500 U/ml) caused a time-dependent >95% decrease in tryptophan and a greater than twentyfold increase in kynurenine concentrations **(Fig 1*B*)**. These findings indicate that IFN-γ induced the expression of IDO, resulting in markedly enhanced degradation of tryptophan and consequent depletion of this essential amino acid from the culture media and a corresponding accumulation of kynurenine.

To examine the role of protein synthesis in modulation of type I collagen and IDO gene expression by IFN-γ, some cultures were pretreated with cycloheximide (10 μg/ml) 2 h prior to addition of IFN-γ (1000 U/ml), and mRNA amounts were determined following a further 72-h incubation. As shown in **Fig 2**, cycloheximide almost completely prevented the inhibition of type I collagen mRNA expression; in contrast, the induction of IDO mRNA by IFN-γ appeared to be only moderately decreased at 72 h. These results suggest that IDO mRNA induction in IFN-γ–treated fibroblasts may be a primary response, whereas the delayed inhibition of type I collagen mRNA expression requires *de novo* protein synthesis.

Tryptophan Depletion Inhibits Type I Collagen mRNA Expression To examine the influence of tryptophan concentration on type I collagen mRNA expression, some fibroblasts were switched from complete media (containing 50 μM tryptophan, as indicated by the manufacturer) to tryptophan-depleted media and dialyzed (therefore tryptophan-depleted) FBS at confluence. After 48 h, some of the cultures received exogenous tryptophan for an additional 24 h of incubation. No differences in viability or total protein synthesis between fibroblasts cultured in tryptophan-depleted or tryptophan-containing media were detected at the end of the incubation period (data not shown). Northern analysis of total RNA indicated that type I collagen mRNA levels were markedly reduced in cultures maintained under tryptophan-free conditions **(Fig 3)**. In contrast, mRNA levels for fibronectin, a major fibroblast product, or GAPDH, a "housekeeping enzyme," remained unchanged in tryptophan-depleted fibroblasts, indicating the lack of significant cellular toxicity under these conditions.

Tryptophan Supplementation of IFN-γ–Treated Cultures Fails to Abrogate Inhibition of Type I Collagen mRNA Expression Because IFN-γ induced the degradation of tryptophan in the culture media, and fibroblasts under tryptophan-free conditions displayed reduced collagen mRNA levels, we speculated that inhibition of collagen mRNA expression by IFN-γ was caused by tryptophan depletion. To test this hypothesis, we examined whether addition of exogenous tryptophan to IFN-γ–treated (and therefore tryptophan-depleted) cultures would restore basal expression of type I collagen mRNA. For this purpose, IFN-γ was added to confluent fibroblasts maintained in complete media and was then followed by various concentrations of tryptophan. Incubation was continued for a further 28 h. At the end of the experiment, media were harvested and the concentrations of tryptophan determined. As shown in **Table I**, addition of exogenous tryptophan to the IFN-γ–treated fibroblast cultures resulted following a 28-h incubation in concentrations that exceeded those in untreated cultures. Total RNA was harvested at the end of the experiments and subjected to Northern analysis. As shown in **Fig 4**, addition of tryptophan in concentrations sufficient to exceed normal levels to the IFN-γ–treated cultures failed to prevent the

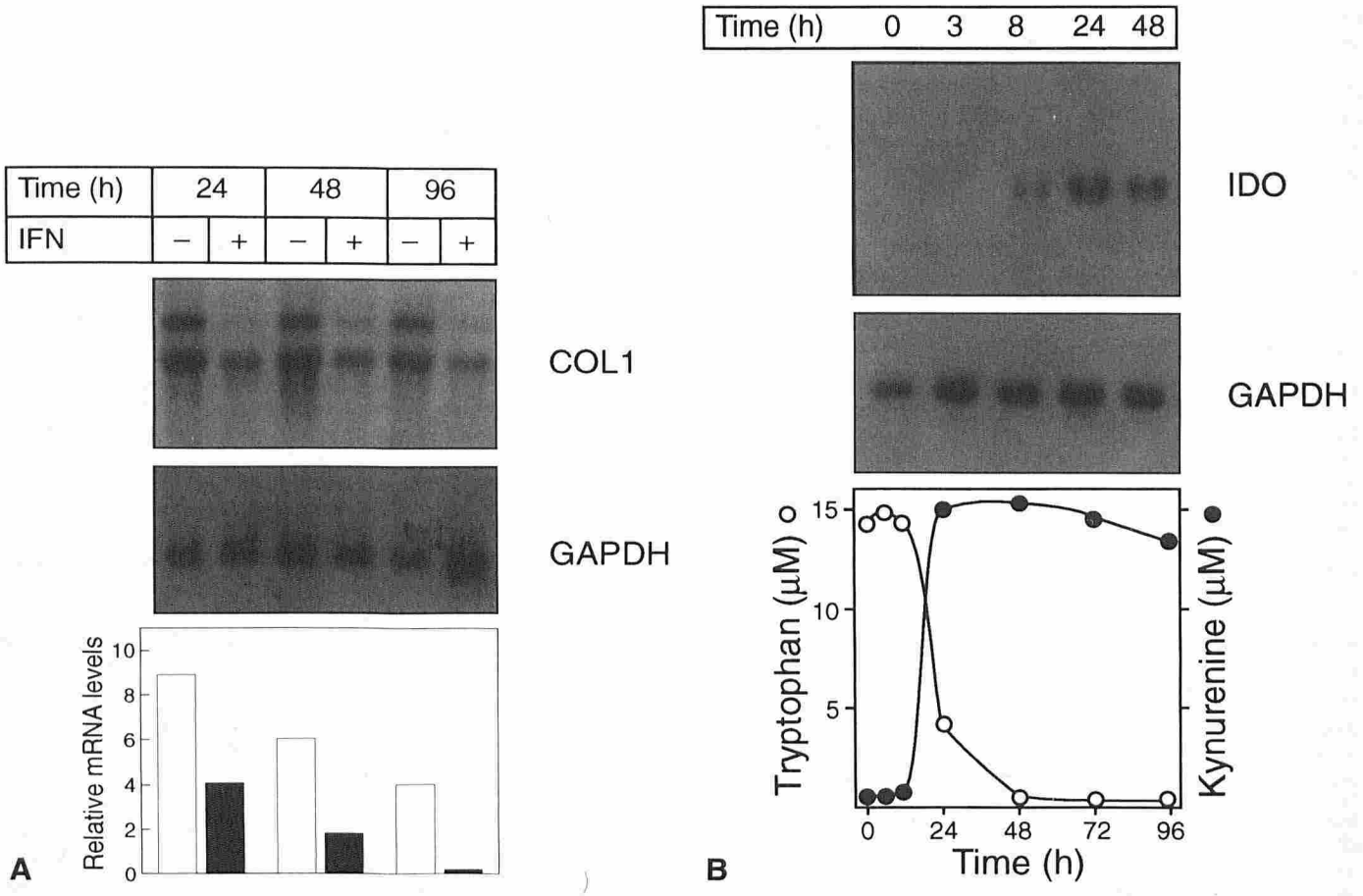

Figure 1. Interferon-γ modulates collagen and indoleamine 2,3-dioxygenase (IDO) gene expression in dermal fibroblasts. Confluent cultures of normal human skin fibroblasts were incubated with interferon-γ (IFN-γ, 500 U/ml) for various periods. At the indicated times, total RNA was isolated, and steady-state mRNA levels were determined by Northern hybridization (10 μg/lane) to [^{32}P]-labeled (*A*) type I collagen and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNAs, or (*B*) IDO cDNA, as described in *Materials and Methods*. Abundance of mRNA was quantitated by scanning densitometry of the autoradiograms, and the values are shown after correction for variations in GAPDH mRNA levels (*A, bottom*). Results represent the mean from two separate experiments. *B*. Modulation of IDO gene expression. Confluent cultures were exposed to IFN-γ for various periods as described above. At the end of the incubation period, cellular IDO mRNA levels (*upper panels*), and tryptophan and kynurenine in the culture media (*lower panels*) were quantitated, as described in *Materials and Methods*.

IFN-γ–induced decrease in type I collagen mRNA. As previously reported [23,27], treatment with IFN-γ markedly increased IDO and HLA-DR mRNA, which was unaffected by the concentration of tryptophan in these cultures.

Tryptophan Metabolites Do Not Influence Type I Collagen mRNA Expression Confluent human fibroblasts exposed to IFN-γ (500 U/ml) for 48 h in complete media *in vitro* produce ~15 μM kynurenine, the principal tryptophan metabolite **(Fig 1*B*)**. To examine the possibility that decrease in type I collagen mRNA in IFN-γ–treated fibroblasts was due to direct inhibition of collagen gene expression by tryptophan oxidation products, we examined the influence of kynurenine on fibroblast collagen synthesis. As shown in **Table II**, addition of kynurenine to confluent fibroblasts, in concentrations similar to or exceeding those attained in IFN-γ–treated fibroblast cultures, did not result in a significant change in type I collagen mRNA levels. These findings indicate that accumulation of kynurenine in the culture media following treatment with IFN-γ cannot account for the decrease in collagen mRNA expression.

DISCUSSION

The present results indicate that in human dermal fibroblasts, IFN-γ caused a time-dependent decrease in type I collagen mRNA, with a marked increase in IDO mRNA and enzyme activity. Consistent with results from a recent study [28], the inhibition of collagen gene expression, but not the induction of IDO gene expression, was sensitive to cycloheximide. Induction of IDO expression occurred more rapidly and at lower concentrations of IFN-γ than inhibition of collagen gene expression. IFN-γ, a key mediator of inflammation, induces several enzymes involved in tumoricidal and antimicrobial activities of target cells [29]. The mechanisms involved in many of these cellular responses have recently been illuminated. It has been shown that binding by IFN-γ to distinct surface receptors is followed by activation of the JAK family of intracellular tyrosine kinases [30]. This leads to activation of cytoplasmic proteins that translocate into the nucleus and bind to recognition sequences in the regulatory regions of certain IFN-γ–inducible genes [31]. Evidence has been presented indicating that IFN-γ–induced IDO transcription is mediated through IFN-γ–responsive elements in the 5′ flanking region of the IDO gene [2]. The modulation of other cellular genes by IFN-γ appears to proceed through indirect mechanisms involving, for example, the activation of cellular arginine metabolism *via* nitric oxide synthetase, or the activation of tryptophan metabolism *via* IDO [4,11]. In the present studies, IFN-γ caused a virtually complete depletion of tryptophan and a parallel increase in kynurenine in the culture

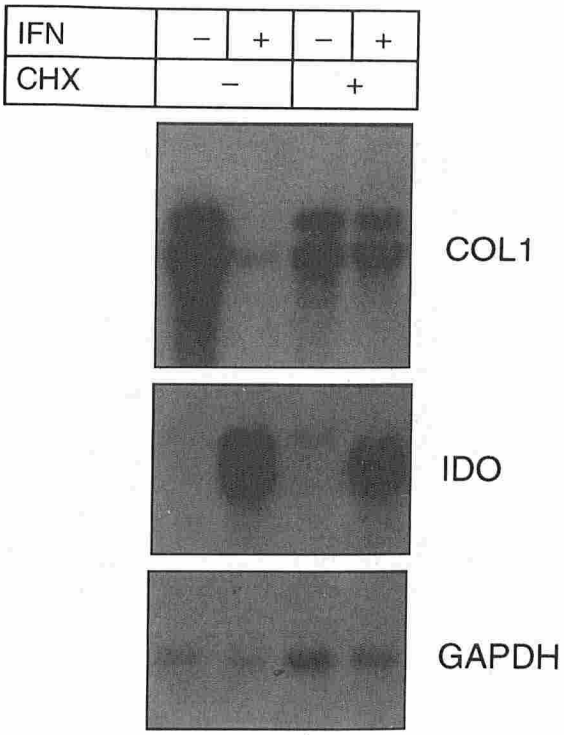

Figure 2. Cycloheximide prevents the inhibition of collagen, but not induction of IDO mRNA by IFN-γ. Cycloheximide (CHX, 10 μg/ml) was added to confluent fibroblasts maintained in complete media, followed 2 h later by IFN-γ (1000 U/ml). At the end of an additional 70 h incubation, cultures were harvested. RNA was analyzed by Northern hybridization with type I collagen, IDO, and GAPDH cDNA.

media, indicating a marked enhancement of tryptophan catabolism in the dermal fibroblasts. Because changes in intracellular tryptophan concentration follow closely the changes in the extracellular pool in IFN-γ–treated fibroblasts [32], the findings suggest that IFN-γ induced tryptophan deprivation of these cells.

Because the inhibitory effects of IFN-γ on type I collagen mRNA expression were associated with induction of IDO and resultant tryptophan catabolism, we speculated that depletion of tryptophan in the cultures may have interfered with basal collagen gene expression. Indeed, fibroblasts cultured in tryptophan-depleted media displayed markedly reduced type I collagen mRNA levels, whereas fibronectin mRNA levels were not altered. The mechanism whereby tryptophan deprivation of fibroblasts selectively interferes with their expression of type I collagen mRNA is unknown. Tryptophan may be required for the synthesis or DNA-binding activity of nuclear transcription factors mediating the basal activity of type I collagen gene. Indeed, conserved tryptophans ("tryptophan clusters") have been shown to be necessary for sequence-specific DNA binding activity of the c-myb proto-oncogene [33]. Therefore, we examined whether repletion of tryptophan in cultures rendered depleted of tryptophan by prolonged incubation with IFN-γ could "restore" normal type I collagen mRNA levels. The results showed that addition of tryptophan, in amounts sufficient to achieve greater than control concentrations at the end of the incubation period, to fibroblasts pre-treated with IFN-γ failed to reverse the inhibitory effects of IFN-γ.

Taken together, these observations indicate that although tryptophan appears to be required for basal expression of the type I collagen gene in dermal fibroblasts, tryptophan degradation is not directly involved in inhibition of collagen mRNA expression by IFN-γ. Tryptophan, an essential amino acid, is required for protein synthesis and serves as a precursor for biologically important amines

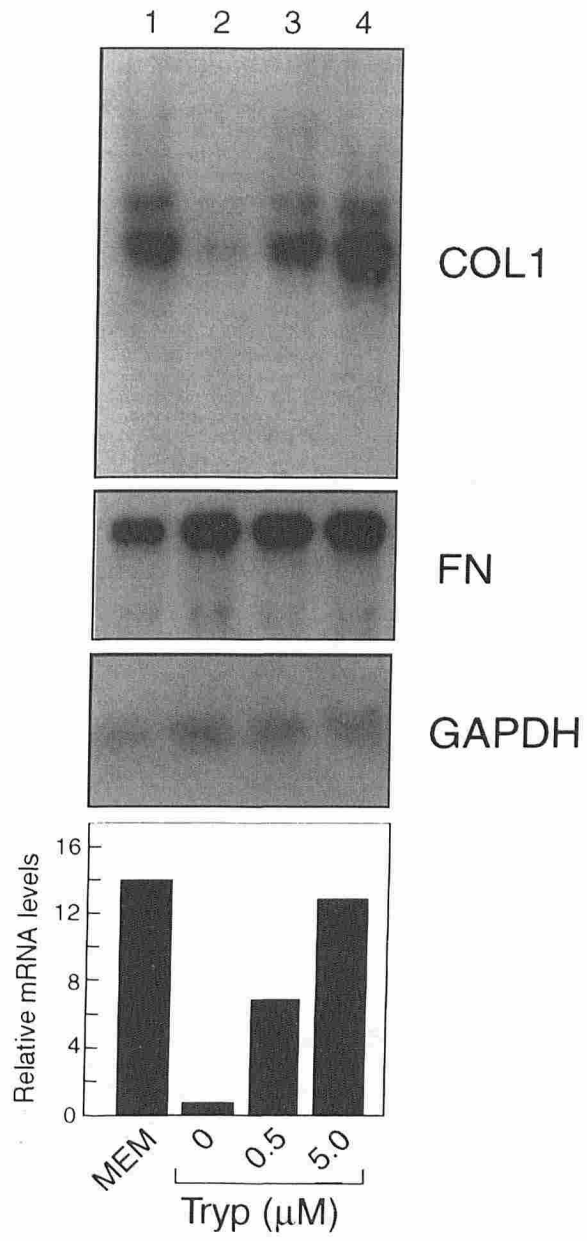

Figure 3. Tryptophan depletion down-regulates collagen mRNA levels. Confluent fibroblasts were maintained in complete MEM, then washed extensively in PBS and switched to tryptophan-free MEM and 5% FBS (*lanes 2–4*), or returned to complete MEM (*lane 1*), at confluence. After 48 h, tryptophan (0.5 or 5 μM) was added to cultures, as indicated. At the end of an additional 24 h incubation, cultures were harvested. RNA was analyzed by Northern hybridization with type I collagen, fibronectin, and GAPDH cDNA (*top*). Collagen mRNA abundance was quantitated, and the values (mean of two separate experiments) are shown after correction for variations in GAPDH mRNA levels (*bottom*).

[34]. Furthermore, tryptophan metabolites may function as powerful anti-oxidants [35]. We recently reported that in supraphysiologic concentrations tryptophan, but not other amino acids, caused marked stimulation of collagenase gene expression in dermal fibroblasts *in vitro* [20]. In contrast, IFN-γ–induced depletion of tryptophan in fibroblast cultures effectively prevented the stimulation of collagenase expression by IL-1 [12]. Because tryptophan appears to be important in the regulation of several biologic processes, its degradation and ensuing cellular starvation may have pleiotropic effects.

Table I. Effect of the Addition of Tryptophan to IFN-γ–Treated Dermal Fibroblast Cultures on Tryptophan Concentration in the Culture Media[a]

Concentration of Tryptophan Added (μg/ml)	Final Concentration of Tryptophan (μM)
Control[b]	31
0	5
100	127
500	1151

[a] Tryptophan was added to confluent fibroblasts that had been pre-incubated with IFN-γ (500 U/ml) in complete media. At the end of the incubation (28 h), media were harvested, and tryptophan concentrations were determined as described in *Materials and Methods*. The values shown represent the mean of two separate experiments.
[b] Control cultures received neither IFN-γ nor exogenous tryptophan.

Table II. Tryptophan Metabolite Kynurenine Does Not Affect Type I Collagen mRNA Levels or Viability of Dermal Fibroblasts *In Vitro*[a]

Kynurenine (μM)	Relative mRNA level[b]	Cell Viability[c] (%)
0	4.99	88
100	5.44	90
250	5.33	94
500	5.43	87

[a] Kynurenine was added to confluent cultures of fibroblasts maintained in complete media. Cultures were harvested following a 48-h incubation period, and type I collagen mRNA levels were determined by Northern analysis, as described in *Materials and Methods*.
[b] Relative levels of type I collagen mRNA, expressed as densitometric units, corrected for variations in GAPDH mRNA levels in the same samples.
[c] Viability was determined by counting cells in a hemocytometer, and assessing trypan blue dye exclusion.

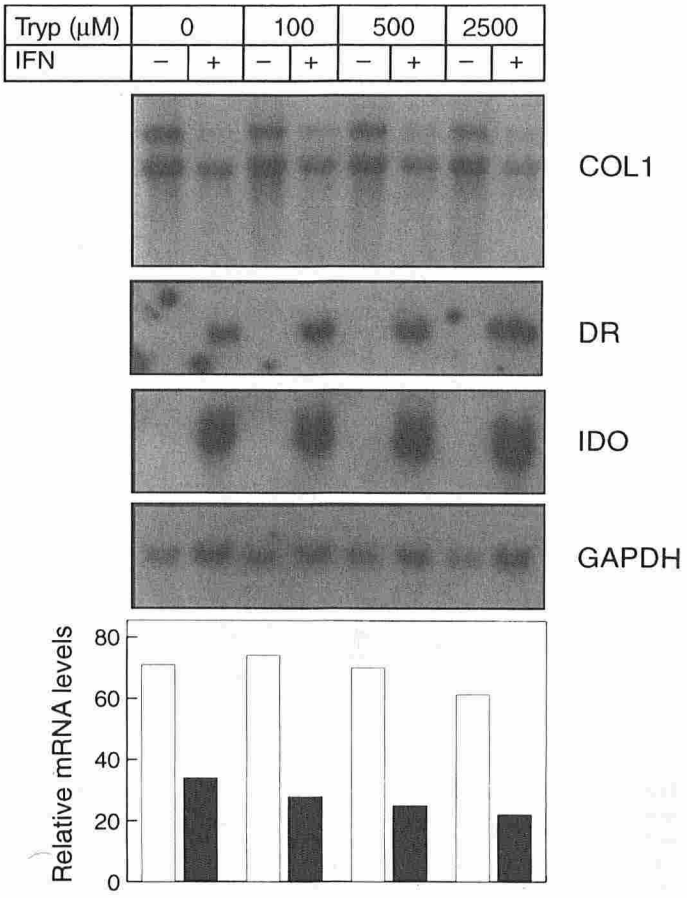

Figure 4. Repletion of tryptophan in the cultures fails to abrogate inhibition of collagen mRNA expression in IFN-γ–treated fibroblasts. IFN-γ (500 U/ml) was added to fibroblasts maintained in complete media, followed 8 h later by various amounts of tryptophan, as indicated. At the end of an additional 28 h incubation, cultures were harvested, and RNA was analyzed by Northern hybridization with type I collagen, HLA-DR alpha chain (DR), IDO, and GAPDH cDNA, as described in *Materials and Methods*. Abundance of mRNA was quantitated, and the values are shown after correction for variations in GAPDH mRNA levels (lower panels). Values shown represent the mean of results from two separate experiments.

The present findings indicate that tryptophan depletion results in a marked and selective decrease in type I collagen mRNA expression in cultured fibroblasts. The results suggest, however, that induction of oxidative tryptophan catabolism and ensuing tryptophan depletion are not directly responsible for the decrease in type I collagen mRNA in IFN-γ–treated fibroblasts. The effects of IFN-γ on collagen gene expression may be direct; indeed, several IFN-γ–response elements (TTnCnnnAA) are located in the upstream regulatory region of the human COL1A1 gene (unpublished). Alternately, intracellular pathways distinct from tryptophan catabolism, such as inducible nitric oxide production, may be involved in mediating the effects of IFN-γ on type I collagen gene expression. In this regard, it is of great interest that recent observations indicate a complex functional interrelationship between arginine and tryptophan catabolism, two important IFN-γ–inducible cellular pathways [36,37]. The relatively delayed inhibition of type I collagen mRNA expression following exposure of fibroblasts to IFN-γ, and the sensitivity of this response to cycloheximide, suggest that the effect of IFN-γ is a secondary phenomenon. A precise understanding of the molecular mechanisms through which IFN-γ inhibits collagen production in dermal fibroblasts may facilitate the application of IFN-γ in anti-fibrotic therapy.

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