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Tumor necrosis factor-α inhibits collagen synthesis in human and rat granulation tissue fibroblasts

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Abstract. The purpose of the study was to examine the effects of tumor necrosis factor-α (TNF-α) on collagen gene expression in rat and human granulation tissue fibroblast cultures. The cells were exposed to 0.1, 1, 10, or 100 ng/ml of TNF-α, and the rate of collagen synthesis was measured as synthesis of protein-bound ^{3}H-hydroxyproline. Total cellular RNA was isolated from fibroblasts, and measurements of specific cellular RNAs from fibroblasts were performed by Northern blot hybridizations using ^{32}P-labeled cDNA probes. In cultures of rat granulation tissue fibroblasts TNF-α decreased ^{3}H-hydroxyproline production to about 75% of that in controls and it also decreased proα1(I) and proα1(III) collagen mRNA levels, maximally to 33% and 23% of the control levels, respectively. In cultures of human granulation tissue fibroblasts a similar inhibiting effect in the production of collagen was seen. TNF-α decreased the production of ^{3}H-hydroxyproline to 56% of the control value with a dose of 100 ng/ml also having an inhibiting effect on proα1(I) collagen mRNA levels of up to 43% of the control level. However, no effect was seen on proα1(III) collagen mRNA levels.
Key words. Tumor necrosis factor-α; wound healing; granulation tissue; collagne; collagen synthesis; fibroblast.

Excessive accumulation of collagen in healing wounds results in the formation of hypertrophic scars of keloids[1,2]. The most abundant collagens expressed in skin in vivo, and by dermal fibroblasts in vitro, are type I, III and VI collagens[3–5]. Of these, type I collagen represents 80–90% of the dermal collagen[6]. Tumor necrosis factor-α (TNF-α) is known to inhibit collagen synthesis and increase collagenase production[7,8]. The addition of TNF-α to fibroblast cultures has produced variable responses in terms of collagen metabolism depending on the experimental system and the source of fibroblasts[9–12]. In dermal fibroblasts TNF-α has been shown to suppress type I procollagen mRNA levels[12]. The responsiveness of fibroblasts cultured from granulation tissue to TNF-α has not been studied although these cells are known to differ in their response to various inflammatory mediators[13]. Using a subcutaneously implanted viscose sponge as a wound model in the rat we have shown a decrease in wound collagen content with daily injections of TNF-α into the sponge[14].

The purpose of the present study was to examine the effects of various doses of TNF-α on collagen synthesis by human and rat granulation tissue-derived fibroblasts in culture.

Materials and methods

Cell culture studies. Fibroblasts were released from human and rat experimental granulation tissue as described earlier for synovial cells[15]: granulation tissue was cut into small pieces and digested by collagenase (0.5 mg/ml collagenase type IV, No C-5138, Sigma) and DNase (0.15 mg/ml DNase type I, No 260912, Calbiochem) in Hanks's balanced salt solution containing 5 mM $CaCl_2$. After shaking at 37 °C for two to three hours the cell suspension was filtered through cotton gauze and the cells collected by centrifugation. The cells were grown in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 10% fetal calf serum. For passaging the cells were detached with trypsin and divided 1:3. The rate of collagen synthesis was measured as synthesis of protein-bound ^{3}H-hydroxyproline. Confluent cell cultures were first preincubated for 24 h in the presence of ascorbic acid (50 μg/ml) and 1, 10, or 100 ng/ml of recombinant human TNF-α (produced in *E. coli* and purified to homogeneity by BASF Company, Germany). Rat granulation tissue fibroblasts were also treated with 0.1 ng/ml of TNF-α. After preincubation fresh ascorbic acid, β-aminopropionitrile (50 μg/ml), and TNF-α were added with ^{3}H-proline (2.5 μCi/mmol, New England Nuclear, Boston, MA, USA) and the amount of peptide-bound ^{3}H-hydroxyproline in the labeling media was measured after 24 h[16]. The number of cells in culture dishes was counted with a Bürker's hemocytometer after detaching the cells with trypsin treatment.

Isolation of RNA. Isolation of total cellular RNA from fibrobasts was performed as described by Chirgwin et al.[17]. The cells were lysed in 3 ml of 4 M guanidinium thiocyanate, 25 mM sodium acetate and 0.14 M β-mercaptoethanol. The lysates were layered onto a CsCl

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cushion (5.7 M CsCl, 25 mM sodium acetate) and centrifuged in a SW-55 rotor (Beckman) for 21 h at 35,000 rpm at 20 °C. Thereafter the aqueous guanidinium thiocyanate solution and CsCl were removed and the RNA pellets were washed with 200 µl of 95% ethanol. The pellets were dissolved in 300 µl of RNase-free water, RNA was extracted with 300 µl of phenol/chloroform (1:1) and the aqueous phase precipitated with 1:10 vol of 3 M sodium acetate and 750 µl of 95% ethanol at −20 °C. The RNA pellet was dried and finally dissolved into RNase-free water.

Hybridizations. 12 µg aliquots of total cellular RNA were fractionated electrophoretically on 0.75% agarose gel after denaturation with glyoxal, and transferred to Pall Biodyne nylon membrane (Pall Process Filtration Ltd, Portsmouth, UK). RNA was immobilized to the membrane by baking at 80 °C for 2 h. The membrane was prehybridized for 2 h in a solution containing 50% formamide, 5 × SSC, 0.05 M sodium phosphate, pH 6.5, 0.02% bovine serum albumine, 0.02% polyvinylpyrrolidone, 0.02% ficoll, 250 µg/ml denatured calf thymus DNA, and 0.1% sodium dodecyl sulphate[18]. Hybridization was performed for 20 h in an identical solution containing in addition a cDNA probe labeled by nick-translation with α-^{32}P-dCTP (800 Ci/mmol, Amersham, UK) to a specific activity of approximately 5×10^7 cpm/µg. Plasmids used in hybridizations were pα1R2, specific for rat proα1(I) collagen mRNA[19], pRGR5 for rat proα1(III) collagen mRNA[20], pRGAPDH specific for rat glyceraldehyde-3-phosphate dehydrogenase mRNA[21], probe 341-1 for 28S ribosomal RNA[22], pHCAL1U for human proα1(I)[23], and pHFS 3 for human proα1(III)[24]. After hybridizations the membrane was washed three times for 5 min in 2 × SSC/0.1% SDS at room temperature and two times for 30 min in 0.1 × SSC/0.1% SDS at 55 °C. Kodak X-Omat films were exposed to membranes at −70 °C with intensifying screens and the extent of hybridization was quantified densitometrically from the X-ray films.

Results

Collagen production in cell culture. In all the experiments performed with rat granulation tissue fibroblasts, TNF-α was found to decrease collagen synthesis (as determined by hydroxyproline radioactivity). This decrease was observable with 0.1 ng/ml of TNF-α (−16%) and reached its maximal value, (−31%) with 100 ng/ml of TNF-α (table 1). However, in human granulation tissue fibroblast cultures TNF-α was able to decrease collagen synthesis only at a concentration of 10 and 100 ng/ml (table 2). The maximum decrease (−45%) was observed with 100 ng/ml of TNF-α.

Cellular mRNA levels. Measurement of cellular mRNA levels was performed by Northern blot hybridizations using ^{32}P-labeled cDNA probes specific for rat and human proα1(I) and proα1(III) collagen mRNA. Cellular mRNA levels of a non-collagenous 'house-keeping' protein, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were measured as a reference mRNA in rat granulation tissue culture examinations and 28S rRNA in human granulation tissue cultures. In the cultures of rat granulation tissue fibroblasts TNF-α was found to reduce proα1(I) and proα1(III) collagen mRNA levels maximally by 67% and 77% from the control level, respectively, when corrected for cellular GAPDH and mRNA levels (table 3; fig. 1). Correspondingly, in the

Table 1. Effect of various doses of tumor necrosis factor-α (TNF-α) on ^{3}H-hydroxyproline production in rat granulation tissue fibroblasts.

Group	Cells × 10^3	Radioactive hydroxyproline (d.p.m./10^3 cells)
Control	528 ± 30	0.73 ± 0.05
TNF-α		
0.1 ng/ml	561 ± 17 (+6%)	0.61 ± 0.02 (−16%)
1 ng/ml	551 ± 37 (+4%)	0.54 ± 0.04 (−26%)
10 ng/ml	495 ± 24 (−6%)	0.56 ± 0.03 (−23%)
100 ng/ml	474 ± 24 (−10%)	0.50 ± 0.03 (−31%)

Twenty culture dishes of confluent fibroblasts were randomly divided into the four groups. Thus each measurement was performed in quadruplicate (mean ± SEM).

Table 2. Effect of various doses of tumor necrosis factor-α (TNF-α) on ^{3}H-hydroxyproline production in human granulation tissue fibroblasts.

Group	Cells × 10^3	Radioactive hydroxyproline (d.p.m./10^3 cells)
Control	152 ± 9	0.73 ± 0.06
TNF-α		
1 ng/ml	147 ± 12 (−3%)	0.73 ± 0.06 (±0%)
10 ng/ml	138 ± 20 (−9%)	0.49 ± 0.09 (−32%)
100 ng/ml	163 ± 7 (+7%)	0.40 ± 0.03 (−45%)

Sixteen culture dishes of confluent fibroblasts were randomly divided into the four groups. Thus each measurement was performed in quadruplicate (mean ± SEM).

Table 3. Cellular mRNA levels of proα1(I) and proα1(III) collagen in rat granulation tissue fibroblasts.

Group	proα1(I)/GAPDH	proα1(III)/GAPDH
Control	100	100
TNF-α		
1 ng/ml	56	31
10 ng/ml	33	23
100 ng/ml	47	30

The values shown in relative densitometric units are from analysis of a representative Northern blot.
proα1(I)/GAPDH represents proα1(I) collagen mRNA values corrected for cellular GAPDH mRNA levels.
proα1(III)/GAPDH represents proα1(III) collagen mRNA values corrected for cellular GAPDH mRNA levels.

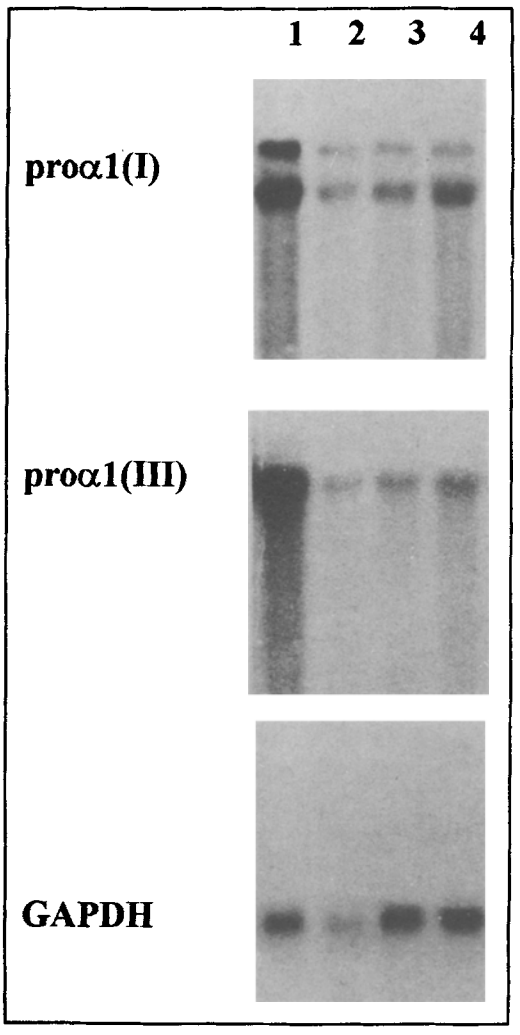

Figure 1. Northern analysis of procollagen mRNA levels in developing rat granulation tissue after TNF-α treatment. The mRNAs were analyzed separately in quadruplicate cultures. The filters were hybridized with rat cDNA probes for proα1(I) collagen, proα1(III) collagen and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Lines from 1 to 4 represent TNF-α concentrations of 0, 1, 10 and 100 ng/ml, respectively.

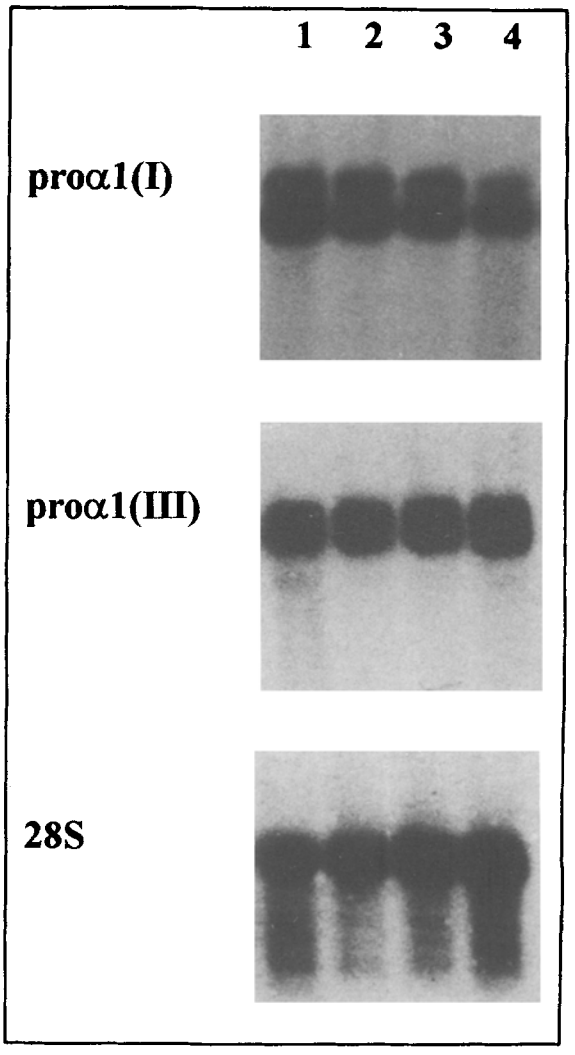

Figure 2. Northern analysis of procollagen mRNA levels in developing human granulation tissue after TNF-α treatment. The mRNAs were analyzed separately in quadruplicate cultures. The filters were hybridized with human cDNA probes for proα1(I) collagen, proα1(III) collagen and 28S for ribosomal RNA. Lines from 1 to 4 represent TNF-α concentrations of 0, 1, 10 and 100 ng/ml, respectively.

Table 4. Cellular mRNA levels of proα1(I) and proα1(III) collagen in human granulation tissue fibroblasts.

Group	proα1(I)/28S	proα1(III)/28S
Control	100	100
TNF-α		
1 ng/ml	94	96
10 ng/ml	74	100
100 ng/ml	43	99

The values shown in relative densitometric units are from analysis of a representative Northern blot.
proα1(I)/28S represents proα1(I) collagen mRNA values corrected for cellular 28S mRNA levels.
proα1(III)/28S represents proα1(III) collagen mRNA values corrected for cellular 28S mRNA levels.

cultures of human granulation tissue fibroblasts proα1(I) collagen mRNA levels decreased dose-dependently with TNF-α (up to 43% with 100 ng/ml of TNF-α) compared to the control cultures (table 4; fig. 2). In contrast, the differences observed in proα1(III) collagen mRNA levels with TNF-α were small and insignificant.

Effect on cell proliferation. The results summarized in tables 1 and 2 show that the effects of TNF-α on proliferation of rat and human granulation tissue fibroblasts were marginal (between +7% and −10% of the controls), and clearly smaller than the changes in hydroxyproline and procollagen mRNA levels.

Discussion

TNF-α has been shown to induce a broad range of activities in vivo and in cultures of several different cell types, some of which are related to wound healing. The level of wound fluid TNF-α within a subcutaneously

implanted polyurethane sponge has been found to be significantly higher than basal serum levels in non-wounded mice but remained clearly under 0.1 ng/ml[25,26]. Local application of 50 µg of TNF-α is detrimental to wound healing as it decreases activity of the type I collagen genes[27]. Similarly, we have previously shown an inhibitory effect with 200 ng, but not with 50 ng, of TNF-α on the formation of new granulation tissue in rats[14]. In culture conditions, the effects of TNF-α on cell proliferation seem to depend on the origin of the fibroblasts and on experimental conditions[9-12]. While TNF-α has been shown to stimulate the proliferation of normal human fibroblasts[28] it inhibits the proliferation of keloid fibroblasts[29]. In this study, TNF-α did not particularly stimulate cell proliferation in rat or human granulation tissue fibroblasts suggesting that normal wound fibroblasts respond differently to TNF-α than fibroblasts from normal skin or keloids. Besides inhibiting collagen synthesis and increasing collagenase production[7,8,30], TNF-α has been shown to cause ~50% decrease in the quantity of procollagen α1(I) mRNA levels in dermal fibroblasts[7,31]. In the present study, 10–100 ng/ml of TNF-α decreased collagen synthesis in both rat and human granulation tissue fibroblasts. TNF-α produced a related reduction of procollagen α1(I) mRNA levels in both cell types demonstrating that TNF-α inhibits type I collagen production at pretranslational level. Furthermore, TNF-α has also been shown to reduce procollagen III mRNA levels 2-fold in human skin fibroblast cultures[32]. This is in accordance with present results where procollagen α1(III) mRNA levels decreased 3-fold in rat granulation tissue fibroblasts. However, in human granulation tissue fibroblasts procollagen α1(III) mRNA levels remained unchanged. This suggests that fibroblasts cultured from different species and/or locations differ in their responsiveness. Our finding of an increase in the ratio of α1(III)/α1(I) mRNA levels is in agreement with an elevated level of type III collagen in healing wounds when compared with the surrounding skin[33]. In most cases the mRNA levels and hydroxyproline synthesis seem to behave similarly. However, the TNF-α-induced reduction in procollagen mRNAs was generally greater than the effects on medium hydroxyproline. This probably reflects the fact that the latter represents all collagen which has accumulated into the culture medium in 24 h while the former represents the mRNA level at the end of the period.

This study provides additional evidence that TNF-α acts as an agent against excessive scar formation and keloids. It is in agreement with previous studies where hypertrophic scarring was suggested to be related to low levels of TNF-α[34]. It is of note that the ratios of the different collagen types may vary in different types of scars[5,35,36]. Markedly elevated levels of procollagen α1(I) and α1(III) mRNA have been observed in hypertrophic scars but not in normal scars[37]. Similarly, the relative concentration of type I collagen is suggested to be significantly higher in keloid tissue than in normal skin[35]. TNF-α is thus a potentially useful agent to inhibit the development of hypertrophic scars, keloid formation and fibrosis. More information is still needed about different doses of TNF-α and mechanisms of its action on normal and pathogenic wound healing.

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