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# Tumor necrosis factor inhibits collagen and fibronectin synthesis in human dermal fibroblasts

A. Mauviel, M. Daireaux, F. Rédini, P. Galera, G. Loyau and J.-P. Pujol

*Laboratoire de Biochimie du Tissu Conjonctif, CHU Côte de Nacre, 14040 Caen Cedex, France*

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Tumor necrosis factor (TNF) caused inhibition of collagen production by confluent cultures of human dermal fibroblasts in a dose-dependent manner. Concomitant increase of prostaglandin $E_2$ release was observed as a result of TNF-induced cell activation. However, a blockade of the cyclooxygenase pathway of arachidonate metabolism by indomethacin did not abrogate the inhibitory effect of TNF on collagen synthesis, suggesting that this effect could be independent of prostaglandin metabolism. Gel electrophoresis of the newly synthesized macromolecules from the culture media showed that both type I and type III collagens as well as fibronectin were affected by the inhibition. Electrophoresis of cell layer-associated proteins demonstrated that the reduction in amounts of collagen and fibronectin in the medium did not result from an intracellular accumulation of these macromolecules. Production of procollagens was reduced in parallel to that of collagens, suggesting that the effect of TNF is exerted before the processing steps of procollagens. These results clearly show that TNF could play a role in modulation of matrix deposition by fibroblasts during inflammatory processes.

Tumor necrosis factor; Collagen; Fibronectin; Prostaglandin $E_2$; (Dermal fibroblast)

## 1. INTRODUCTION

Tumor necrosis factor, cachectin, is a 17 kDa polypeptide released by macrophages and monocytic cell lines stimulated with bacterial endotoxin [1] playing a crucial role in inflammatory response and tumor lysis. Besides cytotoxic and cytostatic activities on mice and human tumor cell lines [2,3], TNF induces multiple responses in several cell types, including connective tissue cells. For example, TNF has been shown to stimulate both prostaglandin $E_2$ ($PGE_2$) and collagenase release in cultured synovial cells [4]. It also enhances resorption of proteoglycans and inhibits their synthesis in cartilage explants [5]. In contrast to its cytostatic effect on tumor cells, TNF exerts growth-stimulating action on normal human fibroblasts [6].

Thus, in several cases, TNF shares many biological activities with interleukin-1 (IL-1) [7–9]. The role of this latter cytokine as a potent stimulant of extracellular matrix degradation and its implication in both inflammatory and degenerative joint diseases have been well documented (review in [10]). We and other authors have also reported that IL-1 could modulate the production of collagen in cultured fibroblasts [11,12], synovial cells [13–15] and articular chondrocytes [14,16]. Since TNF has been demonstrated as a mediator of inflammation [17], it was of interest to determine whether this monokine could also act on the production of extracellular matrix proteins as does IL-1. Attempting to address this hypothesis, we report here that recombinant TNF can inhibit in vitro the synthesis of collagen and fibronectin by dermal fibroblasts. We also provide evidence that this effect is not due to stimulation of prostaglandin formation.

*Correspondence address:* A. Mauviel, Laboratoire de Biochimie du Tissu Conjonctif, CHU Côte de Nacre, 14040 Caen Cedex, France

## 2. MATERIALS AND METHODS

### 2.1. *Fibroblast culture*

Human dermal fibroblasts obtained by explants from foreskin samples, were cultured in Dulbecco's modification of

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Eagle's minimum essential medium (DMEM) supplemented with 10% heat inactivated fetal calf serum (FCS), penicillin (100 IU/ml), streptomycin (100 μg/ml) and fungizone (2.5 μg/ml). The cells were grown at 37°C in a 5% $CO_2$ atmosphere and experiments were performed on confluent cultures to minimize the growth-stimulating effect of TNF.

2.2. *TNF preparation*

Throughout this study we used recombinant human TNF-α, produced by *E. coli* and purified to homogeneity by BASF, FRG.

2.3. *Experimental procedure*

For the experiments, cells were grown in 9.6 $cm^2$ dishes. After reaching confluency, the cultures were preincubated for 24 h in DMEM supplemented with 2% FCS and 50 μg/ml ascorbic acid (Sigma). Then the media were removed and fresh medium containing also 50 μg/ml β-aminopropionitrile (Sigma) was added, with or without TNF. After a 24 h incubation, the media were collected and used to assay $PGE_2$ content whereas the cell layers were washed twice with phosphate-buffered saline (PBS) and dissolved in 0.2 M NaOH for subsequent protein determination. Estimation of total collagen production was performed under the same conditions, in the presence of 2 μCi/ml [$^3$H]proline (CEA, France, 30–40 Ci/mmol). For determination of the amounts of type I and III collagens and fibronectin by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), radiolabelling was performed with 25 μCi/ml [$^3$H]proline in 25 $cm^2$ flasks.

2.4. *Assay of labelled collagen and non-collagenous proteins*

In the experimental conditions used, more than 95% of the newly synthesized collagen was recovered in the culture medium. Therefore, only collagen from the medium was assayed, as collagenase-digestible protein [18], using purified enzyme from Advance Biofactures Corp., Lynnbrook, NY. Results were expressed as dpm/μg of cell protein. The amount of non-collagenous protein was estimated from the radioactivity remaining after collagenase digestion. Proportion of collagen among other radioactive proteins was calculated with the following formula that takes into account the enrichment of proline in collagen compared with other proteins [19]:

$$\%\ \text{of collagen} = \frac{\text{dpm in collagenase digest} \times 100}{(5.4 \times \text{dpm in residue}) + \text{dpm in collagenase digest}}$$

2.5. *Analysis of radiolabelled proteins by gel electrophoresis*

At the end of experiments, the media were collected and one half of each sample received proteinase inhibitors to final concentrations of 20 mM EDTA, 10 mM *N*-ethylmaleimide and 1 mM phenylmethylsulfonyl fluoride. Both fractions were dialyzed against 1 mM ammonium carbonate buffer (pH 7.5) and lyophilized. The sample half without inhibitors was then digested with pepsin (0.1 mg/ml, Sigma, 2 × cryst.) in solution containing L-proline (400 μg/ml) and acetic acid (0.5 M) at 4°C for 15 h. After lyophilization, the samples were run through a 6% polyacrylamide gel containing SDS and 2 M urea as described by O'Farrell [20]. The cell layers were directly lysed in O'Farrell's sample buffer and the extracts analyzed in the same way. The samples were reduced or not by β-mercaptoethanol (0.1 M). After electrophoresis, the gels were fixed and prepared for fluorography as described previously [21]. The amount of radioactivity present in the bands corresponding to fibronectin and collagen chains was estimated by densitometry.

2.6. *Prostaglandin $E_2$ assay*

$PGE_2$ was assayed in the culture medium by specific radioimmunoassay [22] with antiserum from Institut Pasteur Production, Marnes la Coquette, France.

2.7. *Cell protein determination*

After dissolution of cell layer with 0.2 M NaOH, the amount of protein was assayed by the method of Hartree [23], using albumin as a standard.

2.8. *Statistical analysis*

All the results presented are expressed as mean ± SE unless stated otherwise. The Student's *t*-test was used to evaluate the difference of the means between groups.

## 3. RESULTS

### 3.1. *TNF inhibition of collagen and non-collagenous protein synthesis*

There were no obvious differences in cell morphology under the phase contrast microscope between control and TNF-treated cultures. Fibroblasts were exposed to three concentrations of TNF for 24 h: 1, 10 and 100 ng/ml. As little as 1 ng/ml was enough to reduce markedly both col-

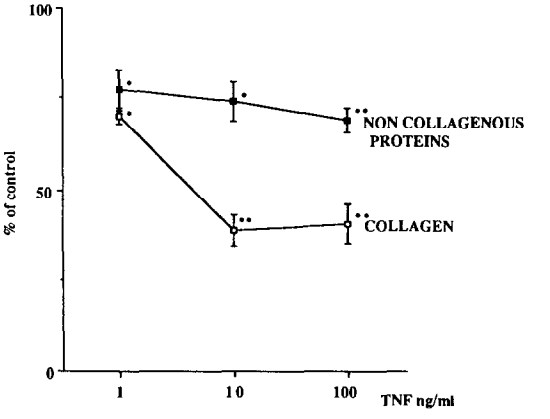


Fig.1. Effect of TNF on collagen and non-collagenous protein synthesis. Confluent fibroblasts were incubated for 24 h in the presence of varying concentrations of TNF and [$^3$H]proline as described in section 2. Amounts of radiolabelled collagen and non-collagenous protein are expressed in dpm/μg of cell layer protein and represented as a percentage of controls (values for controls: collagen, 184 ± 12 dpm/μg cell protein; non-collagenous protein, 279 ± 14 dpm/μg cell protein). The results are means ± SE of triplicate dishes. * $p < 0.01$, ** $p < 0.001$.

lagen and non-collagenous protein production by 30 and 23%, respectively (fig.1). Maximum inhibition of collagen synthesis was observed for 10 ng/ml (60%) and reached a plateau. The production of non-collagen was less affected since only 30% inhibition was registered for the highest concentration of TNF used, 100 ng/ml. The relative proportion of collagen among the total newly synthesized protein, taking into account the enriched imino acid content of collagen, was therefore reduced from 11.2% in control cultures to 10.0, 6.1 and 6.7% in the cultures exposed to 1, 10 and 100 ng/ml TNF, respectively.

Despite fibroblasts being used after reaching confluency, a growth-enhancing action of TNF was observed for concentrations of 10 and 100 ng/ml, whereas 1 ng/ml was without significant effect. Increases of cell proliferation, measured in terms of cell protein, reached 16 and 24% above controls for 10 and 100 ng/ml TNF, respectively ($p < 0.05$, $n = 3$).

### 3.2. *Effect of TNF on the production of $PGE_2$ by fibroblasts*

To investigate further the mechanism of fibroblast activation by TNF and to what extent the inhibitory effect on collagen synthesis could be related to arachidonate metabolism, we assayed the level of $PGE_2$ in the culture medium in parallel to collagen labelling. Results shown in table 1 clearly demonstrate that TNF markedly stimulated the secretion of $PGE_2$ by dermal fibroblasts in a dose-dependent manner. Enhancement reached 4.5 to 12.8-fold the values of control cultures when TNF concentrations varied from 1 to 100 ng/ml.

Table 1

$PGE_2$ production by dermal fibroblasts treated with TNF

| Sample | Prostaglandin $E_2$ | |
|---|---|---|
| | pg/ml | % of control |
| Control | 658 ± 127 | 100 |
| TNF, 1 ng/ml | 2968 ± 94[a] | 451 |
| TNF, 10 ng/ml | 4673 ± 145[a] | 710 |
| TNF, 100 ng/ml | 8416 ± 774[a] | 1279 |

Experimental conditions were the same as for fig.1. Results are means ± SE of triplicate dishes. [a] $p < 0.001$

### 3.3. *Addition of indomethacin does not abolish the TNF-induced inhibition of collagen synthesis*

Given that the depression of collagen synthesis induced by TNF occurred concomitantly to a huge increase of $PGE_2$ released in the medium, and that $PGE_2$ has been reported to inhibit collagen synthesis [23], it was of interest to determine whether addition of a blocker of the cyclooxygenase pathway of arachidonate metabolism could modify the outcome. The results shown in fig.2 indicate that addition of indomethacin (Indo) did not abrogate the inhibitory effect of TNF on collagen synthesis, the difference between the value for 'TNF + Indo' compared to that of 'Indo' remaining statistically significant. These data suggest that TNF exerts its effect on collagen production through mechanisms that seem unrelated to prostaglandin metabolism.

### 3.4. *SDS-PAGE analysis of radiolabelled collagens and fibronectin*

To investigate further the effect of TNF on matrix deposition, newly synthesized proteins from the media of cultures treated with increasing concentrations of TNF were analyzed by slab SDS-

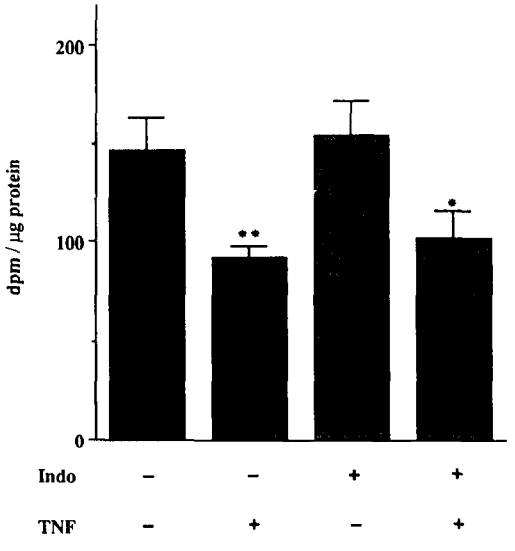


Fig.2. Effect of indomethacin (10 µM) on the inhibition of collagen synthesis by TNF (10 ng/ml). Experimental conditions were the same as for fig.1. Results, expressed in dpm/µg of cell protein, are means ± SE of triplicate dishes. * $p < 0.01$, ** $p < 0.001$.

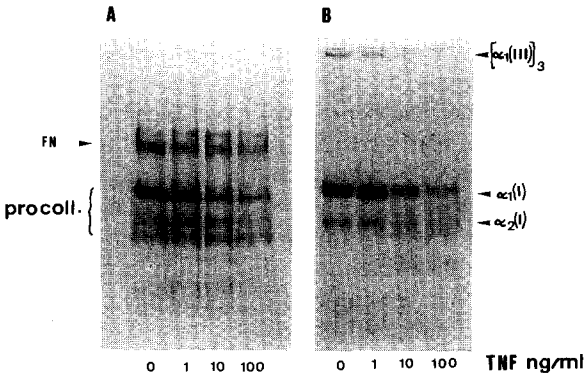


Fig.3. SDS-PAGE fluorograph of medium proteins. (A) Total proteins after reduction with $\beta$-mercaptoethanol (0.1 M). FN, fibronectin; procoll., procollagens. (B) Pepsin-digested proteins without reduction. Collagen chains are indicated by arrowheads.

PAGE and subsequent fluorography, with and without pepsin digestion. The fluorograms are shown in fig.3A and B and the scannings of procollagen, collagen and fibronectin bands are presented in table 2 as arbitrary densitometric units. These results confirm the inhibitory effect of TNF on collagen synthesis and show that both type I and type III were affected. The proportion of type III collagen produced by our cultures was small, so that we could not quantify it as dissociated $\alpha_1$(III) chains after interrupted electrophoresis (not shown). The band corresponding to the trimeric form $[\alpha_1(III)]_3$ (fig.3A) was too faint to really determine the amount of type III collagen with any accuracy. Therefore we cannot deduce from these experiments whether the inhibition extent is the same for both collagens or not. Fibronectin production was also decreased in a dose-dependent manner as can be seen in fig.3A. In addition, SDS-PAGE analysis of the cell-layer associated proteins demonstrated that the reduction of collagen and fibronectin in the culture media did not result from accumulation of these proteins in the cell layer since no increase in radioactivity could be observed in the corresponding bands (fluorographs not shown). Moreover, the amounts of cell-associated fibronectin and collagen were also reduced by TNF treatment to the same extent as in culture media.

Table 2

Scannings of fluorographs presented in fig.3

| | Control | TNF | | |
|---|---|---|---|---|
| | | 1 ng/ml | 10 ng/ml | 100 ng/ml |
| Fibronectin | 990 | 563 | 460 | 278 |
| | (100) | (56) | (46) | (28) |
| $\alpha_1(I) + \alpha_2(I)$ | 4116 | 4161 | 2441 | 1824 |
| | (100) | (101) | (59) | (44) |
| $[\alpha_1(III)]_3$ | 255 | 117 | 41 | 28 |
| | (100) | (46) | (16) | (11) |
| Procollagens | 2820 | 2671 | 2460 | 1500 |
| | (100) | (95) | (87) | (53) |

Results are expressed in arbitrary densitometric units and % of respective controls are indicated in parentheses

## 4. DISCUSSION

Many similarities have been shown between the activities of TNF and IL-1, two major monocyte-macrophage-derived cytokines (review in [25]). Both exert broad effects on various target cells, including cells from connective tissue. Their ability to induce matrix-resorbing activity has been well documented and their implication therefore suggested in the cartilage breakdown occurring in osteoarticular diseases [8,10]. Besides the resorptive effects of IL-1 exerted through stimulation of metalloprotease release [8], a modulating action of this cytokine on the synthesis of matrix molecules has also been demonstrated [11–16]. Concerning collagen metabolism, we have previously shown that IL-1 could augment the steady-state level of procollagen I and III mRNAs in human synovial cells [15] and dermal fibroblasts [26]. However, in spite of this increase in mRNA level, a reduction of the amount of collagen released into the culture medium was very often observed, suggesting that post-transcriptional events can influence the production of collagen in IL-1-treated cells, at least in in vitro systems [14,15,26]. In the present paper, we demonstrate that recombinant TNF is capable of inhibiting collagen and fibronectin synthesis in human dermal fibroblasts. Interestingly, a similar effect of TNF has been recently observed on collagen synthesis in fetal rat bone cultures [27]. Our results suggest that TNF displays the same modulating function as IL-1 on collagen production by connective tissue cells.

The effects observed here on the radioactivity present in collagen from fibroblasts exposed to

TNF could be due to decreased collagen synthesis, increased collagen degradation or both. It is difficult to rule out an eventual degradation of the collagen after secretion in the medium since TNF, like IL-1, was shown to stimulate collagenase release by synovial cells and fibroblasts [4]. However, we believe that it is unlikely that this process could account for the reduction of collagen recovered from the medium since collagenase is produced as an inactive form. Moreover, our cultures contain serum that would block any collagenase activity if present. For example, we have never found any spontaneously active collagenase in our earlier studies with IL-1, using the same experimental conditions [28]. Enhanced intracellular degradation of newly synthesized collagen molecules has also been suggested to play a role in the reduction of collagen production under the influence of IL-1 [14,15]. This hypothesis is based on the fact that activation of target cells by cytokines such as IL-1 and TNF generally provokes a rise in the concentration of intracellular cAMP, a messenger which has been reported to stimulate the degradation of newly synthesized collagen [29,30]. However, this remains to be confirmed on an experimental basis. It is more likely that the reduction of collagen caused by TNF was predominantly due to decreased synthesis. We do not know yet what the mechanism is by which TNF exerts this inhibitory effect. Work is in progress to determine whether procollagen mRNA level is altered by the factor and to what extent there is a similarity with the mode of action of IL-1. Previous reports have shown that the inhibition of collagen synthesis induced by IL-1 may be related in part to prostaglandin generation since addition of indomethacin frequently reversed the effect and produced an increase of collagen synthesis [13,14]. In support of this interpretation, it should be noted that exogenous $PGE_2$ added to cultures of fibroblasts causes a decrease in the level of procollagen mRNA [24] whereas indomethacin acts in the opposite way [31]. TNF has been reported to activate phospholipase $A_2$ in osteoblasts, synovial cells and fibroblasts [32] and the consequent formation of $PGE_2$. This was confirmed in the present studies since a great increase in $PGE_2$ release was observed. However, our results are not consistent with a role of $PGE_2$ in TNF action on collagen synthesis as no change was noticed in the presence of indomethacin, a cyclooxygenase inhibitor. Further research is needed to clarify the mechanism of TNF effects on collagen synthesis.

Recently, it has been demonstrated that fibroblasts can produce cytokines when stimulated with immunomodulators such as lipopolysaccharide or phorbol myristate acetate [33] or with cytokines themselves [34]. Therefore, it cannot be excluded that the effects observed in our experiments could be partly due to cytokines secreted by the fibroblasts as a result of TNF stimulation. For example, IL-1 or TNF-stimulated fibroblasts produce interferon-$\beta_2$ (IFN-$\beta_2$), a cytokine with antiviral properties [35]. However, we have recently shown that purified IFN-$\beta_2$ was without effect on both collagen and $PGE_2$ formation by fibroblasts (unpublished). Nevertheless, we do not know whether IL-1 or IFN-$\gamma$ could be expressed by TNF-activated fibroblasts and act as an amplification loop on the collagen synthesis.

In conclusion, we have shown that TNF is one factor responsible for inhibition of collagen synthesis that may play a role in the modulation of matrix turnover during inflammatory diseases.

## ADDENDUM

While this manuscript was being submitted, a paper was published on the same subject by Solis-Herruzo et al. [36]. Our study is in accordance with their data and extends them further to fibronectin.

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