

## Cytokine interactions promote synergistic fibronectin accumulation by mesangial cells

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### Cytokine interactions promote synergistic fibronectin accumulation by mesangial cells.

**Background.** The development of glomerulosclerosis has been associated with the presence of the cytokines transforming growth factor- $\beta_1$  (TGF- $\beta_1$ ), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and/or interleukin-1 $\beta$  (IL-1 $\beta$ ), at some stage in the glomerulus. To better understand the role of these cytokines in the scarring process their effect on rat mesangial cell fibronectin production was investigated.

**Methods.** Mesangial cells were exposed to 10 ng/ml of either TGF- $\beta_1$ , TNF- $\alpha$ , or IL-1 $\beta$  or to TGF- $\beta_1$  in combination with either TNF- $\alpha$  or IL-1 $\beta$ . Tissue culture supernatants and cell lysates were assayed for fibronectin. Supernatants were also assayed for TGF- $\beta_1$ . Northern blot analyses probing for fibronectin, transin, TIMP-1 and TGF- $\beta_1$  were carried out on RNA extracted from mesangial cells exposed to individual and combinations of cytokines.

**Results.** Individually these cytokines were only able to induce modest increases in fibronectin protein levels. However, when mesangial cells were exposed to TGF- $\beta_1$  in combination with either TNF- $\alpha$  or IL-1 $\beta$  then fibronectin levels were synergistically up-regulated approximately fivefold over unstimulated levels. Northern analysis demonstrated that fibronectin mRNA levels in the combination were also synergistically increased. In contrast, rat transin gene expression in the combinations was reduced to well below levels induced by TNF- $\alpha$  and IL-1 $\beta$  individually. In addition, synergistic up-regulation of both TGF- $\beta_1$  protein and message by the cytokine combinations was also observed. TGF- $\beta_1$ :TNF- $\alpha$  and TGF- $\beta_1$ :IL-1 $\beta$  induced additive increases in TIMP-1 (tissue inhibitor of metalloproteinases-1) mRNA levels.

**Conclusions.** These data illustrate that complex interactions can occur between cytokines within the glomerulus modulating both matrix synthetic and degradation pathways. These could initiate the scarring process and the development of glomerulosclerosis.

The effects of individual cytokines on the modulation of glomerular cell proliferation, matrix synthesis and immunoinflammatory modulation has been extensively studied [1, 2]. The research has usually focused on the properties and actions of a single cytokine within any one particular disease model.

Transforming growth factor- $\beta$  (TGF- $\beta$ ), for example, has been shown to play a major role in the stimulation of matrix

production by glomerular cells [3]. The level TGF- $\beta$  mRNA and the number of cells producing the protein has been shown to be higher in glomeruli isolated from anti-Thy-1 nephritic rats than in glomeruli from normal rats [4]. Furthermore, administration of a neutralizing antibody to TGF- $\beta$  in the same model has been shown to result in significant suppression of extracellular matrix production with a concomitant reduction in the degree of histological damage [5]. Up-regulated TGF- $\beta$  expression has also been reported in other models of glomerulonephritis including Habu snake venom-induced mesangioproliferative glomerulonephritis [6], anti-glomerular basement membrane (GBM) nephritis [7] and in puromycin aminonucleoside nephrosis (PAN) [8]. Further support for a pathogenetic role of TGF- $\beta$  in renal scarring has been provided by the observation that *in vivo* transfection of the TGF- $\beta$  gene increases the production of extracellular matrix deposition in the kidney [9].

One potential source of this cytokine within the glomerulus is the infiltrating macrophage. Ding et al demonstrated that glomerular macrophages isolated from PAN nephrotic or diet induced hypercholesterolemic rats expressed TGF- $\beta$  mRNA. Similarly, peritoneal macrophages from nephrotic or diet induced hypercholesterolemic rats also exhibited augmented TGF- $\beta$  mRNA levels compared to normal rats [10].

Macrophages are also potent sources of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin (IL)-1 $\beta$ . TNF- $\alpha$  has been shown to be secreted by glomerular macrophages isolated from rabbits with nephrotoxic nephritis [11] while anti-TNF- $\alpha$  antibodies have been shown to dose dependently reduce proteinuria and glomerular necrosis in the same model [12]. Macrophage secretion of IL-1 $\beta$  has also been demonstrated in nephrotoxic nephritis [13, 14]. In addition, IL-1 receptor antagonist treatment has proved beneficial in suppressing crescentic glomerulonephritis [15]. The presence of TNF- $\alpha$  and IL-1 $\beta$  has also been demonstrated within the glomeruli of rats with acute PAN nephrosis. Furthermore, maneuvers to reduce the macrophage infiltrates in these animals using an EFAD diet resulted in a

**Key words:** mesangial cells, fibronectin, TGF- $\beta$ , TNF- $\alpha$ , IL-1 $\beta$ , glomerulosclerosis, matrix development, scarring, injury.

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reduced number of cells staining positive for TNF- $\alpha$  and IL-1 $\beta$  in parallel with the number of ED1 positive cells [16].

Thus, under both physiological and pathological conditions mesangial cells *in vivo* are potentially exposed to a number of different cytokines acting in concert at any one time. This may be particularly pronounced in glomeruli containing large numbers of macrophages. Support for an important role of macrophages in the pathogenesis of glomerulosclerosis is provided by the observations that macrophage conditioned medium (MPCM) promotes a series of responses in cultured mesangial cells conducive to the initiation of the sclerotic process inducing the up-regulation of the genes and/or proteins for fibronectin, laminin and collagen IV, the modulators of matrix turnover TIMP-1 and transin, and the pro-fibrogenic cytokines TGF- $\beta$  and platelet-derived growth factor (PDGF) [17].

It is unlikely that a single cytokine would act in isolation, and thus the aim of the present study was to assess the contribution of, and the potential interactions between, cytokines known to be associated with the injured glomerulus, in the initiation of the scarring process.

Since an increase in the magnitude and distribution of fibronectin is the hallmark of most human and experimental models of glomerulosclerosis [18, 19], mesangial cell production of this matrix protein was chosen as a marker of scarring in the current study.

## METHODS

Unless otherwise stated chemicals and reagents were obtained from the Sigma Chemical Company, UK (Poole, England, UK).

### Culture of rat mesangial cells

Glomerular cells were cultured from the glomerular explants of adult Wistar rat kidneys using standard techniques [20]. The cells were cultured in RPMI 1640 supplemented with 20% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin (Gibco), 100  $\mu$ g/ml streptomycin (Gibco), 5  $\mu$ g/ml bovine insulin and 2 mM glutamine (Gibco). Cultured cells were characterized by their typical stellate fusiform morphology, their positive staining for the Thy-1 antigen [21] and their resistance to the toxic effects of D-valine [22].

Mesangial cells of passages 2 to 10 were cultured in 24 well plates (ICN Flow) or 25 cm<sup>2</sup> flasks (Corning), allowed to grow to confluence and then rendered quiescent in medium containing 0.5% FCS for 72 hours prior to use.

### Culture of mesangial cells in the presence of cytokines

Confluent quiescent mesangial cells were exposed to: (a) 10 ng/ml of either platelet derived-human TGF- $\beta$ <sub>1</sub>, recombinant murine TNF- $\alpha$  or recombinant murine IL-1 $\beta$  (R&D Systems) or medium alone; (b) 10 ng/ml TGF- $\beta$ <sub>1</sub> in combination with either 10 ng/ml TNF- $\alpha$  or IL-1 $\beta$ ; (c) 10 ng/ml of TGF- $\beta$ <sub>1</sub> in combination with 10 ng/ml TNF- $\alpha$  and 10

ng/ml IL-1 $\beta$ ; (d) 10 ng/ml of either TNF- $\alpha$  or IL-1 $\beta$  in combination with decreasing doses of TGF- $\beta$ <sub>1</sub> (10, 1, 0.1, 0.001, 0 ng/ml) for up to seven days. Mesangial cell culture supernatants and cell lysates were assayed for fibronectin.

For Northern blot analysis confluent, quiescent mesangial cells were exposed to 10 ng/ml TGF- $\beta$ <sub>1</sub>, TNF- $\alpha$  or IL-1 $\beta$  individually or in combination or to medium alone for 24 hours.

### Preparation of cell lysates

Following removal of tissue culture supernatants, cell monolayers were washed with PBS, scraped into 1% Nonidet P40 in wash buffer (PBS containing 0.3 M NaCl and 1% Tween 20) and then incubated at room temperature for approximately 30 minutes. The cell scrapings were then transferred into 2 ml tubes and sonicated for 5 seconds and centrifuged for 30 seconds at 11600 g. Sonication and centrifugation were repeated following which the lysate supernatants were assayed for fibronectin and total cell protein.

### Fibronectin ELISA

Culture supernatants or cell lysates were assayed for fibronectin using an inhibition ELISA, modified from the method of Rennard et al [23]. Briefly, 60  $\mu$ l of rat plasma fibronectin standard (19 to 5000 ng/ml; Calbiochem, UK), supernatant sample or cell lysate was incubated with an equal volume of rabbit anti-rat fibronectin diluted 1:2000 in wash buffer at 4°C overnight. Fifty microliters of this reaction mixture was then transferred to each well of a 96-well micotiter plate (Nunc Immunoplate, Denmark) which had been precoated at 4°C overnight with rat plasma fibronectin (1  $\mu$ g/ml in 0.05 M carbonate buffer, pH 9.6) and blocked with 2% BSA in wash buffer for one hour at room temperature. The plate was incubated with the reaction mixture at room temperature for two hours. After washing each well four times, 50  $\mu$ l of goat anti-rabbit IgG conjugated to horseradish peroxidase (HRP; Dako) was added to the well and incubated at room temperature for one hour. After a further four washes 50  $\mu$ l of 0.67 mg/ml 1,2 phenylenediamine dihydrochloride in 0.03 M citrate buffer pH 5.0, containing 0.012% H<sub>2</sub>SO<sub>4</sub> and absorbance read at 492 nm on a Titertek Multiskan Plus microtiter plate reader (Flow Laboratories).

### Transforming growth factor- $\beta$ ELISA

TGF- $\beta$  ELISAs were carried out using a commercially available assay on acid activated tissue culture supernatants according to the manufacturer's instructions (Promega).

### Protein determination

The protein content of cell lysates dissolved in 1% Nonidet P40 was determined by a BioRad DC protein assay using BSA standards.

## Northern blotting

Total RNA was extracted using TRIzol reagent (Gibco), a monophasic solution of phenol and guanidinium thiocyanate, based on a method of Chomczynski and Sacchi [24], according to the manufacturer's instructions. Thirty microgram aliquots of RNA were electrophoresed on a 1% agarose gel containing 1.9% formaldehyde, in MOPS (3-[N-morpholino]propane-sulfonic acid). The resolved RNA was transferred on to Hybond-N nylon membranes (Amersham) by capillary action using 20× SSC (1 × SSC = 15 mM tri-sodium citrate, 150 mM sodium chloride). The membranes were prehybridized for four hours at 37°C or 42°C (depending on probe) with 200 μg/ml of denatured salmon sperm DNA in 50% formamide, 1% SDS, 5× Denhardt's, 5× SSPE (1 × SSPE = 11.5 mM sodium phosphate, 150 mM sodium chloride, 1 mM EDTA). The membranes were then hybridized overnight with a <sup>32</sup>P-dCTP cDNA probe that had been Klenow DNA polymerase labeled using a random primer labeling system (Prime-a-Gene; Promega) in fresh hybridization buffer (same composition as prehybridization buffer). Following hybridization the membranes were washed twice with 1% SDS, 2× SSPE at room temperature, twice with 0.2% SDS, 0.2% SSPE at 65°C then exposed to X-Omat LS film (Kodak) with intensifier screens at -70°C. Membranes were subsequently stripped in boiling 5% SDS, 0.5× SSPE before reprobing. Densitometric analysis of the transcripts was carried out on a BioRad GS 700 imaging scanner. RNA loading was normalized using a cDNA probe for cyclophilin.

## Probes

All of the cDNA probes were generous gifts. cDNA for rat fibronectin was from Dr. R.O. Hynes (Massachusetts Institute of Technology, Boston, MA, USA) [25], murine TIMP-1 cDNA was from Dr. D.T. Denhardt (Department of Biochemistry, Rutgers University, Piscataway, NJ, USA) [26], rat transin cDNA was from Professor R. Breathnach (Laboratoire de Recherche, Nantes, France) [27], murine TGF-β was from R. Akhurst (Dept. Medical Genetics, Glasgow University, Glasgow, Scotland, UK) [28], and human cyclophilin was from SmithKline Beecham Pharmaceuticals.

## Statistics

To allow for variation in mesangial cell fibronectin production between multiple experiments, the results of the effects of cytokines or their combinations are presented as fold increase over control (medium alone). Absolute levels of fibronectin and TGF-β protein corrected for cell protein concentration are also given in the text where appropriate.

Data were expressed as means ± SEM. For comparison of means between two groups an unpaired *t*-test was used. To compare values between multiple groups an analysis of

variance (ANOVA) with a Bonferroni correction was applied. Statistical significance was defined as  $P < 0.05$ .

## RESULTS

### Effect of individual cytokines on fibronectin production

Under basal conditions (no added cytokines) the fibronectin concentration in mesangial cell culture supernatants was  $132.6 \pm 31$  ng/μg cell protein. Exposure of mesangial cells directly to TGF-β<sub>1</sub>, TNF-α or IL-1β at 10 ng/ml resulted in small but significant increases in secreted and cell-associated fibronectin levels ( $P < 0.04$  vs. medium alone, Fig. 1). For each of these cytokines, dose response curves of up to 25 ng/ml demonstrated that 10 ng/ml of the individual cytokine produced a maximal response (data not shown).

### Effect of combinations of cytokines on mesangial cell fibronectin production

To assess the effects of combinations of cytokines on fibronectin production mesangial cells were exposed to 10 ng/ml TGF-β<sub>1</sub> added in combination with either 10 ng/ml of TNF-α or IL-1β. This resulted in synergistic increases in fibronectin levels to at least fivefold over unstimulated levels (Fig. 1). Moreover, this effect was dependent on the dose of TGF-β<sub>1</sub>; titrating TGF-β<sub>1</sub> against constant doses of either TNF-α or IL-1β resulted in a dose dependent reduction in fibronectin accumulation (Fig. 2).

The combination of all three cytokines together did not produce any greater increase in fibronectin levels compared to that stimulated by combinations of just two cytokines (fold increase over medium alone in secreted fibronectin of  $4.19 \pm 1.5$  vs.  $4.14 \pm 0.65$  vs.  $6.4 \pm 2.3$  for combinations of TGF-β<sub>1</sub>:TNF-α:IL-1β vs. TGF-β<sub>1</sub>:TNF-α vs. TGF-β<sub>1</sub>:IL-1β, respectively).

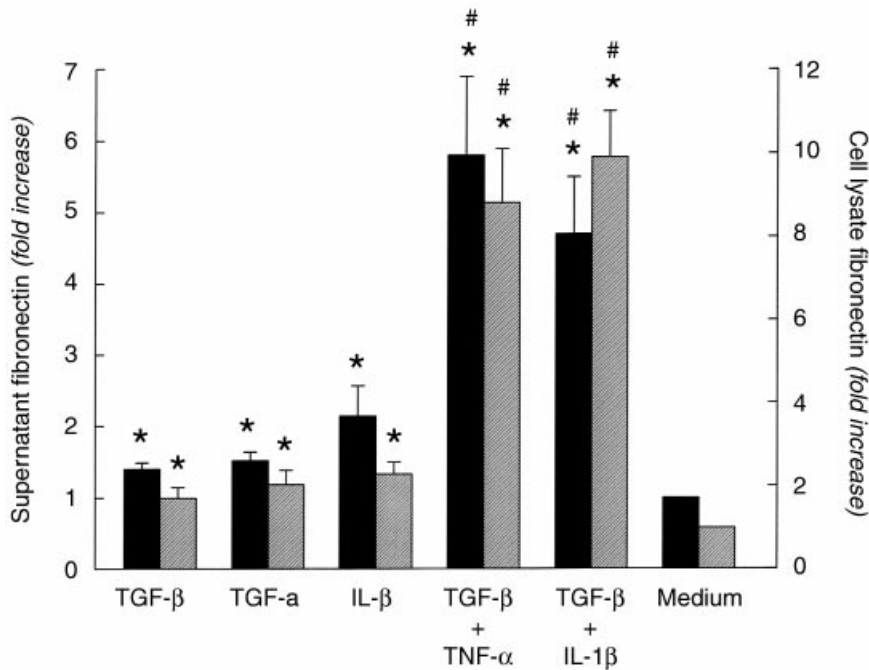
### Effect of combinations of cytokines on fibronectin gene transcription

Northern blot analysis demonstrated that individually each cytokine induced fibronectin gene transcription above control levels. However, a combination of TGF-β<sub>1</sub> and TNF-α stimulated synergistic increases in fibronectin message to levels greater than the sum of the individual cytokines, respectively (Fig. 3A). The TGF-β<sub>1</sub>:IL-1β combination induced increases in fibronectin message that ranged between additive and synergistic values (Fig. 3B).

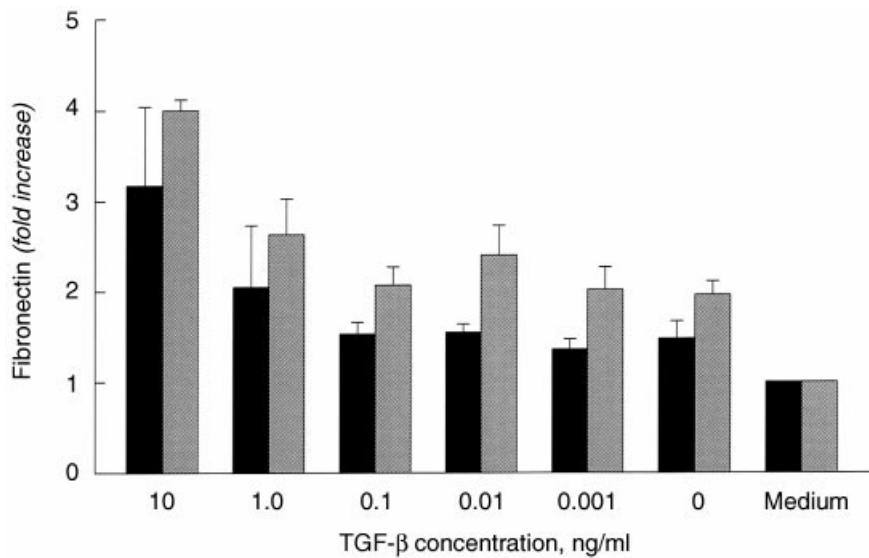
Northern analyses probing for collagen IV (α1 chain) and laminin (B1 chain) gene expression resulted in qualitatively similar results (data not shown).

### Effect of cytokine combinations on the gene expression of transin and TIMP-1

Since the observed increase in fibronectin levels in response to cytokines could also result from decreased



**Fig. 1. Effect of individual and combinations of cytokines on fibronectin production.** Supernatants (■) and cell lysates (▨) from mesangial cells exposed to 10 ng/ml transforming growth factor- $\beta$  (TGF- $\beta_1$ ), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), or interleukin-1 $\beta$  (IL-1 $\beta$ ), or to 10 ng/ml TGF- $\beta_1$  in combination with 10 ng/ml of either TNF- $\alpha$  or IL-1 $\beta$  were assayed for fibronectin. Results are expressed as the fold increase over medium alone (corrected for cell protein) \* $P < 0.04$  versus medium alone, # $P < 0.001$  versus individual cytokines ( $N = 5$  to 22 each carried out in quadruplicate).



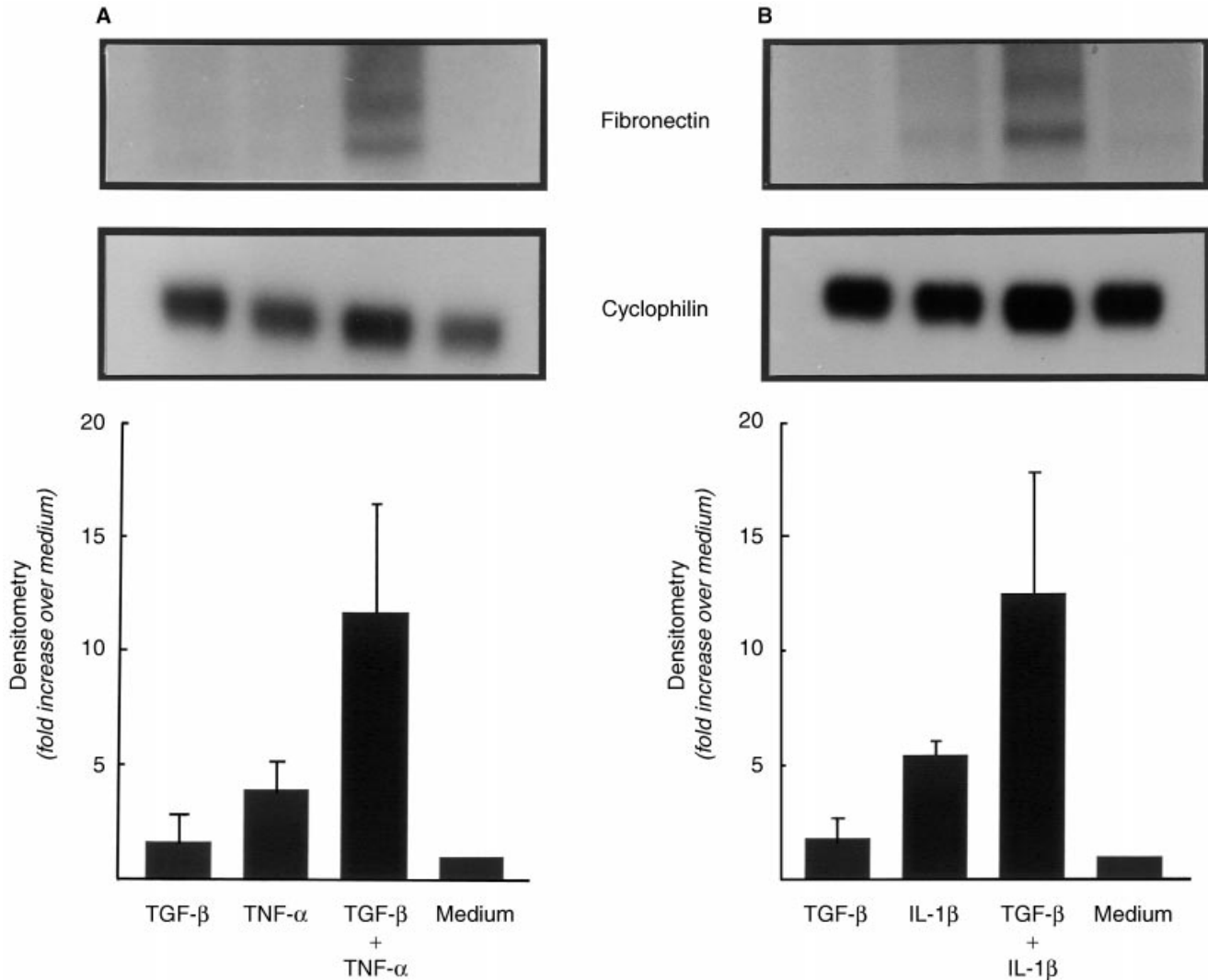
**Fig. 2. Effect of titrating TGF- $\beta_1$  against a constant dose of either TNF- $\alpha$  (■) or IL-1 $\beta$  (▨).** Supernatants from cells exposed to 10, 1, 0.0, 0.01, 0.001, 0 ng/ml TGF- $\beta_1$  in combination with 10 ng/ml of either TNF- $\alpha$  or IL-1 $\beta$  were assayed for fibronectin. Results are expressed as the fold increase over medium alone (corrected for cell protein) ( $N = 3$  each carried out in triplicate).

matrix degradation, Northern blot analysis was utilised to assess the gene expression of the rat matrix metalloproteinase transin and its tissue inhibitor of metalloproteinases TIMP-1.

Individually IL-1 $\beta$  and TNF- $\alpha$  were able to up-regulate the gene transcription of transin over that of control. However, unlike the effects on fibronectin gene transcription, in combination with TGF- $\beta_1$  transin gene expression was reduced below levels stimulated by TNF- $\alpha$  and IL-1 $\beta$  individually; the TGF- $\beta_1$ :TNF- $\alpha$  combination reduced transin mRNA levels to  $13.4 \pm 7.8\%$  of

the predicted additive values of mRNA induced by individual cytokines ( $P < 0.001$ , vs. TNF- $\alpha$  alone,  $N = 4$ ), while the TGF- $\beta_1$ :IL-1 $\beta$  combination reduced expression to  $30.8 \pm 12.8\%$  ( $P < 0.001$ , vs. IL-1 $\beta$ ,  $N = 4$ ; Fig. 4 A, B).

TIMP-1 mRNA was found to be up-regulated in response to all cytokines, although expression was strongest in response to IL-1 $\beta$  and TNF- $\alpha$ . In the cytokine combinations expression of TIMP-1 message was equivalent to that induced by the predicted sum of the individual cytokines (Fig. 5 A, B).



**Fig. 3. Effect of cytokine combinations on fibronectin gene transcription.** Northern blot analysis of mesangial cell RNA following 24 hours of exposure to 10 ng/ml TGF- $\beta_1$ , TNF- $\alpha$  (A) or IL-1 $\beta$  (B), or to TGF- $\beta_1$  in combination with TNF- $\alpha$  (A) or IL-1 $\beta$  (B). A representative blot from four experiments is shown. Densitometric analysis incorporates data from all four experiments normalized for RNA loading and expressed as the fold increase over control (medium alone).

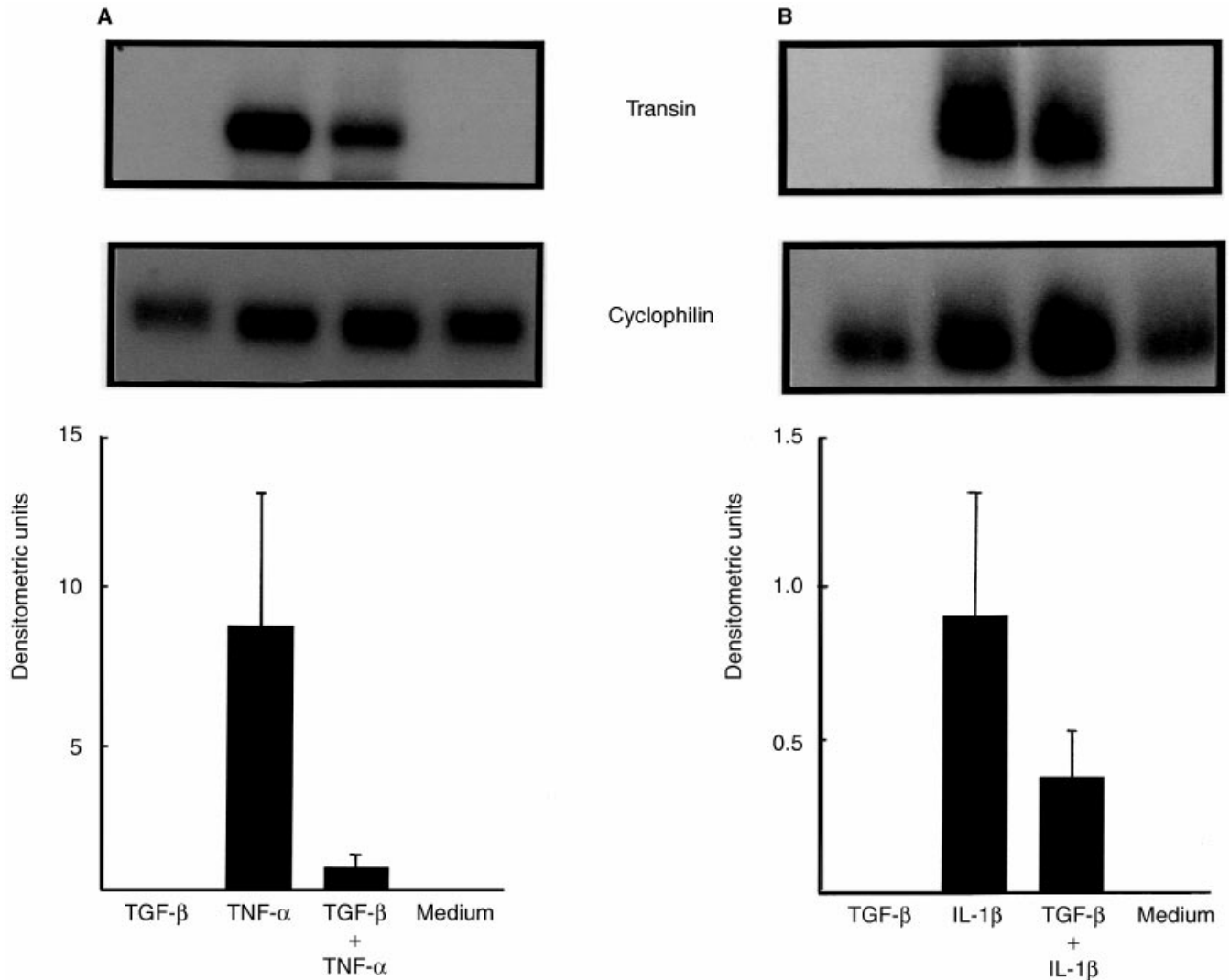
### Effect of cytokine combinations on TGF- $\beta_1$ mRNA and protein

Northern analysis was performed to investigate whether any changes in TGF- $\beta_1$  expression occur in the cytokine combinations that could possibly explain our observations. Northern blot analysis demonstrated that both the TGF- $\beta_1$ :TNF- $\alpha$  and TGF- $\beta_1$ :IL-1 $\beta$  combinations synergistically up-regulated TGF- $\beta_1$  message over levels induced by individual cytokines (Fig. 6 A, B). These data were further supported by the observation that TGF- $\beta_1$  protein secretion was found to be increased by the combinations over levels induced by individual cytokines (Table 1). [Under basal conditions (no added cytokines) the mesangial cell supernatant contained  $37.9 \pm 10.0$  pg/ $\mu$ g cell protein of TGF- $\beta_1$ .] When the TGF- $\beta$  assays were performed on non-activated tissue culture supernatants TGF- $\beta_1$  could not

be detected (data not shown). This would suggest that the increase in TGF- $\beta_1$  in the tissue culture supernatants in response to the cytokines was as a result of increased endogenous production by mesangial cells and not a result of simply measuring exogenously added TGF- $\beta_1$  (which was added in an activated state).

### DISCUSSION

TGF- $\beta$ , TNF- $\alpha$  and IL-1 $\beta$  have all been reported to be present at some stage in the diseased glomerulus, and are all known to be secreted by activated macrophages. This study has demonstrated that individually these cytokines have modest effects on mesangial cell fibronectin production. However, in combination (as is likely to be found *in vivo* when secreted by macrophages) they synergistically



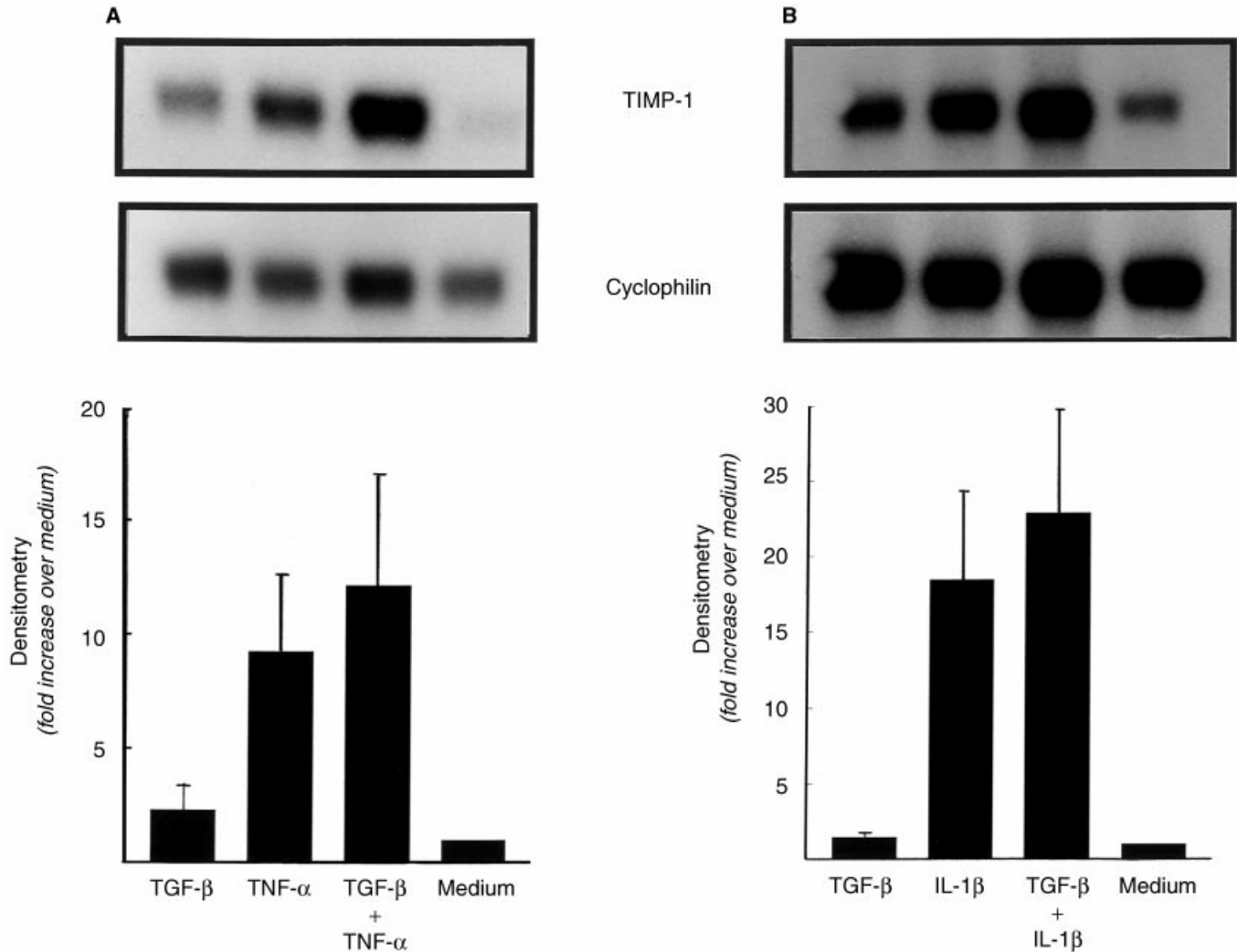
**Fig. 4. Effect of cytokine combinations on transin gene transcription.** Northern blot analysis of mesangial cell RNA following exposure to 10 ng/ml TGF- $\beta_1$ , TNF- $\alpha$  (A) or IL-1 $\beta$  (B) or to TGF- $\beta_1$  in combination with TNF- $\alpha$  (A) or IL-1 $\beta$  (B). A representative blot from four experiments is shown. Densitometric analysis incorporates data from all four experiments normalized for RNA loading and expressed as the fold increase over control.

up-regulate the production of this matrix protein. Synergistic interactions between bioactive peptides is not an uncommon phenomenon: TNF- $\alpha$  has previously been shown to synergize with interferon- $\gamma$  to greatly enhance fibronectin production by human melanocytes and malignant melanoma cells [29]. However, this is the first report of synergistic interactions between cytokines to promote extracellular matrix accumulation by glomerular mesangial cells. The increase in fibronectin levels is due, at least in part, to an increase in message for fibronectin over and above the levels that might be expected from an additive effect. Whether the enhanced message is due to increased message stability or increased rate of transcription is yet to be investigated.

In the current study fibronectin was chosen as the marker of the scarring process. Fibronectin itself has many prop-

erties which could further modulate the pathogenesis of glomerulosclerosis. For example, exogenously added fibronectin has been shown to modulate constitutively produced levels of cultured mesangial cell fibronectin [30, 31], to stimulate the secretion of cytokines such as TNF- $\alpha$  and IL-6 [30, 31], as well as altering certain functional characteristics of mesangial cells such as phagocytosis [32]. *In vivo*, circulating plasma fibronectin has been shown to specifically accumulate in end-stage renal lesions of graft versus host disease [33], while plasma fibronectin treatment of animals with proliferative glomerulonephritis has been shown to decrease the amount of fibronectin in glomerular lesions [34].

Matrix accumulation can also occur as result of a decrease in matrix degradation. Down regulation of specific matrix proteinases or up-regulation of their inhibitors



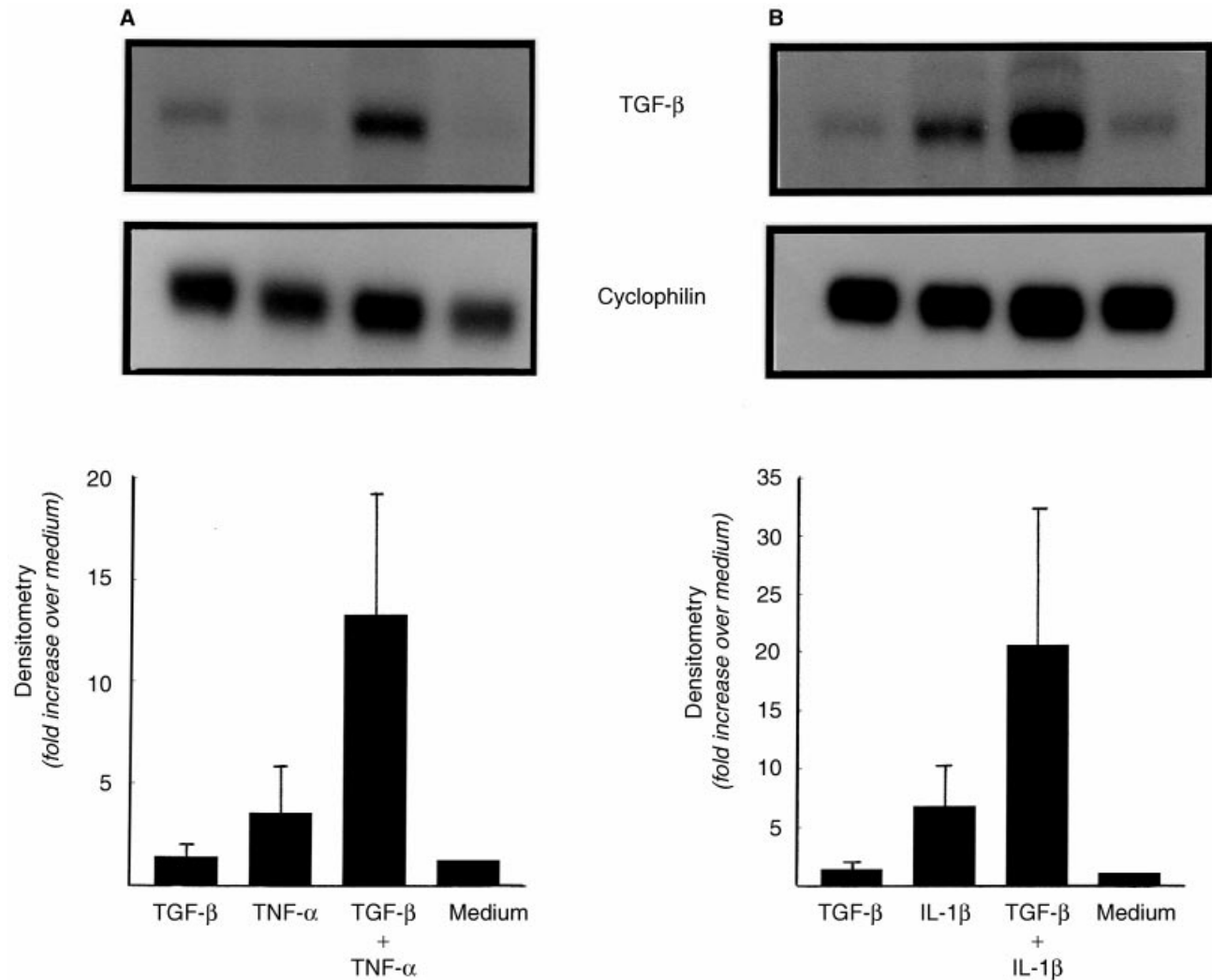
**Fig. 5. Effect of cytokine combinations on TIMP-1 gene transcription.** Northern blot analysis of mesangial RNA following exposure to 10 ng/ml TGF- $\beta_1$ , TNF- $\alpha$  (A) or IL-1 $\beta$  (B) or to TGF- $\beta_1$  in combination with TNF- $\beta$  (A) or IL-1 $\beta$  (B). A representative blot from four experiments is shown. Densitometric analysis incorporates data from all four experiments normalized for RNA loading and expressed as the fold increase over control.

would also be expected to result in an accumulation of matrix. In the current study the gene expression of the matrix metalloproteinase transin was markedly reduced by the cytokine combinations compared to the mRNA levels induced by TNF- $\alpha$  or IL-1 $\beta$  individually. These data suggest that TGF- $\beta_1$  in the combination inhibits the transcription of transin induced by either TNF- $\alpha$  or IL-1 $\beta$  possibly by inhibiting transin gene expression via an upstream sequence in the transin promoter called the TGF- $\beta$ -inhibitory element [35]. TGF- $\beta$  is one example of a factor that regulates the induction of TIMP-1 and the protease transin in opposite directions, the net effect of which is to inhibit the degradation of matrix. TGF- $\beta$  is known to be a potent stimulator of the synthesis of matrix components exerting dual effects on matrix accumulation. However, TGF- $\beta$  alone, in this study, did not cause the marked increases in fibronectin by rat mesangial cells that were induced by the

cytokine combinations. This may possibly be explained by the fact that the cytokine combinations also up-regulated the production of TGF- $\beta_1$  mRNA and endogenous TGF- $\beta_1$  protein by mesangial cells. This would be expected to result in a further suppression of the transin gene and hence fibronectin accumulation as a result of decreased degradation.

Taken together, these data suggest that accumulation of fibronectin in response to cytokine combinations occurs as a result of the combined effects of increased fibronectin synthesis and decreased matrix degradation, the latter occurring as a result of a decrease in transin expression and an additive increase in TIMP-1 expression. A contribution from a degree of post-translational modification of protein expression cannot be excluded at this stage.

The observations in this study also illustrate the pitfalls in looking at one cytokine in isolation in a biological system.



**Fig. 6. Effect of cytokine combinations on TGF- $\beta_1$  gene transcription.** Northern blot analysis of mesangial cell RNA following 24 hours of exposure to 10 ng/ml TGF- $\beta_1$ , TNF- $\alpha$  (A) or IL-1 $\beta$  (B) or to TGF- $\beta_1$  in combination with TNF- $\alpha$  (A) or IL-1 $\beta$  (B). A representative blot from four experiments is shown. Densitometric analysis incorporates data from all four experiments normalized for RNA loading and expressed as the fold increase over control.

**Table 1.** Mesangial cells were exposed to 10 ng/ml TGF- $\beta_1$ , TNF- $\alpha$  or IL-1 $\beta$ , or to TGF- $\beta_1$  in combination with TNF- $\alpha$  or IL-1 $\beta$

| Cytokines                  | TGF- $\beta$ | TNF- $\alpha$ | IL-1 $\beta$ | TGF- $\beta$ + TNF- $\alpha$ | TGF- $\beta$ + IL-1 $\beta$ | Medium |
|----------------------------|--------------|---------------|--------------|------------------------------|-----------------------------|--------|
| Fold increase over control | 1.77 ± 0.2   | 1.24 ± 0.2    | 1.88 ± 0.7   | 3.13 ± 0.5                   | 2.87 ± 0.65                 | 1.0    |

Tissue culture supernatants were assayed for TGF- $\beta_1$ . Results are expressed as the fold increase in TGF- $\beta$  over medium alone after correction for cell protein ( $N = 3$ ).

Abbreviations are: TGF, transforming growth factor; TNF, tumor necrosis factor; IL- interleukin.

Such an approach will miss potentially important interactions with other cytokines or the effects of one cytokine could be misinterpreted. The role of cytokines in disease is contextual and depends on a wide variety of factors including the presence of other growth factors, cytokines and hormones, which can act both synergistically and antagonistically with respect to one another, and on cell type and environment. Alterations in the local cytokine network, such as would be experienced during a macrophage influx,

are crucial. Alterations in cytokine secretion could change the nature of cytokine interactions within the local environment of the resident glomerular cells and ultimately perturb the fine balance between matrix synthesis and degradation. This would in turn alter the morphology and functions of these cells within the glomerulus.

In conclusion, these data suggest one way cytokines may interact with each other within the glomerulus to initiate the scarring process.

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