Fibronectin attachment is permissive for IL-1 mediated gene regulation

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Abstract **This study examines the effect of cell–matrix interaction on IL-1 induced gene regulation. In fibroblasts and smooth muscle cells attached to fibronectin, IL-1 caused a pronounced reduction in proteoglycan synthesis, while no reduction occurred in cells plated on bare plastic. Further, fibronectin attachment was permissive for IL-1 mediated suppression of both versican and collagen mRNA levels, initially noted after 4–6 h of IL-1 incubation. Attachment to vitronectin was less potent in influencing regulation, and collagen had no effect, suggesting specificity of the matrix modulation of the IL-1 induced response. Similar fibronectin induced dependence was demonstrated for IL-1 regulation of IL-6 gene expression, supporting the notion of a general effect of fibronectin receptor engagement on IL-1 induced signal transduction.**

Key words: Fibronectin; Integrin; Cytokine; Gene regulation

1. Introduction

The presence of an extracellular matrix has been shown to influence cell behavior and various aspects of growth factor effects on cellular responses [1]. Attachment of cells to the extracellular matrix is mediated through integrins, a family of cell surface glycoproteins connecting the extracellular matrix and the cytoskeleton [2]. Binding of integrins to various extracellular matrix components is specific [3] and has been shown to induce signal transduction [4,5] involving activation of tyrosine kinase [6].

IL-1 is a central mediator of inflammation that has pronounced effects on a variety of inflammatory repsonses including modulation of extracellular matrix metabolism [7] as well as cytokine and growth factor gene regulation [8.9]. Our earlier studies on binding and internalization show that IL-1 receptors are located at focal adhesions [10] and that ligand receptor binding results in rapid alterations at focal adhesions and in the cytoskeleton [11]. Recently it has been shown that both known IL-1 activated pathways, Map-2 kinase and NF-κB, are induced by integrin engagement through the fibronectin receptor [12–14] and further, that IL-1 induced calcium fluxes are dependent on cell-matrix interaction [15]. This data suggest an involvement of structural elements in regulation of IL-1 activated signal transduction, and indicate a possible coregulation of biological responses through integrins and the IL-1 receptor.

In this report we analyze the effect of cell–matrix interaction on IL-1 induced biological responses in two types of attached cells with different integrin profiles, smooth muscle cells and fibroblasts. We determine the effect on regulation of proteoglycan synthesis, particularly on versican mRNA levels, which are significantly reduced in IL-1 stimulated cells surrounded by an abundant extracellular matrix [16]. The data show that attachment to fibronectin is permissive in IL-1 induced suppression of both proteoglycan and collagen gene expression, as well as for IL-1 regulation of IL-6 mRNA levels. The influence on diverse IL-1 induced responses supports the notion of a general effect of fibronectin attachment on IL-1 action, possibly involving ligand receptor binding and/or IL-1 activated signal transduction.

2. Materials and methods

2.1. Cell cultures

Human gingival fibroblasts (4 cell lines) (16), transfer 9–14, or monkey arterial smooth muscle cells (2 cell lines), transfer 7–12, (kindly provided by Dr. Russell Ross and Elaine Raines, University of Washington, Seattle) were used. Cells were detached with EDTA (5 mM), plated in 35 mm dishes (4×10^5 cells/dish) or in 10 cm dishes (3×10^6 cells/dish), on bare tissue culture plastic or on dishes coated with fibronectin (FN 10 μg/ml, Gibco, Gaithersburg, MD) as described [12,17] for 3–4 h in 10% FCS (fetal calf serum), unless stated otherwise. After attachment, cells were incubated with or without IL-1β (10^{-9} M) (kind gift of Dr. Steven Dower, Immunex Corp.) for various times as indicated.

2.2. [^{35}S]Sulfate incorporation into proteoglycans

Cells were incubated without or with IL-1β (2×10^{-9} M) for various times (4, 8, 12, 24, 48, 72 h) in DV-medium containing 10% FCS. During the last 4 h of incubation medium was replaced with DV medium with and without IL-1, containing [^{35}S]sulfate (carrier-free, 200 μCi/ml) (ICN, Irvine, CA). Subsequently, supernatants were removed, the cultures rinsed with PBS, and supernatants and rinses pooled. Cell-layer proteoglycans were extracted in 8 M urea containing 0.25 M NaCl, 2 mM EDTA, 0.3% Triton X-100, and protease inhibitors (5 mM benzamidine, 100 mM 6-aminohexanoic acid) in Tris-HCl (50 mM) buffer, pH 7.4 (urea extraction buffer). Quantitative changes in the rate of incorporation of label was determined by precipitation with cetyl pyridinium chloride (CPC) as described by Wasteson et al. [18]. Briefly, aliquots (50 μl) of medium and extracted cell layer samples from triplicate cultures were spotted in duplicates on Whartman 3MM chromatography paper, dried and washed (5 × 1 h; 1% CPC, 0.05 M NaCl). In some experiments radiolabeled samples were analyzed by DEAE sephacel chromatography and elution in 8 M urea in 50 mM Tris-HCl containing 2 mM EDTA, 0.3% Triton X-100 and 3 M NaCl [16]. Radioactivity remaining was determined by liquid scintillation counting (Hewlett Packard, Model 3255, Laguna Hills, CA). Results of duplicate determinations of each triplicate were averaged, the total radioactivity per sample was normalized to cell number, and data expressed as percentage of control. Control values of the various strains ranged from 44,000–83,000 cpm/100,000 cells.

2.3. RNA extraction and Northern transfer analysis

Total RNA was isolated by the single step method as described [19]. The yield was between 3–5 μg of RNA/10^5 cells. The ratio of the optical density at absorbance 260 nm to 280 nm of the RNA samples was 1.5 or higher.

RNA (20 μg/lane) were electrophoresed overnight (15–16 h) in 1% (w/v) agarose gels containing 16.7% (v/v) of 37% (w/w) formaldehyde (Fisher Laboratories, Inc.) [20]. Following electrophoresis, RNAs were

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transferred from agarose gels to nitrocellulose filters (Bethesda Research Laboratories Life Technologies, Inc.) according to Thomas [21]. After transfer, the filters were baked at 80°C for 2 h. Before hybridizing specific cDNA probes, the filters were prehybridized for at least 2 h at 42°C in a solution containing 50% (v/v) formamide (Bethesda Research Laboratories Life Technologies, Inc.), 6 × SSPE 1 × SSPE = 0.15 M NaCl, 0.2 M NaH_2PO_4 and 0.02 M Na_4 EDTA), 5 × Denhardt's solution (1 × Denhardt's solution = 0.02% Ficoll, 0.02% polyvinylpyrrolidone and 0.02% bovine serum albumin), 0.05% (w/v) SDS, 100 μg/ml salmon testes DNA (Sigma) and 100 μg/ml Torula yeast RNA (Calbiochem Corp.). Hybridizations of the filters with ^{32}P-labeled (nick translation using Bethesda Research Nick Translation System; and 5′[α-^{32}P]dCTP, from Amersham Corp.) cDNA probes (specific activity 0.2–1.0 × 10^8 cpm/μg/DNA) were carried out at 42°C using the same solution as above. In addition, probes were labeled by random priming using the Prime-it II Random Primer kit (Strategene) and according to manufacturers instructions, and incubated 1 h at 37°C. The hybridization time was at least 16 h, whereafter the filters were washed twice with 2 × SSPE/0.1% (w/v) SDS and twice with 0.1 × SSPE/0.1% (w/v) SDS for 15–20 min/time at 42–52°C, depending on the cDNA probe used for the analysis. After washing, the filters were airdried and the autoradiographed at −70°C using Kodak-XAR2 film for 8, 16 or 96 h. After autoradiography, fluorograms were scanned using a densitometer (Model GS 300, Hoefer Scientific Instruments, San Francisco, CA), or analyses was performed on a Macintosh (Model IIsi) computer and scanner (ScanJet IIcx, Hewlett Packard, Boise, Idaho) using the public domain NIH Image program (Wayne Rasband at the U.S. National Institutes of Health and available from the Internet by anonymous ftp from zioppy.nimh.nih.gov). Hybridization of the filters with a cDNA probe for 28S ribosomal RNA was used for normalization of the loading of RNA onto the gels [22].

2.4. cDNA probes

The following cDNA probes were used: for large CS PG, cDNAs encoding the carboxy terminal region and the middle region (corresponding to the sequences between base pair 6412 through 8125 and base pair 2607 through 6092, respectively) of the large human fibroblast PG, versican, kindly provided by Dr. E. Ruoslahti and Dr. R. Le Baron, La Jolla, CA [23]. For collagen type I, probes corresponding to basepairs 247–861 and 787–270 including the 3′ end untranslated regions [24], respectively of pro_α I collagen were used, kindly provided by Dr. Linda Sandell, Veterans Adm. Hosp., Univ. Washington, Seattle, WA. For IL-6 [25], a cDNA probe, kindly provided by Steve Dower at Immunex corporation, Seattle, was used. For 28S ribosomal RNA, cDNA fragment of 280 base pairs of bovine 28S ribosomal subunit was used (Clontech, Palo Alto, CA).

3. Results and discussion

The presence of an extracellular matrix has long been known to effect cell behavior, including growth factor induced proliferation and regulation of protein synthesis (see review [1]). In this study we show that IL-1 induced effects on proteoglycan synthesis in both fibroblasts and smooth muscle cells are dependent on cell–matrix interaction (Fig. 1). Thus, IL-1 stimulation of cells attached to fibronectin resulted in a pronounced reduction to about 80% and 60% after 48 and 72 h respectively (Fig. 1a,b). In contrast, cells plated on tissue culture plastic and incubated with IL-1 for the same time period showed no effect with values corresponding to between 90 and 100% of control (Fig. 1a,b).

This type of IL-1 induced decrease in proteoglycan synthesis in fibroblasts surrounded by an endogenous matrix is due to a pronounced reduction in mRNA levels for versican [16], a large chondroitin sulfate proteoglycan [23]. Similarly, smooth muscle cells attached to an endogenous matrix after 5 days in culture and subsequently incubated with IL-1 for 12–48 h, showed a successive reduction in versican mRNA over time (Fig. 2).

To analyze the mechanism of the effect of the extracellular matrix, various matrix components were examined for the capacity to modulate IL-1 responses at the level of mRNA for versican and collagen (Fig. 3a). These experiments showed that in fibroblasts plated on fibronectin, IL-1 caused a pronounced reduction in both versican and collagen mRNA while no effect were seen in cells plated on tissue culture plastic (Fig. 3a). The effect was similar to that induced in the presence of an endogenous matrix [16] with levels in IL-1 treated cultures to between 10 and 30% of the control. Quantitation showed that the reduction in versican mRNA induced under these conditions corresponded to about 24 ± 1% in fibroblasts (Fig. 3b). The effect of vitronectin attachment was much less pronounced resulting in a reduction to about 57 ± 23%. In contrast, fibronectin (53 ± 3%) and vitronectin (60 ± 18%) had similar effects on smooth muscle cells. Further, attachment to collagen (FB 96 ± 23; SMC 75 ± 6) had little or no effect on the IL-1 response in either cell type, compared to that induced on plastic (95 ± 10% and 77 ± 16%) (Fig. 3b). Similar data were obtained for regulation of collagen mRNA (Data not shown). The difference in the response induced by various extracellular matrix components suggests integrin specificity. The lower effect of the matrix modulation in smooth muscle cells could simply

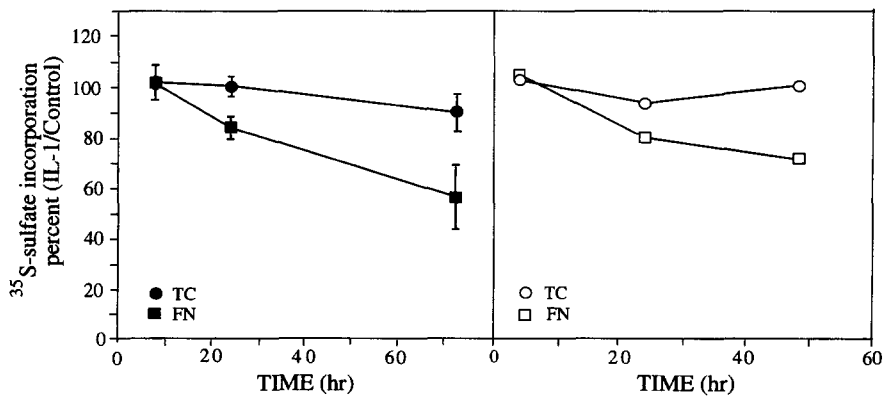

Fig. 1. Effect of fibronectin attachment on IL-1 induced changes in sulfate incorporation in fibroblasts (a) and smooth muscle cells (b). Cells were plated on fibronectin coated plates or on bare tissue culture plastic as stated in section 2 and stimulated with IL-1β (2×10^{-9} M) for the indicated times. Proteoglycan synthesis was determined by precipitation assay following ^{35}S-labeling, as described and data expressed relative to control. Graphs shows average and S.E.M. of 2–5 experiments. Fibronectin attached cells (squares), Cells on tissue culture plastic (circles), Fibroblasts (filled symbols), Smooth muscle cells (unfilled symbols).

reflect a lower number of $\alpha_5\beta_1$ receptors. This could lead to engagement of the vitronectin receptor ($\alpha_v\beta_3$) for cell–fibronectin interactions [26], consistent with the similarity of the responses induced by fibronectin and vitronectin in these cells.

The alterations induced by fibronectin attachment were rapid, and while no effect was seen at 2 h (110% of control), a pronounced reduction of mRNA levels occurred after 4 h of IL-1 incubation (Fig. 4a), suggesting a direct effect on IL-1 regulation. The effect successively became more pronounced, resulting a reduction to about 50% and 24% after 4–6 and 24 h, respectively, in fibronectin attached cells. In contrast, in cells on tissue culture plastic IL-1 had no effect over the same time period. Similar data were obtained using smooth muscle cells although the effect of fibronectin attachment was consistently less pronounced (data not shown).

Experiments, determining the influence of cell–matrix interaction on IL-1 induced up-regulation of IL-6 mRNA, similarly showed a pronounced effect of fibronectin attachment. Thus, in cells plated on fibronectin, IL-1 incubation for 2 h caused about a 5-fold increase in IL-6 mRNA which after 4–24 h resulted in an enhancement averaging between 8- to 10-fold. In comparison, IL-1 incubation of cells attached to bare plastic showed an increase of at most about 2-fold over the same time period (Fig. 4b). The pronounced effect of fibronectin attachment on a diverse set of genes supports the notion of coregulation of biological responses through the IL-1 receptor and the integrins. Such influences of fibronectin attachment correlates well with effects via the fibronectin receptor on other IL-1 regulated genes, such as collagenase and stromelysin [27,28]. The notion of the matrix modulation involving a direct effect is further supported by the early onset of induction of the biological response for both the matrix and cytokine gene regulation. The effects could thus, stem from alterations in ligand binding, influencing kinetics of cytokine/receptor interactions. We have earlier shown that IL-1 receptors are located at focal adhesions [10,29], at sites of cell–matrix interaction through integrins. Preliminary studies determining potential effects of integrin engagement on IL-1 receptor binding indicate that fibronectin attachment causes an increase in receptor mediated binding of IL-1 and in addition has pronounced effects on binding kinetics (Qwarnstrom unpublished).

In addition, the effect of the extracellular matrix could involve activation of additional signal transduction pathways, potentially related to cytoskeletal alterations. Thus, IL-1 receptor binding causes rapid phosphorylation of talin, a transmembrane linkage protein and subsequently reorganization of the actin cytoskeleton [11]. Such alterations, induced by cell matrix interactions, have been found to cause changes in expression of IL-1 responsive genes such as collagenase and stromelysin [27]. These types of structural alterations could involve cytoskeletal regulated GTP-binding protein such as Rac and Rho [30,31] or influence activation of $pp^{125}FAK$ [6] at focal adhesions, or alter IL-1 induced calcium regulation [15], and subsequently affect known signal transduction pathways activated by IL-1, such as map-2 kinase and NF-κB [12–14].

Such effects could involve alterations in Map-2 kinase phosphorylation sites during activation [32]. In addition, conse-

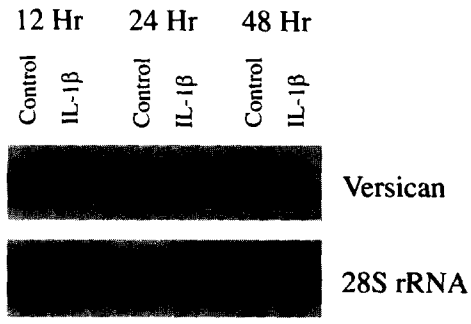

Fig. 2. Effect of IL-1 incubation on versican mRNA in smooth muscle cells surrounded by an endogenous matrix. Cells were grown to confluence (5 days) on tissue culture treated plates and subsequently incubated with IL-1β (2×10^{-9} M) for the times indicated, and extraction and separation of RNA and probing using the versican and collagen cDNA's were done as described in section 2.

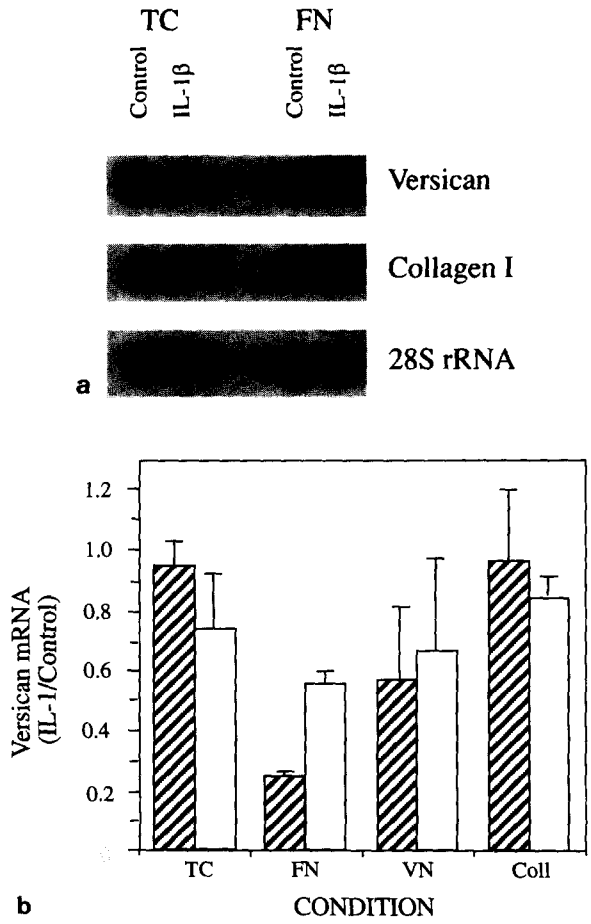

Fig. 3. (a) Fibronectin attachment is a prerequisite for IL-1 induced reduction of versican and collagen mRNA. Cells were plated on fibronectin coated plates (FN) or on bare tissue culture plates (TC) as described, and incubated with or without IL-1 for 24 h, and Northern analyses for versican and collagen I carried out as described in section 2. (b) Effects of various matrix proteins on IL-1 induced regulation of versican mRNA. Cells were plated on bare tissue culture plates (TC) or on non-treated plates coated with fibronectin (FN), vitronectin (VN) or collagen I (Coll) as described in section 2. Cells were treated with IL-1β for 24 h and levels of versican mRNA were determined by Northern analyses as above. Quantitation was done by scanning of the autoradiographs, as described in section 2 and data expressed as relative to control for each condition. Graph shows average and S.E.M. for 2–5 experiments. Fibroblasts (hatched bar), smooth muscle cells (open bars).

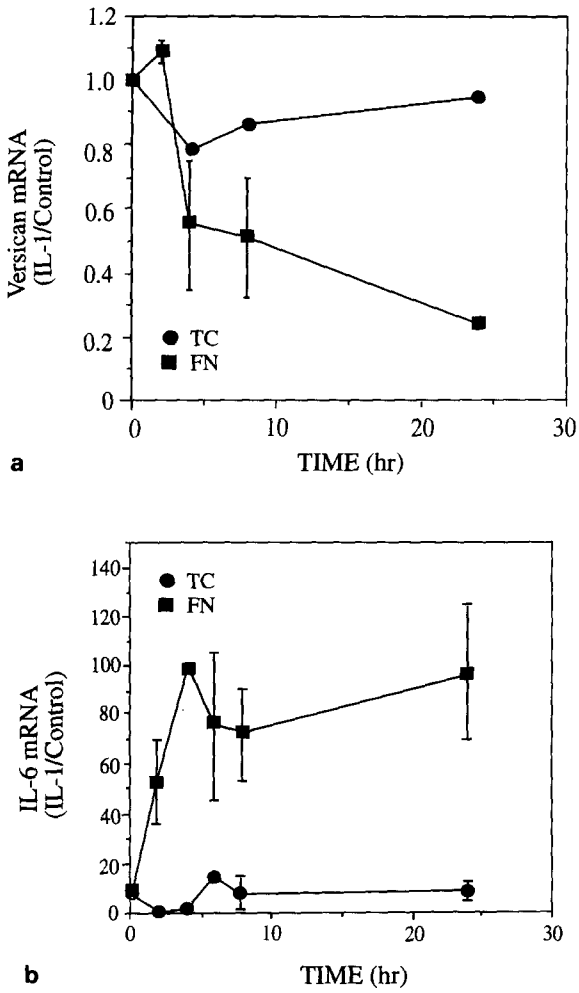

Fig. 4. Time course of fibronectin modulation of IL-1 induced regulation of versican (a) and IL-6 (b) mRNA. (a) Cels were plated on tissue culture plastic (●) or on fibronectin coated plates (■) as above and incubated with IL-1 for various times as indicated and versican mRNA levels for determined by Northern analyses, as above and data expressed relative to control. Graph shows average and S.E.M. for 3 experiments. (b) Cells were plated and incubated with IL-1 as above and IL-6 mRNA levels were determined by Northern analyses as described in section 2. Data are expressed as percent of the peak value (fibronectin attached cells 4hr IL-1). Cells on tissue culture plastic (●); Fibronectin attached cells (■), Graph shows mean and S.E.M. for 4 experiments.

quences of NF-κB activity could be influenced by alterations in participating dimers [12,33], or possibly through induction of HMG-like proteins resulting in switch to a transcriptional suppressor [34]. An effect on either of the two pathways could influence regulation of the collagen gene, containing both AP-1 and NF-κB binding sites [35]. Further, influence of cell–matrix interaction on additional pathways is indicated by the presence of unrelated negative regulatory domains in the versican promoter sequence [36].

In summary, these data show an effect of attachment through the fibronectin receptor on IL-1 mediated biological responses in fibroblasts and smooth muscle cells, and suggest a co-regulation by integrins and the IL-1 receptor of responsive genes, during inflammation and wound healing.

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