Vol. 162, No. 1, 1989 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 14, 1989 Pages 144-150

TRANSFORMING GROWTH FACTOR-BETA CAUSES PARTIAL INHIBITION OF INTERLEUKIN 1 - STIMULATED CARTILAGE DEGRADATION IN VITRO

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Received May 31, 1989

We show that purified human transforming growth factor-beta (1-10ng/ml) inhibits interleukin 1-stimulated loss of proteoglycan from cartilage in vitro. Inhibition is incomplete, as interleukin 1 retains the ability to cause a dose dependent stimulation of proteoglycan release in the presence of high levels of transforming growth factor-beta (100ng/ml) although both basal and interleukin 1-stimulated levels can be reduced by up to 50 per cent. This observation, together with its ability to stimulate proteoglycan synthesis and to stimulate proteinase inhibitor production, suggests a possible role for transforming growth factor-beta in limiting cartilage proteoglycan loss in inflammatory conditions such as rheumatoid arthritis.

The inflammatory cytokine interleukin 1 (IL-1) is thought to be a key mediator of cartilage matrix degradation in conditions such as rheumatoid arthritis: IL-1 causes proteoglycan loss from cartilage in vitro (1) and in vivo, when injected into rabbit joints (2) and significant levels of IL-1 are found in joint fluids from patients with rheumatoid arthritis (3, 4). IL-1 has also been shown to stimulate the production of the metalloproteinases, stromelysin and collagenase, by a variety of cell types, including chondrocytes (reviewed in 5) and these proteinases may mediate IL-1-stimulated cartilage degradation. In particular, the proteoglycan degradation products released from IL-1-stimulated cartilage in vitro are consistent with the action of a metalloproteinase (6). Previous studies have failed to show that addition of naturally occurring proteinase inhibitors, including tissue inhibitor of metalloproteinases (TIMP), can prevent cartilage degradation in vitro (7), however, agents which stimulate the local production of TIMP or which inhibit the production of the metalloproteinases, might be able to prevent IL-1-stimulated cartilage degradation.

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Abbreviations
IL-1, interleukin 1; TGF, transforming growth factor; TIMP, tissue inhibitor of metalloproteinases; EGF, epidermal growth factor; FGF, basic fibroblast growth factor.

0006-291X/89 $1.50

The transforming growth factor-beta (TGF-β) family of molecules are homo- and hetero-dimeric polypeptides produced by a large number of cells and tissues (8, 9). The majority of cell types investigated so far possess high affinity receptors for TGF-β and show a range of responses to the peptide (reviewed in 10). TGF-β has recently been shown to enhance production of the extracellular matrix components collagen, fibronectin and proteoglycans, by a variety of cell types in vitro (11, 12) and to cause the differentiation of mesenchymal cells to produce the cartilage specific matrix macromolecules, type II collagen and aggregating proteoglycans (13). In addition TGF-β has recently been shown to stimulate the production of proteinase inhibitors, including plasminogen activator inhibitor 1 (14) and TIMP (15) and to inhibit the production of the metalloproteinases stromelysin (16) and collagenase (15), suggesting that it may also have a role in limiting tissue destruction.

We have therefore investigated whether TGF-β is able to prevent IL-1-stimulated cartilage degradation as measured by proteoglycan loss from fragments of porcine articular cartilage in culture.

Materials and Methods

IL-1 and TGF-β: Recombinant human IL-1 alpha was a generous gift from Glaxo Laboratories Ltd., Greenford, UK. The preparation had a specific activity of $1-2 \times 10^8$ units/mg, was >90% pure, and endotoxin free. TGF-β (prepared from human platelets) was >95% pure (R and D Systems Inc., Minneapolis, USA).

Culture medium: Control culture medium was Dulbecco's modification of Eagles medium containing 25mM HEPES (Flow Laboratories), supplemented with sodium bicarbonate (0.5g/l) glutamine (2mM), bovine serum albumin (0.1mg/ml), streptomycin (100µg/ml) and penicillin (100u/ml).

Cartilage degradation assay: Cartilage fragments were removed from the condylar ridge from the metacarpophalangeal joint of the forefeet of pigs. Each slice was cut into about 8 fragments (2mm x 2mm) one fragment from each cartilage slice being represented in control and all treatment groups. Fragments were cultured at 37° C for 24 h in 96 well microtest plates (Nunc, Denmark) containing 200µl/well of control medium, to stabilize them prior to setting up the experiment. Wells were washed in Hanks balanced salt solution (Gibco) and test materials added, diluted in control medium (200µl/well). Incubations were for 3 days, or for 7 days with medium being renewed on days 3 and 5. Medium was harvested and stored at -20° C until assay. In order to determine the total glycosaminoglycan content of the cartilage fragments the remaining cartilage was digested with papain (2.5mg/ml crude papain, Sigma) in 0.1M phosphate buffer pH 6.5 containing 5mM EDTA and 5mM cysteine hydrochloride, incubating at 65° C until digestion was complete (2-4 h).

Medium and papain digests were assayed for sulphated glycosaminoglycans using a modification of the 1,9,-dimethylmethylene blue dye binding assay (17). Sample or standard (40µl) is mixed with dye reagent (250µl) prepared as described (17) in the well of a microtiter plate, and the absorbance at 600nm determined within 5 minutes. Chondroition sulphate from shark fin (5-40µg/ml) is used as a standard. The complex formed with 1,9,-dimethylmethylene blue results in a decrease in absorbance at 600nm. Hyaluronate causes no absorbance change (18). Cartilage degradation is expressed as µg glycosaminoglycan released, or as the percentage of the total glycosaminoglycan of the cartilage released over the incubation period.

Results

A high dose (10ng/ml) of human recombinant IL-1 alpha was used to stimulate the degradation of porcine articular cartilage. This caused a 2-3 fold stimulation of glycosaminoglycan release from the cartilage fragments under the conditions used in these experiments. Purified human TGF-β caused a dose-dependent inhibition of this IL-1-stimulated glycosaminoglycan release (Figure 1). Inhibition was significant at 1ng/ml TGF-β, over a 3 day incubation period (Figure 1a). Maximal inhibition (30-50% inhibition of IL-1-stimulated levels) was achieved with 10-25ng/ml TGF-β (Figure 2). Results were similar whether expressed as µg glycosaminoglycan released or as a percentage of the total glycosaminoglycan released (Figures 2a and 2b). A small decrease in the basal level of glycosaminoglycan release was also seen (Figures 1 and 2). This did not always reach significance, and was more

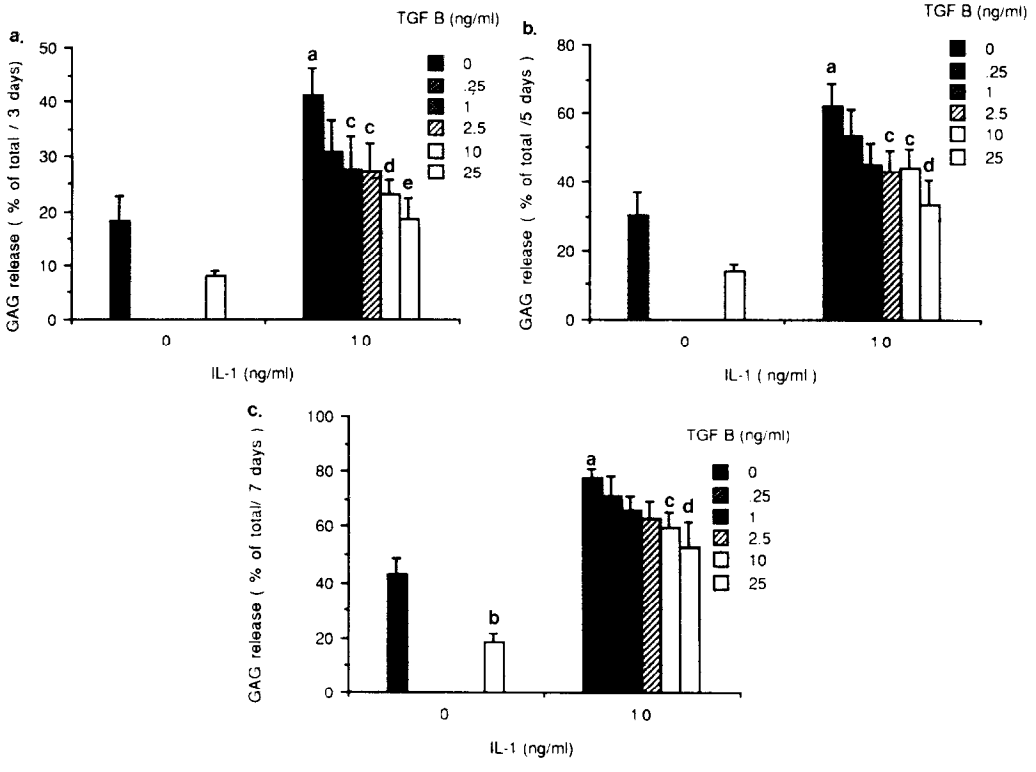

Figure 1 Inhibition of IL-1-stimulated cartilage degradation by TGF-β

Cumulative glycosaminoglycan loss from porcine articular cartilage after a) 3, b) 5, and c) 7 days in culture, expressed as a percentage of the total glycosaminoglycan. Mean ±SEM, n = 6.

a significantly greater than control, P<0.001
b significantly less than control, P<0.01
c significantly less than IL-1 alone, P<0.05
d significantly less than IL-1 alone, P<0.01
e significantly less than IL-1 alone, P<0.001
(Anovar).

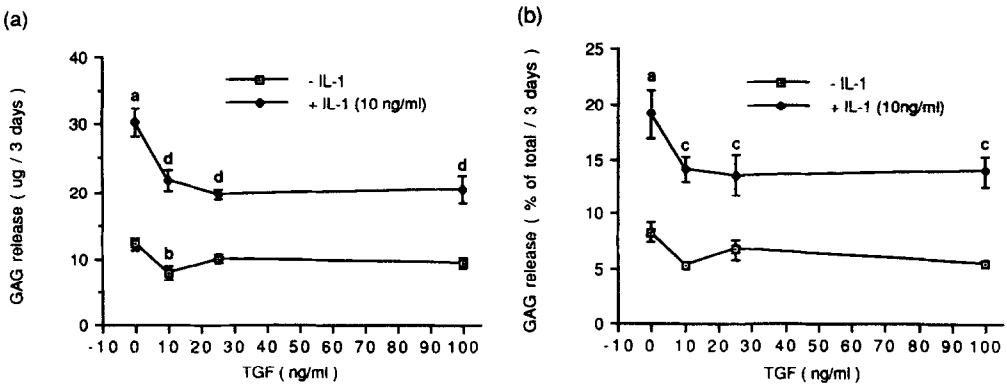

Figure 2 Effect of TGF-β on basal and IL-1-stimulated cartilage degradation
a) µg glycosaminoglycan released and b) glycosaminoglycan loss, expressed as a percentage of the total cartilage glycosaminoglycan, over a 3 day incubation period. Mean ±SEM, n = 6.

a significantly greater than control, P<0.001
b significantly less than control, P<0.05
c significantly less than IL-1 alone, P<0.01
d significantly less than IL-1 alone, P<0.001
(Anovar).

marked in experiments where basal levels of glycosaminoglycan release were relatively high.

Extension of the incubation period from 3 to 7 days did not increase the degree of inhibition achieved by TGF-β (Figure 1). This is highlighted in Figure 3 which shows the response to a single dose of TGF-β (25ng/ml), over a 7 day period, in the presence or absence of IL-1 (10ng/ml). The rate of glycosaminoglycan release with TGF-β alone is less than the basal rate and remains constant over the 7 day period. In the presence of IL-1, the inhibitory effect of TGF-β appears to become less marked suggesting that IL-1 may be able to overide the effect of TGF-β after longer periods of time.

All the data shown indicate that TGF-β is not able to completely prevent IL-1 from stimulating proteoglycan loss from cartilage. This is confirmed in Figure 4 which shows that IL-1 retains the ability to cause a dose-dependent stimulation of glycosaminoglycan release from cartilage in the presence of 100ng/ml TGF-β.

Discussion

The results presented in this paper demonstrate that TGF-β is able to inhibit IL-1-stimulated glycosaminoglycan release from fragments of porcine articular cartilage, in culture. Inhibition is significant with 1ng/ml TGF-β, and maximal with 10-25ng/ml TGF-β. The maximum degree of inhibition achieved is to 50% of IL-1-stimulated or control levels of glycosaminoglycan loss.

A recent study (19) has demonstrated that TGF-β is able to inhibit the basal levels of degradation of newly sysnthesised proteoglycan in cartilage

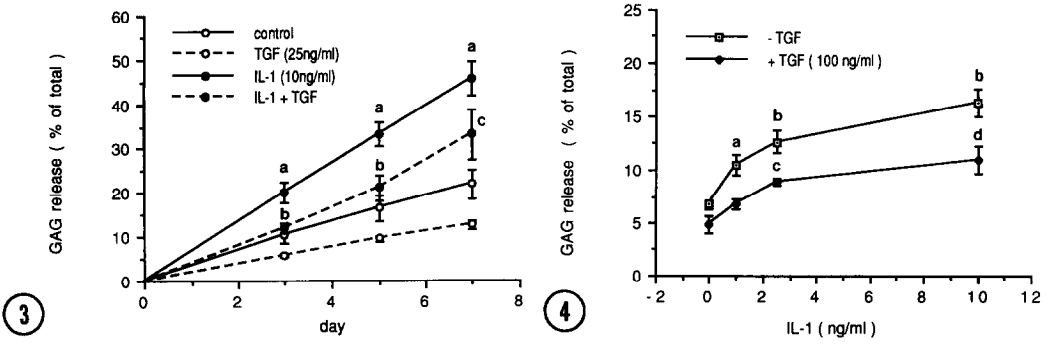

Figure 3 Time course of the response of cartilage to IL-1 and TGF-β
Glycosaminoglycan loss over a 7 day incubation period. Mean ±SEM, n = 6.
a significantly greater than control, P<0.001
b significantly less than IL-1 alone, P<0.01
c significantly less than IL-1 alone, P<0.05
(Anovar).

Figure 4 Effect of a high TGF-β concentration (100ng/ml) on the IL-1 dose
Glycosaminoglycan release during a 3 day incubation period.
Mean ±SEM, n = 6.
a significantly greater than control, P<0.01
b significantly greater than control, P<0.001
c significantly greater than TGF-β alone, P<0.01
d significantly greater than TGF-β alone, P<0.001
(Anovar).
For all concentrations of IL-1 (1-10ng/ml) glycosaminoglycan release in the presence of TGF-β is significantly less than that in the absence of TGF-β (P<.01 Anovar).

fragments cultured for long periods. We have extended these observations to show that the rapid loss of proteoglycan induced by high concentrations of IL-1 can also be inhibited by TGF-β. The proteoglycan fragments released from both untreated and IL-1-stimulated cartilage in culture are consistent with the limited cleavage, close to the hyaluronate binding region, of large aggregating proteoglycans (6, 20). The glycosaminoglycan release measured in these experiments is likely to be largely due to this degradative process although any non incorporated newly synthesised proteoglycan would also be detected. IL-1-stimulated cartilage degradation is widely thought to be mediated by the metalloproteinases, such as stromelysin and collagenase (5, 6), although this has not been directly demonstrated. TGF-β has been shown to stimulate TIMP production (15), to inhibit collagenase and stromelysin production by fibroblasts (15, 16) and to inhibit the IL-1-stimulated production of neutral proteinase (caseinase) activity by rabbit chondrocytes in monolayer culture (21). The observation that TGF-β also inhibits IL-1-stimulated cartilage proteoglycan degradation is therefore consistent with a role for the metalloproteinases in mediating this degradation. Stimulation of the production of TIMP by the chondrocytes themselves may result in high local levels of TIMP and so be a more effective means of inhibiting chondrocyte-

mediated cartilage degradation than the exogenous addition of proteinase inhibitors. Other possible mechanisms for the action of TGF-β on cartilage cannot, however, be ruled out.

TGF-β does not completely block the ability of IL-1 to stimulate glycosaminoglycan release from cartilage. This may indicate that more than one mechanism contributes to proteolgycan loss, or that not all the cells in the cartilage are responding to TGF-β. All experiments were performed in the absence of serum or growth factor supplements but many effects of TGF-β are reported to be dependent on, or modified by, the presence of other growth factors, such as fibroblast growth factor (FGF) or epidermal growth factor (EGF). For example, TGF-β is reported to stimulate TIMP production by fibroblasts only in the presence of EGF or FGF (15). Recent data from our laboratory shows that TGF-β can stimulate TIMP production in the presence of IL-1 alone (J. K. Wright, unpublished observations) however, it may be possible to enhance the response of cartilage to TGF-β with growth factors such as EGF or FGF.

Recent studies have shown that TGF-β, at concentrations similar to those used in this study, is able to stimulate proteoglycan synthesis in cartilage fragments (19) and to restore the ability of IL-1- stimulated chondrocytes, in monolayer culture, to lay down proteoglycan- containing matrix (21). TGF-β therefore appears to oppose the effect of IL-1 on both proteoglycan synthesis and degradation and so may be able to protect cartilage from proteoglycan loss in inflammatory conditions such as rheumatoid arthritis.

Acknowledgments

The authors acknowledge the British Petroleum Company plc and the Arthritis and Rheumatism Council for financial support, Glaxo Laboratories Ltd., for their gift of recombinant human IL-1 and Mrs. S. Smith for preparation of the manuscript.

References

1. Saklatvala, J., Pilsworth, L.M.C., Sarsfield, S.J., Gavrilovic, J. and Heath, J.K. (1984). Biochem J. 224, 461-466.
2. Pettipher, E.R., Higgs, G.A. and Henderson, B. (1986) Proc. Natl. Acad. Sci. USA 85, 8749-8753.
3. Wood, D.D., Ihrie, E.J. Dinarello, C.A. and Cohen, P.L. (1983) Arthritis Rheum. 26, 975-983.
4. Di Giovane, F., Sitanayake, D and Duff, G.W. (1987) Br. J. Rheumatol. 26 (suppl 1), 34.
5. Bunning, R.A.D., Richardson, H.J., Crawford A., Skjodt, H., Hughes, D., Evans, D.B., Gowen, M., Dobson, P.R.M., Brown, B.L. and Russell, R.G.G. (1986) Agents and Actions (suppl) 18, 131-152.
6. Campbell, I.K., Roughly, P.J. and Mort, J.S. (1986) Biochem J. 237, 117-122.
7. Saklatvala, J. and Bird, T. (1987) The Arthritis and Rheumatism Council for Research Conference Proceedings No 2. "The Control of Tissue Damage" p 6.

8. Roberts, A.B., Anzano, M.A., Lamb, L.C., Smith, J.M. and Sporn, M.B. (1981) Proc. Natl. Acad. Sci. USA 78, 5339-5343.
9. Massague, J. (1987) Cell 49, 437-438.
10. Sporn, M.B., Roberts, A.B., Wakefield, L.M., and de Crombrugghe, B. (1987). J. Cell. Biol. 105, 1039-1045.
11. Ignotz, R.A., and Massague, J. (1986) J. Biol. Chem. 261, 4337-4345.
12. Redini, F., Galera, P., Manviel, A. and Pujol, J.P. FEBS Letts. 234, 172-176.
13. Seyedin, S.M., Thompson, A.Y., Bentz, H., Rosen, D.M., McPherson, J.M., Conti, A., Siegel, N.R., Gallappi, G.R. and Piez, K.A. (1986) J. Biol. Chem. 261, 5693-5695.
14. Laiho, M., Saksela, O., Andreason, P.A. and Keski-Oja, J. (1986) J. Cell. Biol. 103, 2403-2410.
15. Edwards, D.R., Murphy, G., Reynolds. J. J., Whitham, S.E., Docherty, A.J.P., Angel, P. and Heath, J. K. (1987) The EMBO Journal 6, 1899-1904.
16. Matrisian, L.M., Leroy, P., Ruhlmann, C., Gesnel, M.-C. and Breathnach, R. (1986) Mol. Cell. Biol. 6, 1679-1686.
17. Farndale, R.W., Buttle, D.J., and Barrett, A.J. (1986) Biochim. Biophys. Acta. 883, 173-177.
18. Ratcliffe, A., Docherty, M., Maini, R.N. and Hardingham, T.E. (1988) Ann. Rheum. Dis. 46, 826-832.
19. Morales, T.I. and Roberts, A.B. (1988) J. Biol. Chem. 263, 12828-12831.
20. Tyler J.A. (1985) Biochem. J. 225; 493-507.
21. Chandrasekhar, S. and Harvey, A.K. (1988) Biochem. Biophys. Res. Commun. 157, 1352-1359.