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Tumour necrosis factor α stimulates resorption and inhibits synthesis of proteoglycan in cartilage

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During inflammatory reactions, activated leukocytes are thought to produce a variety of small proteins (cytokines) that influence the behaviour of other cells (including other leukocytes). Of these substances, which include the interleukins, interferons and tumour necrosis factors (TNFs), interleukin-1 (IL-1) has been considered potentially a most important inflammatory mediator because of its wide range of effects (reviewed in refs 1, 2). *In vivo* it is pyrogenic and promotes the acute phase response; *in vitro* it activates lymphocytes[3] and stimulates resorption of cartilage[4] and bone[5,6]. Cartilage resorption is a major feature of inflammatory diseases such as rheumatoid arthritis, and IL-1 is the only cytokine hitherto known to promote it. TNFs are characterized by their effects on tumours and cytotoxicity to transformed cells[7-9], but share some actions with IL-1. I report here that recombinant human TNFα stimulates resorption and inhibits synthesis of proteoglycan in explants of cartilage. Its action is similar to and additive with IL-1, and it is a second macrophage-derived cytokine whose production in rheumatoid arthritis, or inflammation generally, could contribute to tissue destruction.

Two human TNFs have been isolated: TNFα (refs 8, 9) is a product of activated mononuclear phagocytes, TNFβ (ref. 7) of activated lymphocytes. The proteins show about 50% homology in nucleotide sequence and compete for a common class of receptors on the cervical carcinoma line ME-180 (ref. 10). TNFα is probably identical with cachectin[11], a factor that suppresses production of lipoprotein lipase in cultured adipocytes, and may play a part in the development of cachexia during infection[12]. IL-1 shows some biological similarity to TNF: it is cytotoxic to certain transformed cells[13] and it suppresses production of lipoprotein lipase in adipocytes[14]. Furthermore, cachectin has recently been shown to stimulate production of prostaglandins and latent collagenase by human synovial and dermal fibroblasts in a manner similar to IL-1[15]. In view of these findings, I have investigated the effect of TNFα on both the resorption and synthesis of proteoglycan by explants of cartilage.

Chondroitin-sulphate-rich proteoglycan is an essential component of the matrix of cartilage since it enables the tissue to resist compression during load-bearing. Loss of proteoglycan, such as occurs in rheumatoid arthritis, osteoarthritis and other joint diseases, results in severe impairment of the function of cartilage. IL-1 is the only purified cytokine known to cause cartilage to degrade its proteoglycan[4,16], and to inhibit resynthesis[17].

Figure 1*a* shows the amount of proteoglycan (measured as percentage of total chondroitin sulphate) released from porcine articular cartilage during 6 days of culture in the presence of human recombinant TNFα or pure porcine IL-1. The TNFα caused up to 75% of the proteoglycan to be released, although it was less potent than the IL-1, which was significantly active at a 20-fold lower dose (0.5 pM). Figure 1*b* shows a similar experiment carried out on cartilage from bovine nasal septum which was cultured for a shorter period (48 h): again, the IL-1 was more potent. The time dependence of the release of proteoglycan from bovine cartilage caused by sub-maximal concentrations of the two agents revealed that their effects were additive. Figure 1*c* shows that 50 pM IL-1 or 290 pM TNFα caused a similar rate of release, and that this was approximately doubled when the agents were combined. Maximal stimulation of cartilage by IL-1 caused more rapid release of proteoglycan than did TNFα (Fig. 1*d*): results for two concentrations of each cytokine demonstrate that responses were maximal. Supramaximal doses of the two agents in combination caused a rate of release that was considerably faster than that due to TNFα alone, but was not significantly greater than that seen with IL-1 alone. The failure of TNFα to augment the maximal response to IL-1 may be because the limit of the chondrocytes' ability to degrade their matrix *in vitro* was being approached.

The enzymatic mechanism by which the proteoglycan is degraded in cartilage is not understood. Normally, cartilage proteoglycans aggregate in a specific manner with hyaluronic acid, and it is thought that the large size of these aggregates causes them to be trapped in the matrix. Cartilage stimulated by IL-1 releases fragments of proteoglycan which, as judged by gel filtration, are smaller than normal proteoglycan monomers and are unable to aggregate with hyaluronic acid[18]. There is no evidence of degradation of their chondroitin sulphate chains. These changes suggest that degradation is by limited proteolysis of the protein core. The fragments of proteoglycan that were released by cartilage stimulated with TNF behaved similarly on gel filtration to those generated by stimulation with IL-1 (Fig. 2). The bulk of the fragments generated by stimulation with either agent emerged from a Sepharose 2B column at a region between the elution positions of intact proteoglycan and proteoglycan digested with papain (which consists largely of single-chain chondroitin sulphate peptides). Addition of hyaluronic acid to the proteoglycan fragments before chromatography caused little or no formation of aggregates. This suggested that the hyaluronate binding region was blocked or had been lost. When the proteolgycan fragments were chromatographed under dissociative conditions (4 M guanidine-HCl in the chromatographic buffer) the position of the main peak was unchanged. These experiments showed that chondrocytes activated by TNF or IL-1 caused a similar limited proteolysis of the proteoglycans.

In order to study the effect of TNFα on the synthesis of proteoglycan, cartilage was stimulated for 48 h, and $^{35}SO_4$ was added to the culture medium for the last 6 h. In this procedure the isotope becomes incorporated into newly synthesized sulphated glycosaminoglycan (mainly chondroitin sulphate). At the end of the experiment the medium and cartilage were digested with papain, and glycosaminoglycan was precipitated from the digests with cetylpyridinium chloride. The amount of radioactivity present in the precipitates was a measure of chondroitin sulphate (and, by inference, proteoglycan) synthesis. In experiments made with porcine articular (Fig. 3*a*) or bovine nasal septal (Fig. 3*b*) cartilages, TNFα caused a marked sup-

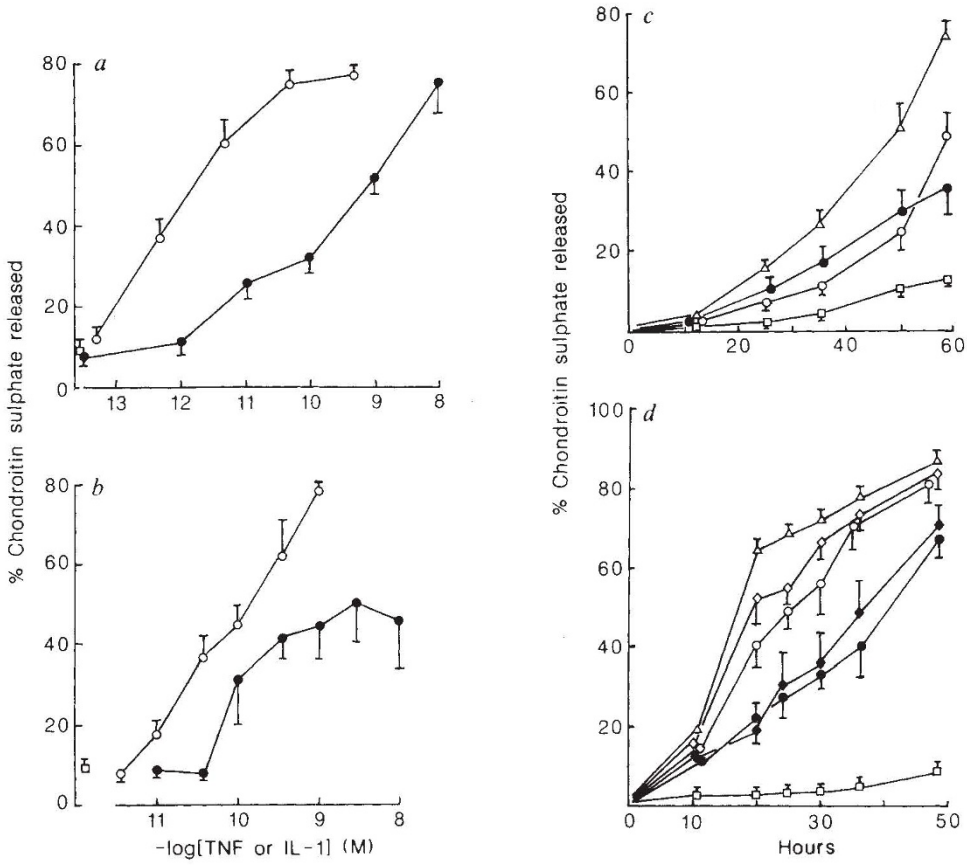

Fig. 1 Stimulation of release of proteoglycan from cartilage by TNFα or IL-1. *a*, Porcine articular cartilage. The amount of chondroitin sulphate released was expressed as a percentage of the total (the content of the medium and tissue combined)[21]. Results are shown as means of quintuplicate cultures ± s.e.m. ●, TNF; ○, IL-1; □, no addition. *b*, Bovine nasal septal cartilage. Symbols as for *a*. *c*, Time course for bovine cartilage disks cultured as in *b* with no addition (□), 50 pM IL-1 (○), 290 pM TNF (●), 50 pM IL-1 and 290 pM TNF in combination (△). Chondroitin sulphate released was measured at the indicated times and results are means ± s.e.m. of eight individual disk cultures. *d*, Time course for bovine cartilage discs cultured as in *c*, with no addition (□), 30 nM TNF (●), 90 nM TNF (◆), 1.5 nM IL-1 (○), 4.5 nM IL-1 (◇) and 90 nM TNF and 4.5 nM IL-1 in combination (△).

Methods. *a*, Pieces of articular cartilage were removed from the metacarpal heads of freshly slaughtered young pigs. Pieces (~4 mg wet weight) were maintained for 48 h at 37 °C in CO_2/air 1:19 in culture medium [Dulbecco's modified Eagle's medium (DMEM)] containing 5% normal bovine serum that had been heat-inactivated at 56 °C for 30 min). Each was then transferred to a well of a 96-well multititre plate and incubated under the same conditions in 0.2 ml of culture medium, either with no addition, or with human TNFα or porcine IL-1 of *p*I 5. The medium was changed at 3 days and the culture was terminated after 6 days. Human TNFα was a recombinant protein expressed in *Escherichia coli* and purified as described previously[7,22]. Porcine IL-1 was a natural leukocyte protein purified to homogeneity as described elsewhere[16]. The *p*I 5 form rather than the *p*I 8 form was used for these experiments. After culture the medium and cartilage were separated. The cartilage was digested completely with papain (see legend to Fig. 3). The chondroitin sulphate content of this digest and culture medium was estimated by use of the metachromatic dye, dimethylmethylene blue (Serva); whale chondroitin sulphate (Sigma) was used as a standard. *b*, Disks (2 × 1 mm) of cartilage were cut from the bovine nasal septa of freshly killed animals. Disks were precultured for 48 h and then stimulated for 48 h with TNFα or IL-1. The chondroitin sulphate content of medium samples and tissue digests was measured exactly as described for pig particular cartilage.

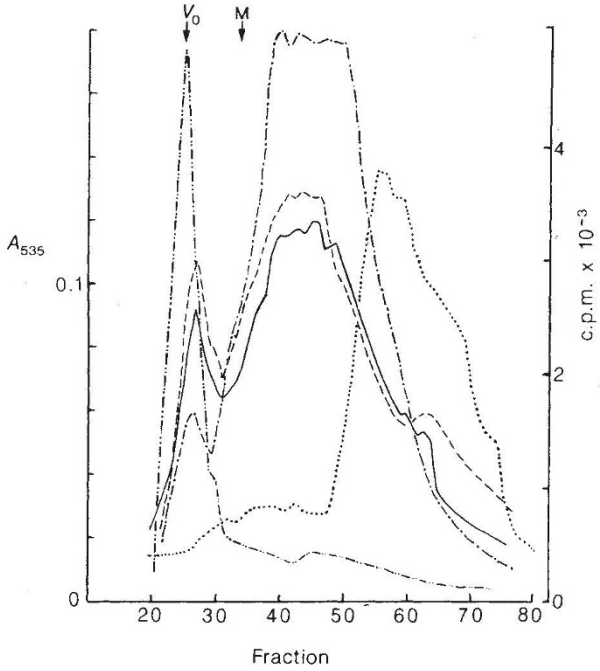

Fig. 2 Gel chromatography of proteoglycans released from stimulated cartilage. Samples of culture supernatants from stimulated bovine nasal cartilage were chromatographed on a column (930 × 6.5 mm) of Sepharose 2B (Pharmacia) eluted with 0.5 M acetate buffer *p*H 5.8. Fractions (0.4 ml) were collected. Proteoglycan was detected as chondroitin sulphate by the dye dimethylmethylene blue (see Fig. 1) and is shown as A_{535}. ——, Culture supernatant from bovine nasal cartilage cultured as in Fig. 1 in the presence of 5 nM TNF; ----, the same supernatant to which hyaluronic acid (2%) had been added before chromatography; -·-·-, culture supernatant from cartilage stimulated with 0.5 nM porcine IL-1, and to which hyaluronic acid had been added before chromatography; ...., a papain digest of proteoglycan extracted from fresh cartilage by 4 M guanidine-HCl. -···-, proteoglycan (+2% hyaluronic acid) extracted by guanidine-HCl (4 M) from unstimulated cartilage that had been biosynthetically labelled with $^{35}SO_4$ for 24 h, measured as c.p.m. Arrows are: V_0, void volume; M, elution position of $^{35}SO_4$-labelled proteoglycan without addition of hyaluronic acid.

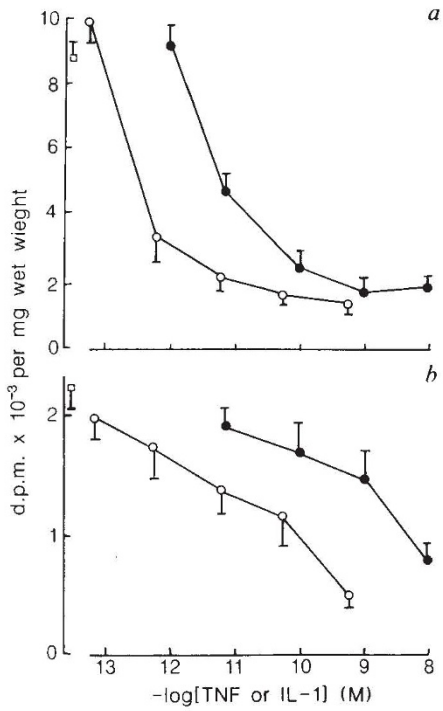

Fig. 3 Effects of TNFα or IL-1 on the incorporation of $^{35}SO_4$ into glycosaminoglycans of cartilage. *a*, Porcine articular cartilage; *b*, Bovine nasal septal cartilage. Each assay point is the mean of five separate explants ± s.e.m. ●, TNFα; ○, IL-1; □, no addition. **Methods.** *a*, Pieces of pig articular cartilage were dissected, precultured and then stimulated either with TNFα or porcine IL-1 for 48 h exactly as described for Fig. 1, except that the culture medium (DMEM) contained 1% normal bovine serum (heat inactivated) during the stimulation period. For the last 6 h the medium was replaced with SO_4-free culture medium (still containing TNFα or IL-1) to which was added 2.5 μCi ml^{-1} of $^{35}SO_4$ (25–40 Ci mg^{-1}; Amersham). After culture, the cartilage pieces were separated from the medium, briefly blotted to remove excess medium, and then weighted. Each piece was digested at 65 °C for 2 h in 0.2 ml of 0.05 M sodium phosphate buffer *p*H 6.5 containing 1 mM EDTA, 2 mM *N*-acetylcysteine, 28 μg ml^{-1} papain (Sigma Type III). Samples of medium (0.2 ml) were also digested with 0.1 ml of the papain solution under the same conditions. Chondroitin sulphate (0.1 ml of 2 mg ml^{-1}) was added to all the samples followed by 0.1 ml of cetylpyridinium chloride (10% w/v). Samples were centrifuged, the precipitates were washed twice in 3% cetylpyridinium chloride, then dissolved in 0.5 ml of formic acid and added to 5 ml of a scintillation mixture (Pico-fluor-30, Packard) and counted in a liquid scintillation counter. The radioactivity of the digests of tissue and medium were added together and the results expressed as d.p.m. per mg wet weight of cartilage. *b*, As for *a* except that disks of bovine nasal septal cartilage were used (see Fig. 1), and the culture medium contained 5% normal bovine serum throughout the experiment.

pression of proteoglycan production as judged the incorporation of $^{35}SO_4$ into glycosaminoglycan, but was less potent than IL-1. The porcine IL-1 inhibited incorporation in porcine articular cartilage in the range 0.1–10 pM; TNFα was 20-fold less active. A similar differential was observed on the bovine cartilage. The lower potency of the human TNFα compared with porcine IL-1 may be due to species differences.

Taken together, the experiments demonstrate that TNFα has a similar action to IL-1 on chondrocytes. It causes them to degrade proteoglycan by limited proteolysis and inhibits their synthesis of new proteoglycan. Exposure of cartilage to either of these leukocyte products during inflammation could lead to loss of proteoglycan and impairment of function; furthermore, their effects may be additive.

Pigs[16], like humans[19], have two different IL-1 proteins: for these experiments the *p*I 5 IL-1 was used rather than the *p*I 8 form. The IL-1s are equipotent on cartilage[16] and compete for the same receptors on porcine synovial fibroblasts: TNFα at 400 times excess over IL-1) did not compete for these receptors (T. A. Bird and J. S., in preparation). The augmentation of the effect of maximal doses of TNFα by IL-1 reported here is consistent with there being different receptors on chondrocytes for the two cytokines. Since they are apparently not homologous[8,16], IL-1 and TNFα would be expected to combine with different receptors. These considerations suggest that chondrocytes (and probably other connective tissue cells) could have two distinct types of receptor (one for IL-1 and one for TNF) whose interaction with ligand promotes resorption of matrix polymers while inhibiting their synthesis. Such a possibility could have important implications for the pharmacological control of inflammatory tissue destruction.

Following submission of this manuscript, Bertolini *et al.*[20] have reported that human TNFs, like IL-1, stimulated Ca^{2+} release from fetal rat bones.

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Post-translational insertion of a fragment of the glucose transporter into microsomes requires phosphoanhydride bond cleavage

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Most eukaryotic secretory and membrane proteins insert co-translationally into the membrane of the rough endoplasmic reticulum (RER), and are targeted there by one or more NH_2-terminal or internal signal sequences (for reviews see refs 1, 2). However, little is known about the actual translocation and membrane integration processes. In particular, any energy requirements for targeting and integration have remained obscure because of the inability to uncouple the processes from concomitant protein synthesis. We