Fibronectin-fragment-induced cartilage chondrolysis is associated with release of catabolic cytokines

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Fibronectin fragments have both catabolic and anabolic activities toward articular cartilage explants *in vitro*. Whereas a 1 nM concentration of an N-terminal 29 kDa fibronectin fragment (Fn-f) increases the proteoglycan (PG) content of cartilage without induction of matrix metalloproteinases (MMPs), 0.1–1 μ M Fn-f temporarily suppresses PG synthesis and enhances MMP release. The higher concentrations cause an initially rapid PG depletion during the first week of culture, followed by much slower PG loss and gradually increasing rates of PG synthesis. To test for the involvement of mediators, human articular cartilage was cultured with Fn-f, and conditioned media were assayed for selected cytokines and factors. With 1 nM Fn-f, the release of the anabolic factors, insulin growth factor-I and transforming growth factor β 1, from cultured cartilage was enhanced by 50–100 % during the entire 28-day culture period and this was associated with both supernormal rates of PG

synthesis and PG content. However, the higher concentrations of Fn-f additionally enhanced release, by at least 10-fold, of the cytokines, tumour necrosis factor α , interleukin-1 α , interleukin-1 β and interleukin-6 while causing depletion of cartilage PG. Release of tumour necrosis factor α , interleukin 1 β and interleukin 1 α peaked at days 2, 3 and 9 during or slightly after the period of maximal PG depletion and decreased to control levels by days 7, 7 and 21 respectively, whereas release of interleukin 6 was enhanced throughout the culture period. Neutralizing antibodies to the catabolic cytokines reduced Fn-f-mediated MMP-3 release and suppression of PG synthesis. The temporal aspects of this interplay between catabolic and anabolic factors are consistent with the kinetics of Fn-f-mediated cartilage damage and attempted repair and may be relevant to cartilage damage and repair *in vivo*.

INTRODUCTION

Proteolytic fragments of fibronectin have very potent cartilage chondrolytic activities [1]. These fibronectin fragments, at concentrations of 0.1–1 μ M, greatly increase expression of matrix metalloproteinases (MMP), rates of proteoglycan (PG) degradation and loss from cartilage tissue in explant cultures [1] and suppress synthesis of cartilage PG [2]. Stromelysin-1 (MMP-3) appears to be a major MMP [3]. The most active fibronectin fragment, the N-terminal 29 kDa fragment (Fn-f), binds to and penetrates intact cartilage [4] to enhance MMP release in a mode that may involve the fibronectin $\alpha_5\beta_1$ receptor, since analogue peptides of the Arg-Gly-Asp-Ser integrin-binding sequence block Fn-f-mediated cartilage chondrolysis [5]. The physiological significance of these activities is supported by observations that injection of fibronectin fragments into rabbit knee joints causes over 50 % removal of articular cartilage PG within 7 days [6] and that synovial fluids of patients with osteoarthritis and rheumatoid arthritis contain elevated levels of fibronectin fragments of a range of sizes [7,8].

Besides enhancement of MMP activities, another major catabolic activity of Fn-f is suppression of synthesis of PG and general protein [2], which occurs within 1 day of addition of Fn-f to cultures in which PG degradation is occurring. However, with time, the rates of synthesis slowly increase and PG degra-

dation slows down to less than control levels. The rates of PG synthesis eventually reach 140 % of control values, suggesting a reversal of earlier catabolic conditions and the initiation of reparative responses [9]. The anabolic response is more obvious at lower concentrations (1 nM) of Fn-f, which cause rates of PG synthesis to increase to 140 % of control levels with no detectable MMP-3 release or PG degradation [9]. Thus Fn-f exerts catabolic or anabolic activity depending on its concentration.

We considered that Fn-f may express these dual catabolic and anabolic properties not directly but by enhancing release or synthesis of both catabolic and anabolic factors. Interleukin (IL)-1 was considered to play a role since it can enhance MMP levels and suppress PG synthesis [10,11] and it is increased in osteoarthritic and rheumatoid synovial fluids [12]. Since our preliminary studies showed that the IL receptor antagonist protein only partially blocked PG degradation (G. A. Homandberg, F. Hui, C. Wen, C. Purple, K. Bewsey, H. Koepf, K. Huch and A. Harris, unpublished work), the additional involvement of tumour necrosis factor α (TNF- α), which has activities similar to IL-1 [13] and is found in high levels in rheumatoid synovial fluids [14], was considered. TNF- α was also considered since it can induce IL-1 β in chondrocytes [13] and in various types of cells [15–17] and since antibodies of TNF- α have been shown to block IL-1 release [16]. IL-6 was considered since it can be induced in chondrocytes by TNF- α [18,19], it is found

Abbreviations used: MMP, matrix metalloproteinase; MMP-3, stromelysin-1; PG, proteoglycan; Fn-f, a specific N-terminal fibrin- and heparin-binding 29 kDa fibronectin fragment generated by thrombin-catalysed proteolysis of human plasma fibronectin; DMEM, Dulbecco's modified Eagle's medium; IL, interleukin; TNF- α , tumour necrosis factor α ; TGF- β , transforming growth factor β ; IGF-I, insulin-like growth factor I; TIMP-1, tissue inhibitor 1 of MMPs.

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at high levels in synovial fluids of patients with rheumatoid arthritis [20], it mediates and blocks certain IL-1 and TNF- α activities [21,22], it induces the tissue inhibitor of MMPs (TIMP-1) [23] and it enhances release of transforming growth factor β (TGF- β) from chondrocytes [24].

The anabolic factor, insulin-like growth factor I (IGF-I), was considered since it is the major PG-synthesis-promoting factor synthesized by chondrocytes [25] and its message is enhanced in osteoarthritic cartilage tissue [26] as well as the density of its respective binding sites [27]. A role for TGF- β was considered on the basis of its many anabolic activities, including promotion of the chondrocyte phenotype [28], restoration of the matrix of IL-1-damaged chondrocytes [29], partial inhibition of IL-1-stimulated cartilage damage [28], suppression of protease release, including MMP-30 [30], and induction of TIMP-1 [31]. Further, since release of TGF- β from chondrocytes is stimulated by IL-1 [24], and TGF- β levels are elevated in synovial fluids of patients with rheumatoid arthritis and osteoarthritis [32], its involvement was considered. Lastly, since both IGF-1 and TGF- β can be liberated by proteinases from complexation with binding proteins [32–34], we considered that Fn-f may have enhanced their release.

We report that low concentrations of Fn-f enhance release of anabolic factors, and high concentrations enhance release of both anabolic and catabolic cytokines. The combination of these anabolic and catabolic factors and the kinetics of their release appear to account for the anabolic and catabolic effects of Fn-f. The differential effects of low and high concentrations of Fn-f are consistent with a role for low concentrations in directly or indirectly promoting tissue repair and a role of high concentrations in amplifying cartilage damage before attempted cartilage repair.

MATERIALS AND METHODS

All common chemicals except where noted were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Na₂³⁵SO₄ (43 Ci/mg S) was from ICN Biomedicals (Costa Mesa, CA, U.S.A.). IL-1 α and IL-1 β assay kits were from Oncomembranes (Seattle, WA, U.S.A.). Predicta IL-6, TNF- α and TGF- β 1 assay kits were from Genzyme Corp. (Cambridge, MA, U.S.A.). The TGF- β 1 kit recognizes both latent and active forms. IGF-I assay kit was from The Nichols Institute (San Juan Capistrano, CA, U.S.A.). Neutralizing rabbit anti-human polyclonal antibodies to TNF- α (catalogue no. IP-300), IL-1 α (LP-710), IL-1 β (LP-712) and IL-6 (LP-716) were from Genzyme Corp. A human MMP-3 ELISA kit was purchased from Fuji Chemicals Industries (Tokyo, Japan).

Isolation of Fn-f from human plasma fibronectin was performed as described previously [1]. Fn-f refers to the well-defined thrombin-generated heparin- and fibrin-binding 29 kDa N-terminal fibronectin fragment that is the most active on cartilage tissue [1]. This Fn-f was shown to be free of several proteolytic activities as described [3]. Assays using IL-1 α , IL-1 β , IL-6, TNF- α , IGF-1 and TGF- β 1 commercially available assay kits with minimal levels of detection of 7.8 pg/ml, 7.8 pg/ml, 35 pg/ml, 15 pg/ml, 300 pg/ml and 0.1 ng/ml respectively did not show detectable levels.

Explant cultures of human articular cartilage

Eight different sources of normal human cartilage from ankle and knee were studied. Knee articular cartilage was obtained from a fetus or from the femoral patella of a 4-year-old female, 45-year-old female or 73-year-old female donor. Ankle articular cartilage was obtained from a 43-year-old female, a 64-year-old male, 77-year-old male or 91-year-old female donor. In all cases,

the cartilage appeared macroscopically normal with no evidence of joint trauma, connective tissue abnormality or arthritic disease. During dissection, care was taken not to remove underlying subchondral bone. Cartilage was cultured as described for bovine tissue [3,9]. Cartilage was routinely equilibrated for 2 days before an experiment; day 0 represents the start of the experiment. Each datum was a mean $\pm$ S.D. based on at least three similar tissue culture wells.

Assay of PG content of cartilage

PG content was determined as described [3,9] and reported as μ g/mg wet weight of cartilage (mean $\pm$ S.D. for at least three cartilage samples). Damage was defined and measured as a decrease in total cartilage PG.

Assay of [³⁵S]sulphate incorporation

Sulphate incorporation was used as an index of synthesis of sulphated PG. Rates were measured in high-density chondrocyte monolayer cultures using a 2 h pulse with 10 μ Ci/ml label in 2 ml of 10 % serum/Dulbecco's modified Eagle's medium (DMEM), followed by a 2 h chase with 10 % serum/DMEM. After removal of the medium and three washes with DMEM, a 2 ml volume of buffer containing 4 M guanidinium chloride, 0.1 % Triton X-100, 10 mM EDTA and 100 mM sodium acetate, pH 5.5, was added to each culture well. After 1 h the extracts were exhaustively dialysed and label was quantified as described [2]. The amount of label was expressed as d.p.m./10⁶ cells based on haemocytometer counting of separate trypsin-treated wells not treated with Fn-f.

Cytokine/factor assays

For assays of cytokines in conditioned medium, cartilage slices were cultured as described above. Aliquots (0.5 ml) of conditioned medium were collected and without freezing, immediately dialysed against 10 mM NaCl/10 mM Tris/HCl, pH 7.4, and concentrated ten times on a Speed-O-Vac concentrator without application of heat. All assays were performed as directed by the manufacturer. The data shown are means $\pm$ S.D. for at least three separate tissue culture wells. Day-0 time points correspond to the amount of material found in the medium at the start of the experiment, immediately after addition of Fn-f. For kinetic points, the contents reported correspond to accumulation over a 1-day period. In order to determine the amount of cytokine retained in the tissue compared with that released into the medium, cartilage tissue in 30–60 mg quantities was extracted with 2 ml of the buffer containing 4 M guanidinium chloride, 0.1 % Triton X-100, 10 mM EDTA and 0.1 M sodium acetate, pH 5.5, for 24 h at 9 °C. The extract was then dialysed against 10 mM NaCl/10 mM Tris/HCl, pH 7.4, and concentrated ten times to reflect the same volume as the concentrate of the respective conditioned medium. This cytokine content of the extract was then compared with that of the corresponding conditioned medium. Addition of exogenous TNF- α , IL-1 α , IL-1 β or IL-6 to extracts caused, in each case, at least 87 % of the expected incremental increases in cytokine content ($n = 3$), confirming that the extraction conditions did not denature the cytokines and reduce their reaction with antibodies used in the ELISA kits.

Preparation of high-density human articular chondrocyte monolayer cultures and tests of neutralizing antibodies

To neutralize cytokine activities, monolayer cultures were used rather than tissue cultures to allow penetration of the antibodies.

Since Fn-f at similar concentrations suppresses PG synthesis and enhances release of pro-MMP-3 in bovine monolayer cultures to the same extent as in bovine explant tissue cultures [35], the monolayer culture system was considered to be a suitable test system. Monolayer cultures of human chondrocytes were prepared by enzymic digestion from cartilage as described [36]. Cells were plated into 24-well culture plates using 500 000 cells/cm² in 10% serum/DMEM. To test the effects of neutralizing antibodies, cultures were adjusted to 5 µg/ml antibody after 4 days. After another day, the medium was changed, fresh antibody solution added and the medium adjusted to 0.1 µM Fn-f. In measurements of the effect of antibodies on incorporation of sulphate, label was added at this time and labelling performed as described above. The neutralizing antibodies used have all been shown to block the effects of the respective cytokines. The anti-TNF-α antibody neutralizes TNF-α bioactivity in L929 cell cytotoxicity assays [37], the anti-IL-1β antibody and anti-IL-1α antibody neutralize the respective cytokine in the thymocyte costimulation assay as well as in several other bioassay systems [38–41] and the anti-IL-6 antibody inhibits IL-6-dependent proliferation of T1165 cells and exogenous recombinant human IL-6 in the B9 cell proliferation assay and in other bioassays [42–44].

MMP-3 ELISA

MMP-3 protein was measured in conditioned medium of human chondrocyte cultures using an ELISA kit from Fuji Chemicals Industries. The medium was not concentrated before assay. This kit recognizes human MMP-3, pro-MMP-3 and MMP-3–TIMP-1 inhibitor complexes as total MMP-3 [45]. To test for MMP-3 trapped in tissue before culture, freshly isolated cartilage (52 mg) was subjected to extraction with 2 ml (the same volume as used in each culture well) of buffer containing 4 M guanidinium chloride, 0.1% Triton X-100, 10 mM EDTA and 100 mM sodium acetate, pH 5.5, for 24 h at 9 °C. The extract was then dialysed against 150 mM NaCl/10 mM Tris/HCl, pH 7.4, and assayed.

RESULTS

Low concentrations of Fn-f enhanced PG content and rates of PG synthesis whereas higher concentrations rapidly decreased cartilage PG content and temporarily suppressed PG synthesis

Normal disease-free articular cartilage obtained *post mortem* from the knees of a 45-year-old woman was cultured in 10% serum/DMEM, with changes of medium every other day. Figure 1(A) shows that the effect of incubation of the cartilage with 1 nM Fn-f was to enhance the PG content of the cartilage above that of control untreated cartilage, whereas 0.1 µM Fn-f caused a rapid depletion of PG during the first week, followed by a much slower rate of decrease in cartilage PG content. Figure 1(B) shows that 1 nM Fn-f enhanced rates of PG synthesis, consistent with the increase in PG content shown in Figure 1(A). In contrast, 0.1 µM Fn-f suppressed PG synthesis during the first week during which, as shown in Figure 1(A), there was an initially rapid decrease in PG content. PG synthesis then slowly increased after the first week during which, as shown in Figure 1(A), there was a very low rate of PG depletion, slower than in control cartilage.

Both low and high concentrations of Fn-f enhanced release of the anabolic factors, IGF-I and TGF-β1

The enhanced rates of PG synthesis suggested that Fn-f may enhance release of anabolic factors such as IGF-I and TGF-β1.

Figure 1 Comparison of the kinetics of cartilage PG degradation and of the effects on PG synthesis in Fn-f-treated cartilage

(A) Effects of Fn-f on cartilage PG content. ○, Untreated control cartilage; ▽, cartilage treated continuously with 1 nM; ●, cartilage treated with 0.1 µM Fn-f. Medium was changed every other day. Fn-f-treated cultures were cultured with fresh dilutions of Fn-f with each medium change. (B) Effects of Fn-f on sulphate incorporation in similar cultures of control cartilage (○), cartilage treated continuously with 1 nM (□) or 0.1 µM (●) Fn-f. The incorporation in control cartilage tissue at day 0, on which the percentage calculations are based, was 1428 ± 97 (*n* = 6) d.p.m./mg of cartilage.

Concentrates of the conditioned media of the above cultures were assayed for IGF-I after an acid dissociation step which inactivates IGF-I-binding proteins as described in the manufacturer's protocol. Figures 2(A) and 2(B) show that control tissue, not treated with Fn-f, slowly released IGF-I and TGF-β1 into the medium. However, the presence of Fn-f enhanced significantly the amount of released material, although this effect appeared to be independent of the concentration of Fn-f.

Only higher concentrations (0.1–1 µM) of Fn-f enhanced release of TNF-α, IL-1β, IL-1α and IL-6

Figure 3 shows that, in contrast with IGF-I and TGF-β, catabolic cytokines were either low or not detected in media from control cartilage not treated with Fn-f or from cartilage treated with 1 nM Fn-f. For TNF-α (Figure 3A), 0.1 µM and 1 µM Fn-f caused enhanced release which peaked at day 2 and then quickly fell to baseline levels by about day 6. For IL-1β (Figure 3B), the higher concentrations of Fn-f caused a concentration-dependent enhanced release with a peak in each case at day 3 and a subsequent decrease to basal levels. For IL-1α (Figure 3C), 10 nM Fn-f caused a slightly elevated release beyond day 9, whereas 0.1 and 1 µM Fn-f enhanced IL-1β release to approximately the same level in both cases, with a peak for release in each case at day 9, rather than day 2 or 3, as for TNF-α and IL-1β respectively. The release remained above control levels until about day 21. For IL-6 (Figure 3D), 10 nM Fn-f enhanced release with a resultant peak at day 12 and elevated levels during the entire culture period. In contrast, 0.1 µM and 1 µM Fn-f

Figure 2 Effect of Fn-f on release of IGF-I and TGF- β 1 into the culture medium

Normal human articular cartilage from a 45-year-old female donor was cultured in 10% serum/DMEM with Fn-f. Media were collected and prepared for assay as described in the Materials and methods section. **(A)** Concentrates were subjected to assays using an IGF-I kit as described by the manufacturer. Curves shown are untreated control cartilage (○) and 0.1 nM (●), 1 nM (▼), 10 nM (□), 0.1 μ M (▽) and 1 μ M (■) Fn-f-treated cartilage. The maximal amount of IGF-I in the conditioned medium for the 1 μ M Fn-f concentration at day 3 was 174 ng/ml. **(B)** The same concentrates were assayed for TGF- β 1. Curves shown are untreated control cartilage (○) and 1 nM (●), 0.1 μ M (▽), and 1 μ M (▼) Fn-f-treated cartilage. The maximum amount in conditioned medium for the 1 μ M Fn-f concentration at day 18 was 2.1 μ g/ml.

immediately enhanced IL-6 release within 2 days to levels that remained elevated throughout the culture period.

Cartilage from several other donors responded to Fn-f with enhanced release of catabolic cytokines

Cartilage tissue from the knees of a 4- and 73-year-old donor and from the ankles of a 43- and 64-year-old donor were also tested at the time points found to show maximal cytokine release for the 45-year-old donor, as described in Table 1. These cartilage samples responded to Fn-f similarly to the cartilage from the 45-year-old donor, in that levels of the four cytokines were greatly elevated at their peak periods as compared with control untreated tissue.

Samples of the cartilages studied above were extracted without culturing as described in the Materials and methods section and the amounts of TNF- α , IL-1 β , IL-1 α and IL-6 determined in order to determine if the cartilage contained trapped cytokines before culturing. None of the tissue extracts had levels significantly greater than those found in control media (results not shown).

To confirm that the cytokines/factors were fully released into the media, cartilage tissue from the 45-, 73- and 43-year-old donors were assayed at the respective time points used in Table 1. The media and tissue extracts were concentrated to the same final volume for direct comparison and calculation of percentage in the medium. The average tissue content for the three donors was only 12, 25, 27 and 25% respectively of the summed TNF-

α , IL-1 β , IL-1 α and IL-6 contents of the media and tissue compartments.

Cycloheximide suppressed Fn-f-mediated cytokine release

In order to determine whether protein synthesis was required for the enhanced release of IGF-I or TGF- β , cartilage cultures were adjusted to 0.1 μ M Fn-f and 0, 10 or 50 μ M cycloheximide at days 1, 7 and 14; conditioned media were then assayed after 24 h. Averaging of the three time points showed that cycloheximide significantly ($P = 0.003$) reduced levels of IGF-I by $22 \pm 3\%$ ($n = 3$) compared with control samples. In the same samples, TGF- β levels were reduced an average of $14 \pm 4\%$ ($n = 3$), an amount that was not statistically significant. Thus cycloheximide was significantly effective only on IGF-I release, although the effect was small.

In order to determine whether protein synthesis was required for the enhanced release of the catabolic cytokines, human cartilage cultures were adjusted to 100 nM Fn-f and at the times used in Table 1 were additionally adjusted to 10 or 50 μ M cycloheximide and the media assayed after 24 h. Cycloheximide at 10 μ M suppressed Fn-f-mediated release of TNF- α , IL-1 β , IL-1 α and IL-6 by 49, 49, 70 and 65% respectively, and 50 μ M cycloheximide suppressed release by 70, 90, 94 and 82% respectively. These effects are in great contrast with those observed for IGF-I and TGF- β . These data, as well as the inability to detect relatively high levels of cytokines in the tissue extracts, suggest that the catabolic cytokines released are synthesized *de novo*.

Neutralizing antibodies to TNF- α , IL-1 α , IL-1 β and IL-6 suppressed the effects of Fn-f on PG synthesis

The ability of neutralizing antibodies to the various cytokines to block a major catabolic activity of Fn-f, namely suppression of PG synthesis, was tested in high-density chondrocyte cultures derived from the 45-year-old donor and from fetal cartilage as described in Table 2. Table 2 shows that 0.1 μ M Fn-f suppressed sulphate incorporation by 43% in the former, a value within the range previously reported for bovine tissue [3,9]. Non-specific rabbit IgG alone, at 5 μ g/ml, did not suppress incorporation. As for the other controls, the cytokine-specific antibodies were tested at 5 μ g/ml for their ability to alter rates of sulphate incorporation in the absence of Fn-f. Table 2 shows that these antibodies alone had no significant effect on sulphate incorporation. However, addition to the Fn-f-treated 45-year-old chondrocyte or fetal chondrocyte cultures of antibodies to TNF- α or to IL-6 or a combination of all of the antibodies significantly blocked the effect of Fn-f, although complete blocking did not occur. The effects of antibodies to IL-1 α and IL-1 β were much less significant on the chondrocytes from 45-year-old cartilage. Individual antibodies to both IL-1 forms or a mixture were more effective on the fetal chondrocytes than on the 45-year-old chondrocytes, whereas antibodies to IL-6 were slightly more effective on the 45-year-old chondrocytes. The complete mixture of antibodies was the most effective on the fetal chondrocytes, totally blocking activity. We conclude that the suppression of PG synthesis by Fn-f is mediated through cytokines.

Neutralizing antibodies to TNF- α , IL-1 α , IL-1 β and IL-6 suppressed the enhanced release of MMP-3 caused by Fn-f

The ability of neutralizing antibodies to the various cytokines to block another major catabolic activity of Fn-f, namely enhanced release of MMP-3 [3], was tested in chondrocyte cultures derived from normal disease-free femoral head cartilage from a 91-year-

Figure 3 Effect of Fn-f on release of TNF- α , IL-1 α , IL-1 β and IL-6 into the culture medium

(A) The same concentrates as described in Figure 2 were assayed for TNF- α . Curves shown are untreated control cartilage ($\circ$) and 1 nM ($\bullet$), 0.1 μ M ($\blacktriangledown$) and 1 μ M (∇) Fn-f-treated cartilage. The maximal amount in the conditioned medium for the 1 μ M Fn-f concentration at day 2 was 1.2 ng/ml. (B) The concentrated media were assayed for IL-1 β . Curves shown are untreated control cartilage ($\circ$) and 10 nM ($\bullet$), 0.1 μ M (∇) and 1 μ M ($\blacktriangledown$) Fn-f-treated cartilage. A concentration of 1 nM Fn-f caused about the same amounts of release as found in control cartilage. The maximal amount in the conditioned medium for the 0.1 μ M Fn-f concentration at day 3 was 60 pg/ml. (C) The concentrated media were assayed for IL-1 α . Curves shown are untreated control cartilage ($\circ$) and 1 nM ($\bullet$), 10 nM (∇), 0.1 μ M ($\blacktriangledown$) and 1 μ M ($\square$) Fn-f-treated cartilage. The maximal amount in the conditioned medium for the 1 μ M Fn-f concentration at day 9 was 300 pg/ml. (D) The concentrated media were assayed for IL-6. Curves shown are untreated control cartilage ($\circ$) and 0.1 nM (∇), 1 nM ($\bullet$), 10 nM ($\blacktriangledown$), 0.1 μ M ($\square$) and 1 μ M ($\blacksquare$) Fn-f-treated cartilage. The maximal amount in the conditioned medium for the 1 μ M Fn-f concentration at day 4 was 0.69 ng/ml.

Table 1 Measurements of catabolic cytokines in Fn-f-cultured human cartilage

Normal human articular cartilage was cultured in 10% serum/DMEM with 0.1 μ M Fn-f for 3 days for assays of TNF- α , IL-1 β and IL-6 and for 9 days for IL-1 α . Medium was changed on day 2 for the TNF- α , IL-1 β and IL-6 assays or on days 2, 4, 6 and 8 for the IL-1 α assay. The conditioned media were then collected on day 3 for the TNF- α , IL-1 β and IL-6 assays or on day 9 for the IL-1 α assay. The concentrations correspond to accumulation between days 2 and 3 for TNF- α , IL-1 β and IL-6 and days 8 and 9 for IL-1 α . Donors 1–4 were respectively, a 45-year-old female (knee cartilage), a 73-year-old female (knee cartilage), a 43-year-old female donor (ankle cartilage) and a 64-year-old donor (ankle cartilage). Units are pg/mg wet weight of cartilage. Means of three wells are shown. S.D. values were typically less than 15% of mean.

Cytokine	Donor 1		Donor 2		Donor 3		Donor 4	
	Control	Fn-f	Control	Fn-f	Control	Fn-f	Control	Fn-f
TNF- α	0.9	41	2.3	32	2.1	27	10	45
IL-1 β	0.1	1.3	0.07	0.91	0.12	0.87	0.15	1.4
IL-1 α	0.7	9.4	0.9	8.2	1.1	6.1	0.7	7.5
IL-6	0.9	18	2.1	23	1.9	22	0.4	27

old donor. Samples of cartilage were extracted as described in the Materials and methods section; levels of 7.7 ± 0.6 nM ($n = 3$ extracts) MMP-3 were obtained. This level can be compared with 19 ± 3 nM ($n = 3$ wells) and 151 ± 27 nM ($n = 3$ wells) found in the media of cartilage cultured respectively with or without 0.1 μ M Fn-f for 3 days. Therefore the amount of trapped MMP-3 was insignificant compared with the enhanced levels. The remainder of the cartilage was digested to obtain chondrocyte cultures for testing of the effects of neutralizing antibodies to the

cytokines. Table 3 shows that media from control tissue not treated with Fn-f had levels of MMP-3 of 17 nM, a concentration over 10-fold lower than the stimulated levels of 214 nM. However, the concomitant addition of antibodies to the cytokines or a mixture of the antibodies suppressed MMP-3 release over a range of values, with IL-1 α release being suppressed least, by 39%, and IL-6 release being suppressed the most, by 86%. These data are consistent with the proposal that this effect of Fn-f is mediated via these catabolic cytokines.

DISCUSSION

Both high and low concentrations of Fn-f slightly enhanced release of the anabolic factors, TGF- β 1 and IGF-1, consistent with the ability of 1 nM Fn-f to enhance PG synthesis [14] and of higher concentrations of Fn-f to enhance PG synthesis after the initial release of catabolic cytokines. Since the release of either factor was not dependent on Fn-f concentration, nor greatly enhanced, Fn-f probably did not induce these factors. In fact, preliminary data from this laboratory (C. Purple, K. Wen and G. A. Homandberg, unpublished work) suggest that in chondrocyte monolayer cultures, devoid of extensive matrix which might trap IGF-I, Fn-f does not enhance synthesis or release of IGF-I. Further, in cartilage cultures, exogenous proteinases such as trypsin or MMP-3 enhance release of IGF-I. Thus the data shown here suggest that Fn-f probably enhanced release of these factors through induced proteolysis. This suggestion is consistent with observations that both IGF-I and TGF- β can be synthesized and stored in the extracellular matrix [32,33] and released by proteolytic damage [32,34]. Such a proteolytic mechanism might also explain how IGF-I is released from bone on addition of IL-1

Table 2 Effect of neutralizing antibodies to catabolic cytokines on Fn-f-mediated suppression of PG synthesis of chondrocytes from 45-year-old femoral patella cartilage and fetal cartilage

Human articular chondrocyte cultures were established in 10% serum/DMEM, and 4 days after the beginning of the culture, the wells were adjusted to 5 µg of the respective antibodies/ml. After another 24 h, media were changed and again adjusted to 5 µg/ml antibody or 5 µg/ml antibody with 0.1 µM Fn-f. After 24 h, sulphate incorporation was measured and compared with control values. Percentage values are based on incorporation into cells, in the absence of Fn-f, of a mean of 2280 d.p.m./10⁶ cells for the 45-year-old cartilage and 1930 d.p.m./10⁶ cells for the fetal cartilage and in each case are defined as 100%. Means ± S.D. values of six wells per experiment are shown. Student's *t* test was used to determine significance; a *P* value of < 0.05 was considered significant. Significance is indicated by (s).

Addition	PG synthesis (% of control)	
	45 years old	Fetal
Fn-f alone	57 ± 4	5 ± 7
Rabbit IgG alone	97 ± 5	97 ± 5
Anti-TNF-α alone	106 ± 9	104 ± 9
Anti-IL-1β alone	109 ± 9	105 ± 9
Anti-IL-1α alone	106 ± 8	102 ± 8
Anti-IL-6 alone	105 ± 4	103 ± 4
Fn-f + rabbit IgG	54 ± 7	55 ± 9
Fn-f + anti-TNF-α	78 ± 8 (s)	100 ± 9 (s)
Fn-f + anti-IL-1α	Not tested	73 ± 3 (s)
Fn-f + anti-IL-1β	Not tested	93 ± 11 (s)
Fn-f + anti-IL-1α, β*	60 ± 4	81 ± 7 (s)
Fn-f + anti-IL-6	78 ± 6 (s)	62 ± 12
Fn-f + anti-TNF-α, IL-1β, α*	Not tested	92 ± 7 (s)
Fn-f + all†	75 ± 5 (s)	98 ± 6 (s)

* Mixture of 5 µg of each antibody/ml.

† Mixture of 5 µg each of anti-TNF-α, anti-IL-1β, anti-IL-1α and anti-IL-6/ml.

Table 3 Effect of neutralizing antibodies of catabolic cytokines on Fn-f-mediated MMP-3 release from femoral head cartilage chondrocytes from a 91-year-old donor

Human articular chondrocyte cultures were established in 10% serum/DMEM and 4 days after the beginning of the culture, the wells were adjusted to 5 µg/ml antibodies. After another 24 h, medium was changed, fresh antibody was added and media adjusted to 0.1 µM Fn-f. After 24 h, the MMP-3 content of media was measured by ELISA. The MMP-3 concentration refers to the concentration in conditioned media normalized to 10⁶ cells per culture well. Means ± S.D. values of six wells per experiment are shown. A *P* value of < 0.05 was considered significant and is indicated by (s). Blocking (%) is defined as ([Fn-f alone] - [Fn-f + antibody]) × 100/[Fn-f alone].

Addition	[MMP-3] (nM)	Blocking (%)
Control cultures	17 ± 4.2	—
Fn-f alone	214 ± 19	—
Fn-f + rabbit IgG	236 ± 28	—
Fn-f + anti-TNF-α	90 ± 4.0 (s)	63
Fn-f + anti-IL-1β	131 ± 5.3 (s)	42
Fn-f + anti-IL-1α	137 ± 8.2 (s)	39
Fn-f + anti-IL-6	45 ± 4.0 (s)	86
Fn-f + all*	41 ± 1.2 (s)	83

* Mixture of 5 µg/ml each of anti-TNF-α, anti-IL-1β, anti-IL-1α and anti-IL-6.

and TNF-α [46], how TGF-β1 is released from chondrocytes on addition of IL-1 and IL-6 [24] and how elastase or trypsin enhance PG synthesis in treated cartilage tissue [47,48]. Although cycloheximide did decrease the release of both factors in the Fn-f-cultures, this may have been through suppression of MMP induction.

In terms of catabolic factors, greatly enhanced release of TNF-α, IL-1β, IL-1α and IL-6 occurred in cultures only with higher concentrations of Fn-f. The cytokines, TNF-α, IL-1β, IL-1α and IL-6, appeared to be induced rather than released from cartilage, as extracts of uncultured cartilage did not show elevated levels and cycloheximide almost totally suppressed release of some of the cytokines. The enhanced release of TNF-α and IL-1β was markedly transient whereas that of IL-1α was more prolonged and that of IL-6 was continuously elevated. The short-term release or peaking of TNF-α and IL-1β, factors that can induce MMPs and suppress PG synthesis, may partly explain why rates of PG degradation decrease and PG synthesis suppression slowly reverses after a few days in culture [9] (Figure 1). If so, this would suggest that the prolonged enhanced release of IL-1α may not be sufficient to continue the enhanced PG degradation and suppression of PG synthesis.

In cultures treated with the higher concentration of Fn-f, the enhanced release of IGF-I and TGF-β1 did not protect against the catabolic effects of IL-1 and TNF-α. This was not surprising, as, although IGF-I antagonizes the effects of IL-1 and TNF-α [49], we have shown elsewhere that exogenous IGF-I delays Fn-f-mediated cartilage chondrolysis only when added to cartilage cultures 2–7 days before the addition of Fn-f [2,3]. Fn-f may damage cartilage simply because the catabolic cytokines enhance catabolic events at a faster rate than IGF-I or TGF-β can evoke anabolic responses.

The catabolic cytokines studied here appear to be involved in effects of Fn-f since both MMP-3 induction and suppression of PG synthesis were partially or completely reversed by neutralizing antibodies to the catabolic cytokines. Addition of antibodies to TNF-α in each case had a greater effect than addition of antibodies to IL-1, consistent with the possibility that TNF-α may be released first in this system, followed by IL-1, and may have initiated a cytokine cascade.

The data suggest a model for the role of Fn-f in cartilage homeostasis, in that at low Fn-f concentrations, as might occur in lightly damaged cartilage, anabolism would be amplified via release of IGF-I and TGF-β1. However, as cartilage damage progresses, the now higher concentrations of Fn-f would cause the additional but short-term release of catabolic cytokines such as TNF-α and IL-1β and a more gradual release of IL-1α. This would temporarily counter the effects of the anabolic factors and promote tissue clearance by enhancing cartilage PG degradation and suppression of PG synthesis. As the cytokine release slows and the effects of the anabolic factors are antagonized to a lesser extent, PG synthesis rates would gradually increase to promote tissue repair and remodelling. Such a scenario might be very relevant to the sequence of catabolic and anabolic events that occur in cartilage damage and repair *in vivo*.

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