

POTENTIAL REGULATION OF CARTILAGE METABOLISM IN OSTEOARTHRITIS BY FIBRONECTIN FRAGMENTS

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1. ABSTRACT

There are few candidates for biochemical pathways that either initiate or amplify catabolic processes involved in osteoarthritis (OA). Perhaps, one of the most likely sources for such pathways may be within the extracellular matrix itself. This review focuses on an example of how specific degradation products of the extracellular matrix of cartilage, produced during proteolytic damage, have the potential to enhance OA-like processes. In this example, these products can induce or activate other factors, such as catabolic cytokines, that amplify the damage. The damage, in turn, enhances levels of the degradation products themselves, as in a positive feedback loop. Since these products are derived from the cartilage matrix, they could be considered barometers of the health of the cartilage that signal to the chondrocyte, through outside to inside signaling, the health or status of the surrounding matrix. The best example and most characterized system is that of fragments of the matrix protein, fibronectin (Fn), although as discussed later, other recently discovered fragment systems may also have the potential to regulate cartilage metabolism. In the case of Fn fragments (Fn-fs), the Fn-fs enhance levels of catabolic cytokines as in OA and, thus, are potentially earlier damage

mediators than catabolic cytokines. The Fn-fs up-regulate matrix metalloproteinase (MMP) expression, significantly enhance degradation and loss of proteoglycan (PG) from cartilage and temporarily suppress PG synthesis, all events observed in OA. However, this Fn-f system may be involved in normal cartilage homeostasis as well. For example, low concentrations of Fn-fs enhance anabolic activities and could play a role in normal homeostasis. This system may also be involved in not only amplifying damage but also coupling damage to repair. For example, high concentrations of Fn-fs that might arise in OA temporarily offset the anabolic response of lower Fn-f concentrations and cause short-term enhanced catabolic events that are followed by slowly increasing anabolic responses. Such effects would be expected for mediators with roles in regulation of metabolism in both normal or diseased cartilage. Other products of matrix degradation have also been shown to regulate cartilage metabolism. A common mechanistic theme to these systems may be that they perturb the cartilage matrix and directly or indirectly alter function of specific receptors involved in metabolism. These concepts illustrate the potential of the cartilage matrix to regulate its composition in both health and

disease.

2. INTRODUCTION

This chapter reviews newer work that gives further insight into previous studies on the capability of the cartilage extracellular matrix, composed of a collagen/PG network as well as accessory proteins, to signal to the chondrocyte the need for reparative processes. Such a scenario has already been established by the numerous observations of outside-in signaling in which signals feed into a cell to regulate metabolism. A newer theme is that while the components of the normal extracellular matrix influence the synthesis, assembly and degradation of macromolecules by chondrocytes, the fragmented components of the damaged matrix alter this influence or feed back regulation and contribute to progression of damage. Although it is known that numerous anabolic and catabolic factors regulate chondrocyte metabolism, little is known of what initiates these pathways. Thus, it is intriguing to question whether the perturbation of the matrix itself, perhaps by matrix fragments, may initiate these pathways. A very important notion is that such a fragment system may be operative not only during damage, but also during normal metabolism and in either case, may shift metabolism in either direction, depending on the concentration of the fragments. Thus, matrix fragments may serve as amplifiers or catalysts for the current metabolic state. This review will focus on discussion of the effects of Fn-fs on cartilage metabolism, the most characterized fragment system.

3. DISCUSSION

3.1. Fn and Fn-fs are elevated in synovial fluids and cartilage in RA and OA

In order to understand how the degradation of the matrix plays a role in regulation of cartilage homeostasis, it is important to recognize that fragments can arise not only from enhanced levels of proteinases that can act on existing pools of precursors, but can also arise from normal levels of proteinases acting on enhanced levels of precursors. Thus, it is important to understand conditions that enhance levels of Fn might also enhance levels of Fn-fs. The role of Fn in cartilage tissue has been reviewed recently (1). The following discussion will focus on the potential causes of enhanced Fn levels in OA.

While some proteins decrease in OA, the precursor of Fn-fs, native Fn, is elevated in the cartilage matrix in human OA cartilage (2-9) with the greatest increase near the articular surface (10-12) or close to eburnated areas (13). Enhanced cartilage Fn levels are also observed in canine (10,14) and rabbit (10) *in vivo* models of OA damage. The increase in Fn levels appears to be greater in the deep than in the superficial cells of human OA cartilage (15). This increase in Fn may be due to both enhanced synthesis and enhanced retention of Fn as shown by Burton-Wurster and Lust (16). This work showed a 3-fold increase in Fn synthesis relative to protein synthesis in canine OA cartilage as well as an increase in the proportion of Fn retained by OA as opposed to normal cartilage

matrix. The increase of Fn within the cartilage matrix is associated with an increase in Fn in synovial fluids of patients with OA and rheumatoid arthritis (RA) (17). The increase may be as much as 3 to 4 fold, with the average concentration of Fn in healthy donor synovial fluids increasing from 171 $\mu\text{g/ml}$ to 721 and 568, in RA and OA synovial fluids, respectively (18). With such high levels of Fn, fragmentation to significant levels is greatly facilitated.

While native Fn can be expressed in various isoforms, due to differential splicing of the precursor mRNA, the population synthesized within cartilage tissue is significantly different than in other tissues and includes relatively high levels of an ED-b(+) form and of a cartilage specific form, (V+C)- which lacks several amino acid sequences found in the isoforms of other tissues, as reviewed (1). The ED-b(+) isoform increases throughout the cartilage matrix in a canine model of OA (19). The mRNA for this Fn isoform is also increased in human OA cartilage (20). In contrast, the isoform found in synovial fluid and likely made by synovial tissue *in vivo* is the ED-a(+) isoform (21) which is present in OA cartilage, but made only at low levels (20).

The cause of the enhanced Fn synthesis in either cartilage or synovial tissue in OA, may be at least partially due to TGF-beta, which has been shown to enhance Fn synthesis in cartilage (22). IGF-1 may also play a role, since IGF-1 enhances Fn synthesis in many cell types, including, but not limited to, preadipose cells (23), rat thoracic aortic smooth muscle cells, glomerular mesangial cells (24) and rat thoracic aortic adventitial fibroblasts (25). Catabolic cytokines are not likely responsible for the enhanced Fn levels. Recent work has shown that IL-1-beta cannot stimulate Fn synthesis in superficial or deep cartilage explants (15). It is interesting to speculate that the effect of the anabolic factors on enhancing Fn synthesis might ultimately lead to higher levels of Fn-fs, which would induce our observed Fn-f mediated chondrocytic chondrolysis. Such a linkage may be a component of "attempted but failing repair" in damaged cartilage.

Biomechanical forces may also influence Fn synthesis. For example, Fn synthesis is increased in canine articular cartilage explants after cyclic impact (26) and in bovine articular cartilage explants after intermittent cyclic loading (27). Compressive loading and unloading has also been shown to affect Fn synthesis (28). It is conceivable that enhanced Fn synthesis may also occur through the effects of altered biomechanical forces on activation and liberation of growth factors stored in cartilage tissue.

With the increases in Fn levels in cartilage and synovial fluids in RA and OA, it would be expected that Fn-f levels would also increase. Griffiths *et al* (29) found Fn-fs which ranged from 200-kDa to as low as about 24-kDa in RA, OA, traumatic arthritis and septic arthritis synovial fluids. These Fn-fs represented a major portion of the total Fn in most cases. Fn-fs in RA synovial fluids have also been confirmed by others (30). We reported Fn-fs in OA synovial fluids, which we estimated to be up to μM

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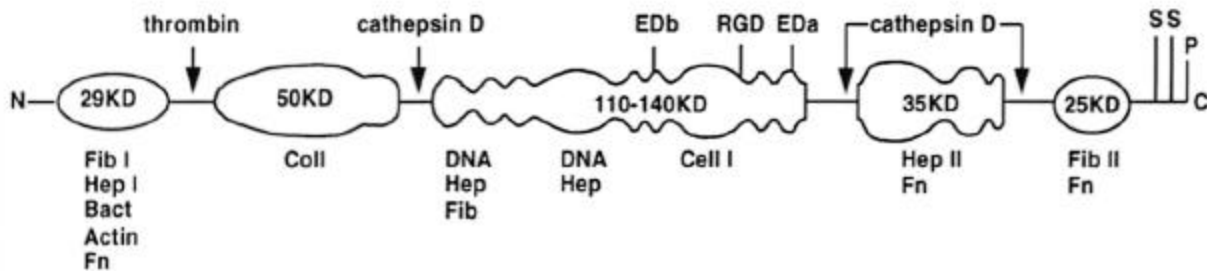


Figure 1. Schematic of Fn domain structure – The Fn dimer consists of two subunits each with separate domains connected by less ordered segments that are easily cleaved by various proteinases. The types of ligands each domain can bind are shown. Note the cleavage sites of the proteinases used in our Fn-f isolations. Our studies focus on tests of the N-terminal 29-kDa segment which is liberated by thrombin. The 50-kDa, cathepsin and thrombin generated Fn-f is less active, while the 110-140-kDa Fn-f family has the least activity. The 35-kDa and 25-kDa segments are not fully characterized by us in terms of cartilage damage.

concentrations, and which ranged from less than 200-kDa down to 30-kDa (31). It is important to emphasize here that Fn-fs have been reported in other pathological body fluids and tissues as well and may also play roles in damage/repair throughout the body (32-39).

Theoretically, the enhanced levels of Fn-fs in OA synovial fluids could be due to proteinases from cartilage or synovial fluids acting on Fn derived from cartilage or synovial fluids. Recent work has shown that the Fn isoform in canine OA synovial fluids is the synovial tissue isoform (21) and different than the cartilage isoforms (19). However, it is possible that there is at least a minor contribution from cartilage-derived Fn-fs in OA synovial fluid since cartilage, if moderately degraded, could theoretically contribute the same mass of Fn-fs as found in OA synovial fluid as discussed by us (40). Furthermore, we have shown that IL-1 treated cartilage explants do release elevated levels of Fn-fs into the culture media (40) and we would expect that in OA, cytokines would also enhance release of Fn-fs from cartilage into the synovial fluid. Nonetheless, regardless of the individual contributions of cartilage and synovial tissue to the Fn-f pool, either pool has the potential to cause cartilage damage. In support of this concept, we have shown that OA synovial fluids contain Fn-fs that can enter cultured cartilage explants *in vitro* and cause severe depletion of PG from the cultured cartilage (40).

3.2 Fn-fs have activities not expressed in native Fn

The presence of high levels of Fn-fs in OA and RA synovial fluids would have been predicted to have some effect on cartilage metabolism, based on observations that Fn-fs often have cryptic properties not shared by native Fn and are often involved in regulation of proteinase levels. For example, Fn-fs enhance elastase release from monocytes (41), enhance protease release from neutrophils (42) and enhance MMPs in synovial fibroblast cultures (43). There is a continually growing list of other activities of Fn-fs not expressed in native Fn. The following references are not comprehensive but rather a short list that illustrates just a few of the various activities Fn-fs express in various types of cells and tissues (33,44-54).

3.3. Fn-fs enhance loss of PG from cartilage explants

We proposed in 1991 (55), that since Fn-fs had been shown to regulate numerous cellular activities of various types of cells, they might affect cartilage metabolism. We discovered very potent effects as described throughout this review. We first investigated the effects of Fn-fs on the kinetics of release of degraded PG into the culture media of 18 month-old bovine metacarpophalangeal cartilage cultured under serum-free conditions. We found that amino-terminal 29-kDa, gelatin-binding 50-kDa and cell-binding 140-kDa Fn-fs were very potent at 0.1 to 1 μ M concentrations (56). The 29-kDa Fn-f was the most active, followed by the 50-kDa and 140-kDa Fn-fs. Figure 1 shows the location of these Fn-fs within the Fn subunit. Subsequently, we found that the Fn-fs penetrate cartilage and surround chondrocytes (57) to cause the release of half of the total PG within a few days when cartilage is cultured in the presence of 100 nM or 1 μ M Fn-fs (56). These concentrations of Fn-fs are at or below measured concentrations in OA synovial fluid (31), consistent with their potential physiologic role. The Fn-fs do not cause significantly increased collagen degradation, nor cause cell death, based on measurement of tissue DNA content (58). This matrix-depleting activity of the Fn-fs is also present in an RGDS synthetic peptide, corresponding to the integrin receptor-binding sequence of Fn, consistent with involvement of a Fn receptor. However, native Fn has only weak matrix-depleting activity, suggesting that this is an activity that might be expressed *in vivo* only in degraded Fn (56). The relative inactivity of native Fn has been confirmed in tests of most of the parameters affected by the Fn-fs. The damaging activities of the Fn-fs require mRNA, as well as *de novo* protein synthesis and metabolic energy, suggesting that the Fn-fs are not acting as proteinases (56). Not only do the Fn-fs enhance proteinase activity, but also they temporarily suppress PG and general protein synthesis, by up to 50% (58,59). However, the effects of the Fn-fs on 18 month-old bovine metacarpophalangeal cartilage are not totally reversible *in vitro*. Upon removal of the Fn-fs from cartilage cultures, the PG synthesis rates increase to values up to 140% of control values. The PG content, however, does not return to normal levels in 18-month old bovine cartilage (58,59). The reversibility has not yet been tested in younger bovine cartilage, but as will be discussed later,

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adolescent rabbit cartilage does recover from Fn-f mediated matrix damage, while mature rabbit cartilage does not.

Most of our work has focused on use of a 29-kDa amino-terminal Fn-f, which is the most biologically potent of the Fn-fs (56). Two other Fn-fs, a gelatin-binding 50-kDa and the cell-binding 140-kDa Fn-f, are slightly less active. The 140-kDa Fn-f is not homogenous but often a mixture of 110 to 140-kDa Fn-fs, due to differential trimming at the carboxyl-terminus. Thus far, all three Fn-fs have been shown to have similar qualitative effects. Therefore, throughout this review the term Fn-f will be used generically. The typical Fn-fs studied by us are generated by sequential cathepsin D and thrombin digestion of human plasma Fn (56,60,61) and are active on bovine (56), human knee (62,65), rabbit and rat cartilage explant cultures (unpublished). Similar cathepsin D and thrombin generated Fn-fs from rabbit plasma Fn, bovine plasma Fn, rat plasma Fn or guinea pig plasma Fn are equally active on bovine cartilage (unpublished). Thus, in general, Fn-fs from one species can cause damage to cartilage of another species. The most potent amino-terminal Fn-fs are not contained within the region of differences between the Fn isoforms discussed earlier. Thus, the Fn-f cartilage chondrolytic activities should be isoform-independent for the amino-terminal Fn-fs and amino-terminal Fn-fs from any type of isoform should be active.

3.4. Fn-fs enhance levels of proteinases

The effects of Fn-fs on induction of proteinases have not been completely characterized, although the up-regulation of several matrix metalloproteinases (MMPs) has been confirmed by gelatin and casein zymography in both human and bovine articular cartilage explants. By these methods, collagenolytic (MMP-1), gelatinolytic (MMP-2, MMP-9) and stromelysin-1 (MMP-3)-like activities are increased by at least several-fold (56,63,64). Up-regulation of protein levels of MMP-3 has been confirmed in both bovine (63,64) and human knee explants (65). At least two MMPs are up-regulated at the mRNA level in a concentration-dependent fashion, MMP-3 and MMP-9 (64). Similar studies on the other MMPs have not yet been performed. Current *in situ* hybridization analysis has confirmed up-regulation of MMP-3 (unpublished). We predict based on our ongoing studies, that most, if not all, of the common MMPs are up-regulated at the mRNA level. Conversely, the Fn-fs have little effect on mRNA levels of tissue inhibitor of matrix metalloproteinases (TIMP-1) (64), a potential regulator of MMP activities in this system.

One interesting observation is that in monolayer chondrocyte cultures nearly all of the MMP-3 protein in the "conditioned" medium is in the zymogen form, while in cartilage explant "conditioned" medium, mostly active MMP-3 is found (64). Thus, either the monolayer cultures do not fully express proteins needed for activation or the cartilage matrix plays a role in activation. The effect of the Fn-fs on proMMP-3 protein levels in monolayer cultures is so significant that 1 μ M concentrations of pro-MMP-3 are found in "conditioned" medium within a few days and pro-MMP-3 is one of the major proteins in the medium (64). MMP-3 appears to have a major role in Fn-f mediated

cartilage matrix damage since antibodies to MMP-3 slow damage to Fn-f treated bovine cartilage explants (63). Because of the importance of MMP-3, we typically measure MMP-3 as a correlate of cartilage matrix damage since we have never observed enhanced degradation of PG and release of PG fragments into the culture medium without up-regulation of MMP-3. However, when PG synthesis is up-regulated by e.g. IGF-1, we still observe both enhanced MMP-3 and enhanced PG degradation, but we do not see a reduced cartilage PG content because of the enhanced PG synthesis.

Although MMP-3 appears to be important, the physiologic site of cleavage of aggregating PG (aggrecan) found in matrix turnover of normal or IL-1 treated cartilage (66, as discussed; references therein) is not the MMP-3 site at Asn³⁶⁰-Phe³⁶¹. Instead, the cleavage site is at residues Glu³⁹²-Ala³⁹³ and is termed the "aggrecanase" site. It should be noted that there is no published work on the identity of "aggrecanase"; it is only an activity based on this observed cleavage site of aggrecan in normal and IL-1 treated cartilage (discussed in ref 66). This activity has not yet been attributed to a specific protein. Nonetheless, this "aggrecanase" type cleavage is found in degraded aggrecan fragments in the "conditioned" medium of Fn-f treated cartilage (66) and the NITEGE³⁹² neoepitope, an epitope on the amino-terminal side of the "aggrecanase" site, containing residues 392 and lower (62), is found in immunostained sections of Fn-f treated cartilage. We have no evidence for MMP-3 mediated aggrecan degradation. This is partly because the "aggrecanase" site is carboxyl-terminal to the MMP-3 site. Thus, it is much more difficult to obtain amino-terminal sequencing evidence for MMP-3 induced cleavage if there is an "aggrecanase" activity present that might act on MMP-3 degraded aggrecan. Our observation of the NITEGE neoepitope in Fn-f treated cartilage could erroneously suggest that there cannot be MMP-3 cleavage because if both MMP-3 and "aggrecanase" acted simultaneously, a 32-residue peptide connecting these two cleavage sites would be generated. Thus, if this peptide diffused out of the cartilage, we would not observe the NITEGE neoepitope. However, we do not yet know if this peptide is generated or whether it escapes the tissue or whether the NITEGE neoepitope in the tissue is lost with time. It is also possible that MMP-3 does not act on aggrecan at all, but performs crucial cleavages on other matrix molecules such as link protein, which might be required for release of "aggrecanase" degraded PG into the medium. Further work will be needed to explore these and other plausible mechanisms. However, it should be noted that the general literature on characterization of the crucial enzymes in normal, OA or cytokine mediated cartilage degradation is not yet unambiguous and that the existence of "aggrecanase" as a new or unique chondrocyte-derived proteinase has not yet been established.

Regardless of the role of MMP-3 in the Fn-f system, we can propose that since MMP-3 can also degrade intact Fn into small Fn-fs (40,67,68), MMP-3 activity found in OA or RA cartilage or synovial fluids may generate Fn-fs, which then amplify MMP-3 expression in a feedback loop. The same might be true of other MMPs.

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Thus, Fn-fs would amplify generation of even higher levels of Fn-fs.

In order to rule out the possibility that the proteolytic activities observed in Fn-f-treated cultures were not due to contamination with other factors or with proteinases, we showed that the activities of the Fn-fs on cartilage tissue were not due to endotoxin. We also showed that the effects required *de novo* mRNA and protein synthesis (56). Furthermore, our Fn-f preparations did not contain detectable levels of IL-1, IL-6 or TNF-alpha (69) or of several different types of proteolytic activities toward synthetic substrates nor activities on zymogram gels (63) and did not cause PG loss from non-living cartilage (59). Synthetic tetrapeptides derived from the 140-kDa Fn-f and certainly devoid of any potential proteinase activity, also enhanced PG depletion from cultured cartilage (56).

It has been reported that at high concentrations of at least 1 μ M, Fn-fs generated by autolytic processing under various conditions have proteolytic activity (70-74). Currently, we study the effects of 1 to 100 nM concentrations of Fn-fs on explants in serum cultures. Innate proteolytic activities at these concentrations would not make significant contributions to our greatly up-regulated MMP activities. These observations of Fn-f proteinase activity might be related to an earlier observation that some, but not all, preparations of Fn contain a plasma gelatinase that can be activated with trypsin or by electrophoresis in SDS (75). We have not detected gelatinases in our Fn-f preparations (63) and, in fact, the gelatinase activities induced by our Fn-f preparations require metabolic energy and mRNA synthesis (56). Furthermore, some of the investigators who reported innate proteolytic activity of Fn-fs (76) have confirmed our observation that the 29-kDa amino-terminal Fn-f, the most active Fn-f in our system, is not very effective at 1 μ M in degrading and releasing PG from non-living cartilage even in serum-free conditions. The same study showed that an auto-activated 42-kDa gelatin-binding Fn-f at 1 μ M was active on non-living cartilage. In contrast, 100 nM concentrations of our counterpart gelatin-binding 50-kDa Fn-f, which is not auto-activated, have been shown to induce MMP-3 protein (63). Thus, while the gelatin-binding Fn-fs activated under certain conditions may have some proteolytic activity, our Fn-fs do not have protease activity at the lower concentrations studied by us and at those concentrations induce MMPs.

3.5. High concentrations of Fn-fs enhance release of catabolic cytokines and decrease cartilage PG content, while low concentrations enhance PG synthesis and PG content

In our initial studies, the kinetics of release of degraded PG from explant cartilage into the culture media was used to measure kinetics of cartilage matrix degradation (56). However, this limited our studies to less than one week due to the extensive matrix damage. In more recent studies, in order to study longer time periods, explant culturing has been performed in 10% serum/DMEM. The serum slows the rate of PG degradation by several-fold and allows an anabolic

response of the cartilage to the damage. Since the kinetics are slowed, it is more difficult to accurately measure PG release into the media. Thus, the cartilage PG content is now measured which does not provide information on rates of degradation but rather on the combined effects of altered anabolism and catabolism. By looking at steady-state metabolism, we discovered a very interesting dose-response effect of the Fn-fs. A 1 μ M concentration caused a 50% decrease in PG content within a few days, followed by a very steady but reduced PG content, as shown in Figure 2A (59). This latter part of the PG content curve was often flatter than in the control, suggesting an anabolic response to the matrix damage. In contrast, in serum-free cultures the PG content decreased by up to 80% within a few days (56). During this early catabolic phase, MMP-3 release into the media plateaued (65) as shown in Figure 2B. A 100 nM concentration caused a slightly slower rate of PG decrease than 1 μ M, and this was also followed by a stable but decreased PG content beyond a few days. However, at 10 nM there was about a 7-day lag period with PG content often higher than controls, followed by a 50% decrease of PG content by day 21. A 1 nM concentration had a surprising effect; the PG content immediately increased from 120 to 150% of control levels and was maintained for up to 21 days in culture. More recent data shows that 1 nM causes a decreased PG content beyond day 35, suggesting that as the Fn-f concentration is lowered the length of the lag period increases, but eventually the same degree of matrix damage occurs eventually. We have no explanation for this observation but it may suggest that as the Fn-fs bind cartilage they slowly concentrate until a minimal tissue concentration is reached which then begins the damaging cascade. Thus, dilute Fn concentrations will require a longer time period for this concentration to be attained.

The early catabolic phase of damage is also associated with enhanced release of catabolic cytokines as described (65,69). Fn-fs added to human knee cartilage explants caused a peak of release of TNF-alpha and IL-1-beta that decreased after a few days as shown in Figure 2C. IL-6 release occurred earlier but continued throughout a 21 day culture while IL-1-alpha release showed a lag period before release began but continued for most of the culture. As shown in Figure 2B, the peak of MMP-3 release occurred with the onset of TNF-alpha and IL-1-beta release and declined as IL-1 α release declined. The cytokines apparently account for the bulk of the protease induction and the temporary PG synthesis suppression since antibodies to these cytokines, when added to human chondrocyte cultures, partially or totally block these two activities (69). The effects likely involve transcriptional gene regulation, since metabolic and RNA synthesis inhibitors suppress the release of these cytokines and these cytokines are not found in un-treated cartilage (69).

3.6. After the damage phase, PG synthesis increases to supernormal levels during a period of decreasing cytokine release and enhanced anabolic factor release

As stated earlier, the Fn-fs suppress PG and protein synthesis up to 50% (58,59). Thus, our observations of enhanced PG content discussed were at

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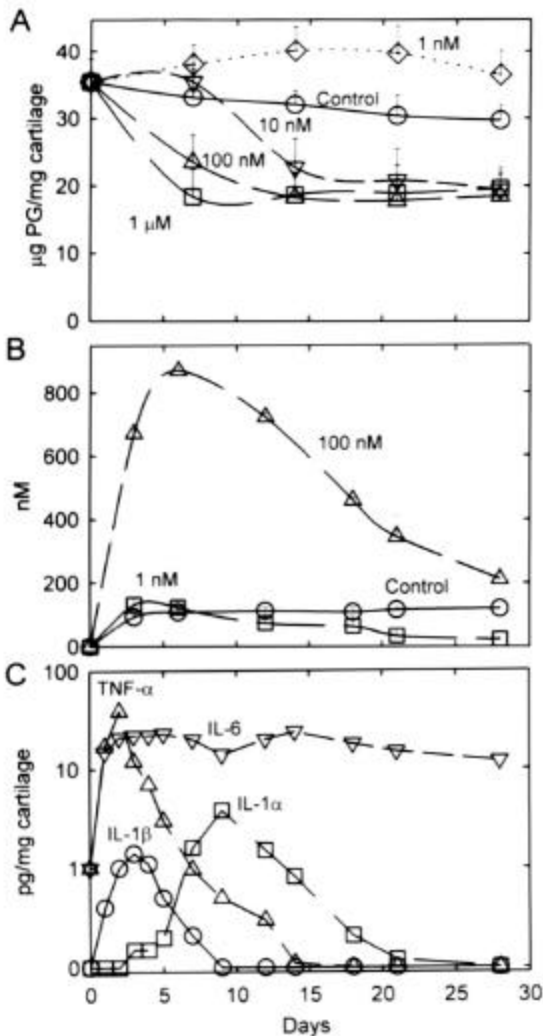


Figure 2. Effect of various concentrations of the 29-kDa Fn-f on PG content, MMP-3 release into the media and release of catabolic cytokines. Panel A; bovine articular cartilage was cultured in 10% fetal bovine serum/DMEM (Dulbecco's modified Eagle's medium) with 1, 10, 100 nM or 1 µM Fn-f or without Fn-f (Control). PG content of cartilage was determined by the DMB assay. Similar kinetics are observed for human knee cartilage with 1 and 100 nM Fn-f. Note the lag period for PG depletion which becomes longer as the Fn-fs are diluted. Also note that the overall extent of PG depletion is similar at the higher concentrations but that 1 nM Fn-f immediately enhances PG content. Panel B; conditioned medium of human knee cartilage was assayed for MMP-3 release. Note that the peak occurs during the period of maximal loss of PG shown in panel A. Panel C; conditioned media of human knee cartilage was assayed for the cytokines shown. The control levels, with no Fn-f addition, are not shown but were typically less than 5% of the peak. Note that only IL-6 does not peak and subside during the period of maximal MMP-3 expression.

odds with the effects on PG synthesis. In order to reconcile these data, we considered that the effect on PG synthesis may have been only temporary. Upon further investigation,

we found that the very slow decrease in PG content after the damage phase and the above normal PG contents found with 1 nM Fn-f treatment occurred with enhanced PG synthesis. Thus, while high concentrations initially suppress PG and protein synthesis, the rates slowly increase to "supernormal" levels. The time period required for the increase to "supernormal" levels was inversely related to the Fn-f concentration as shown in Figure 3A. The greater the Fn-f mediated early matrix damage, the slower the increase in anabolism. This inverse relationship is consistent with what would be expected when positive and negative or anabolic and catabolic effects are summed. While the increase to "superanabolic" levels is very significant at 1 nM Fn-f, we have found that the rates of synthesis and PG content begins to decrease by day 35, suggesting that this "superanabolic" response may precede matrix damage. As discussed earlier, 1 nM Fn-f appears to be "superanabolic" up to day 28 in culture. However, this dilute concentration may simply require a longer period of time before a critical level accumulates within the matrix and initiates damage. The data in Figures 2 and 3 do suggest a loss in elevated activities by day 28 and more recent data show a catabolic effect by day 35.

In order to explain why PG synthesis rates increase to over 100% of control and not merely to control levels in Fn-f treated cartilage, we have tested the possibility that Fn-fs also activate growth factors. The effect on TGF-beta, which stabilizes the chondrocyte phenotype (77,78) and blocks the effects of IL-1 on chondrocytes (78,79) has been tested. IGF-1, which has been shown to be the synovial fluid component (80) and the serum component (81) responsible for maintaining PG synthesis rates and which can antagonize the effects of IL-1 and TNF-alpha (82) has also been tested for the possibility that its release is enhanced by Fn-fs. As shown in Figure 3B,C, the IGF-1 and TGF-beta levels in the media of Fn-f treated cartilage, are elevated over control levels by statistically significant amounts in cartilage exposed to either lower or higher concentrations of Fn-fs (69). The lack of strict dose dependence suggests that the effect may not be transcriptional. In fact, metabolic inhibitors have no effect on release of these factors (69), suggesting that they might be released from cartilage stores. Also, up-regulation in chondrocyte cultures, devoid of matrix, was not detected. Thus, the Fn-fs do not up-regulate synthesis of these factors, but somehow enhance their release and activation.

The reason the Fn-fs do not cause a further decrease in steady-state PG content beyond about one week in culture is that the release of catabolic cytokines has greatly decreased by then and the effects of the anabolic factors have become predominant. Since explant cultures with the Fn-fs continuously present show a pulse-like release of catabolic cytokines, especially in terms of TNF-alpha and IL-1-beta (Figure 2) and not continuously elevated release, we investigated whether this cytokine pulse could be initiated with only short-term cultures. We found that a 7-day exposure to the Fn-fs was sufficient to cause as much cartilage PG depletion, MMP-3 release and enhanced release of TNF-alpha, IL-1 and IL-6 as a continual exposure for up to 28 days (83). This suggests

Effect of Fibronectin Fragments on Cartilage

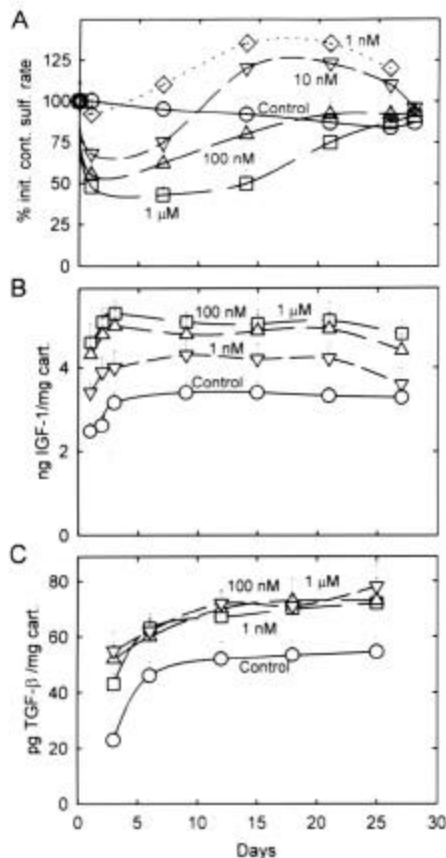


Figure 3. Effect of Fn-fs on PG synthesis and on IGF-1 and TGF-beta release into media of explants of bovine articular cartilage – Panel A, bovine articular cartilage in 10% serum/DMEM was cultured with various concentrations of 29-kDa Fn-f and at various times, incubated in the presence of ^{35}S -sulfate labeling. Values were normalized to % of the initial specific activity at day 0. Note that as the Fn-f concentration is increased, PG synthesis suppression increases during the first two days. However, the synthesis rates slowly increase subsequently. The rate of this increase becomes greater as the Fn-f is diluted. The period of supernormal PG synthesis corresponds to the period of very stable PG content beyond 7 days shown in Figure 2A, which is higher in PG content than in control cartilage. Thus, the pattern of PG synthesis is consistent with the pattern of PG content. Also, note that 1 nM Fn-f causes an immediate increase in PG synthesis, consistent with the immediate increase in PG content shown in Figure 2A. Panel B, human knee cartilage was cultured with 10% serum/DMEM with various concentrations of Fn-f and media analyzed for IGF-1 content using the Nichol's Institute (San Juan Capistrano, CA), IGF-1 assay kit, a kit which includes an acid dissociation step to inactivate IGF-1 binding proteins and which can be used to measure total IGF-1. Similar values were observed for bovine cartilage. Panel C, media used for IGF-1 assays were assayed for TGF-beta content. Both curves suggest an effect that is not dependent on the concentration of the Fn-f. The effects on cytokine release (figure 2C) were concentration dependent.

that the Fn-fs can set into motion a damage cascade with effects that linger long after the Fn-fs are removed from the culture. This may also partly explain why restoration of PG in adult bovine cartilage is not possible (58,59), since the damage cascade is continuing after Fn-fs are removed to allow repair. However, as will be discussed later, restoration of PG is possible, in spite of the damage cascade, under certain conditions in which catabolism is blocked or anabolism enhanced.

3.7. The enhanced anabolic reparative phase triggered by the damage makes cartilage more refractory to subsequent damage

The collective data on cytokine and growth factor release, on PG synthesis and on MMP-3 levels, allow us to describe an interesting scenario, likely to be physiologically relevant. At low concentrations of Fn-fs, only IGF-1 and TGF-beta are released and this apparently causes an enhancement of PG synthesis to above control rates (69). However, as the Fn-f concentration is raised, as might occur in severe OA, the additional release of catabolic cytokines occurs, leading to a masking of the initial enhanced PG synthesis rates with an ultimate suppression of PG synthesis and induction of MMPs. Subsequently, the release of TNF-alpha and IL-1-beta slows, while IL-1-alpha and IL-6 release continues. This apparently allows a slowing of the catabolic processes and allows the anabolic effects of enhanced IGF-1 and TGF-beta release to again predominate. Thus, damage induces reparative responses. This dose dependent effect on PG synthesis can be compared to early OA where PG synthesis is elevated (84; and references therein), as with 1 nM Fn-f, and to severe OA, where PG synthesis suppression continues (84,85), as with higher Fn-f concentrations.

Based on the observations of this "superanabolic" effect of the Fn-fs, we have proposed that low concentrations or short exposures to Fn-fs might help to condition cartilage in a beneficial fashion and that such effects might occur in minimally damaged cartilage *in vivo* (59). To test this possibility, we cultured cartilage with 1 nM Fn-f for 1 week, which slightly increases the cartilage PG content and enhances PG synthesis, and then subjected the cartilage to 100 nM Fn-f. This pre-conditioning significantly slowed the onset of PG depletion as shown in Figure 4A (59). Thus, continuous exposure to low concentrations of Fn-fs may be beneficial over a limited time period. In separate experiments, the effect of exogenous IGF-1 was found to be similar to that of 1 nM Fn-f, consistent with a role for Fn-fs in mobilizing growth factors. In other experiments, the effect of a short exposure to a higher Fn-f concentration, followed by a short recovery period, was tested. This might be analogous to a more traumatic type of cartilage injury *in vivo*. Cartilage was exposed to 100 nM Fn-f for 4 days, a period that does not induce maximal catabolic effects, followed by a 4-day recovery, and then a second exposure to 100 nM Fn-f (83). As shown in Figure 4B, this type of pre-treatment also made the cartilage more refractory to subsequent cartilage PG depletion. Our results collectively show that if the damage period or Fn-f concentration is minimal, the anabolic effect can be triggered to limit damage, making

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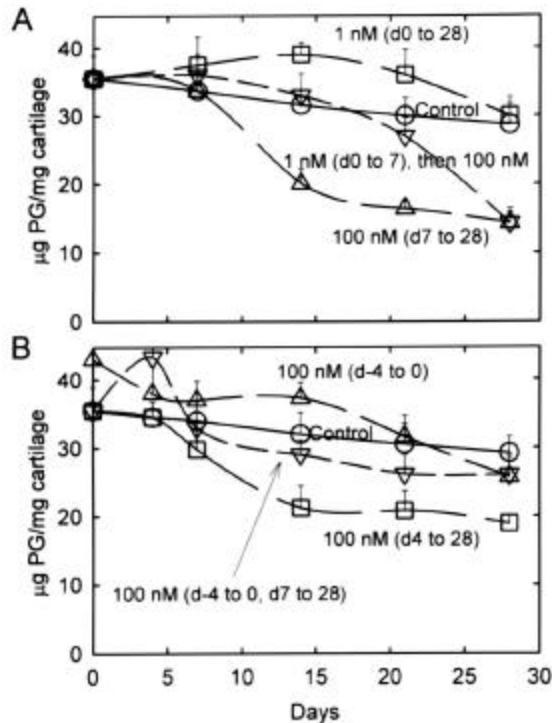


Figure 4. Effect of Fn-fs on conditioning of cartilage – Panel A, bovine articular cartilage in 10% serum/DMEM was cultured with no addition (Control), or with 1 nM 29-kDa Fn-f for days 0-28, or with 1 nM Fn-f for days 0-7, followed by adjustment to 100 nM Fn-f for days 7-28; or with 100 nM Fn-f for days 7 to 28. Cartilage PG content was measured by the DMB assay. Panel B, bovine articular cartilage was cultured in 10% serum/DMEM with no addition (Control), or with 100 nM Fn-f for 4 days before the start of the experiment (d-4 to 0) or with 100 nM Fn-f for days 4 to 28, or with 100 nM Fn-f for days -4 to 0 and days 4 to 28. Note that conditioning with low concentrations of Fn-f for a long period or with high concentrations of Fn-f for a short period, conditioned the cartilage against further damage.

cartilage more refractory to subsequent damage. These data are consistent with a role for moderate tissue damage in normal tissue homeostasis.

We have no evidence for the cause of this "superanabolic" response described above and utilized in our attempts to "condition" cartilage. However, one possibility is that the "conditioning" has led to up-regulation of MMPs to a level sufficient for activation of growth factors. The level required for activation may be much lower than that required for matrix damage. Thus, 1 nM Fn-f treated cartilage may show "superanabolism" without matrix damage. It is known that proteolysis activates TGF-beta (86) that is known to be stored in the extracellular matrix, as well as IGF-1, that is known to be trapped within the cartilage matrix (82,87). In fact, proteinases have been shown to release TGF-beta from the extracellular matrix in other types of cells (88) and proteinases, including MMP-3, can degrade IGF-1 binding

proteins (IGFBPs) (89) that can trap and inactivate IGF-1 within the cartilage matrix. In support of the role of MMPs, we have shown that addition of MMP-3 to cultured cartilage results in the initial suppression of PG synthesis, elevated IGF-1 in the media and, after a few days, enhanced PG synthesis (unpublished), just as do the high concentrations of Fn-fs. We have also shown that Fn-f treatment of chondrocytes enhances release of IGF-1 and IGFBPs from around the chondrocyte matrix (Purple and Homandberg, submitted). While we have no evidence of a role for protease-induced activation of IGF-1 in the IGF system, we have found that one of the IGFBPs, BP4, decreases in concentration in the presence of the Fn-fs. Since there is no effect on mRNA levels for this protein, it is likely that this protein is degraded during Fn-f treatment (90). The observation that proteinases can enhance PG synthesis was first made in 1984 (91,92). Thus, it is very conceivable that proteases induced during the damage phase might account for a linkage between cartilage damage and attempted repair responses.

Other work with the Fn-f system has suggested an additional feature that may also contribute to the "superanabolic" effect. Fn-fs up-regulate expression of some, but not all IGFBPs (90). We propose that as the newly synthesized IGFBPs are released from chondrocytes, they bind IGF-1 and concentrate it around the cells. Thus, during Fn-f induced proteolysis, the active IGF-1 concentration around the cell may be greatly elevated, leading to enhanced PG synthesis. This interesting mechanism is currently under investigation.

3.8. Decreasing catabolism or increasing anabolism blocks the Fn-fs and promotes restoration of PG in Fn-f damaged cartilage

Our earlier studies showed that after treatment of 18 month-old bovine metacarpophalangeal cartilage with Fn-f to cause removal of approximately half of the total cartilage PG, continuous culturing in 10% serum/medium did not allow restoration of PG (59). Therefore, we tested whether agents that either decrease catabolism or enhance anabolism might be effective in shifting the homeostasis sufficiently toward anabolism to restore the PG content. The agents tested were directed toward different points in the pathway for Fn-f mediated activities. A synthetic peptide, RFDS, which presumably blocks Fn receptor activity and Fn-f activity (93), an anti-oxidant, N-acetylcysteine (NAC), that is known to block cytokine pathways and which also blocks Fn-f activities (94,95), and a growth factor, IGF-1, which is known to antagonize cytokine action and enhance PG synthesis, were tested. All three agents partially or fully blocked the cartilage damaging activities of the Fn-fs. Subsequent studies showed that all three also were effective in enhancing restoration of PG (96). Thus, restoration of PG in Fn-f damaged adult bovine cartilage in explant cultures was possible with perturbation of metabolism. These observations also showed that in order to restore PG in cartilage no longer exposed to the Fn-fs, the catabolic events that initiated the PG loss and continued during the attempted repair had to be suppressed or compensated. This was consistent with our earlier observation that the

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damage events are prolonged long after the Fn-fs are removed from the culture. A further conclusion is that therapeutic agents that can inhibit cartilage degradation should have potential as reparative agents.

3.9. Could the enhanced anabolic response be involved in normal homeostasis?

Since Fn-fs at relatively low concentrations have "superanabolic" effects and Fn-fs would be generated during normal cartilage tissue turnover, it might be expected that they would have some role in normal homeostasis. While this is possible, this role cannot be confirmed until more is known of the basal levels of Fn-fs and the turnover rates of native Fn. However, it is possible that the Fn-fs play a role in moderate tissue damage, where their role may be in inducing anabolic processes for tissue remodeling. With more severe matrix damage and higher concentrations of Fn-fs, their role might shift to amplify the tissue damage.

We can speculate that the Fn-fs found in severe tissue damage might be derived not only from the damaged cartilage *per se* but also from Fn that would be induced as part of a repair response. For example, growth factors, such as TGF-beta or IGF-1 that might be released during a mild reparative or remodeling response, might up-regulate Fn levels in an uncontrolled fashion, which could lead to elevated Fn-f levels in the presence of normal baseline levels of proteinases. Thus, Fn-fs could increase dramatically after moderate tissue damage and eventually amplify the damage. As explained earlier, this damage would then initiate anabolic processes and thus, the Fn-fs would sequentially amplify both directions of catabolism and anabolism

3.10. Could the Fn-fs initiate early OA?

Another question is if the Fn-fs are involved in early stages of OA, from where would they originate? Fn-fs could arise from cartilage degradation in early stages of OA, however extensive matrix degradation would be required. On the other hand, Fn-fs could be generated by MMP-3 action on elevated synovial fluid Fn levels early in the disease. For example, it has recently been shown that in an experimental OA model of partial-meniscectomized rabbits, MMP-3 was first up-regulated in the synovium, followed later by up-regulation of MMP-3 in cartilage (97). This was suggested to indicate that the inflammation of synovium was the initiator in the cartilage degeneration. Based on these observations, we could propose that the synovial tissue MMP-3 generated in early OA, prior to an indication of cartilage matrix damage, might generate Fn-fs from synovial fluid Fn that would penetrate non-activated cartilage. In turn, this would lead to generation of cartilage Fn-fs, which would amplify the degradation. In support of this contention, we have shown that MMP-3 generated Fn-f mixtures derived from synovial fluid Fn are as active as those derived from plasma Fn (40). We have also shown that the Fn-fs found in OA synovial fluid are active in cartilage chondrolysis and account for part of the cartilage matrix damaging activities observed when the fluids are added to cultured cartilage (40). Lastly, we have shown that addition of MMP-3 to bovine synovial fluid leads to

generation of Fn-fs (40). Thus, a potential role for Fn-fs in early OA could originate from the initial involvement of synovial tissue. The additional generation of Fn-fs within the cartilage tissue would certainly additionally amplify ongoing cartilage damage.

3.11. Fn-fs damage cartilage in an *in vivo* model that shows biochemical similarities with the *in vitro* model. Both have been used as models of cartilage degradation

In collaboration with Dr. J. M. Williams, we have found that Fn-fs cause articular cartilage damage when injected into rabbit knee joints (98). We have continued to characterize this model and have shown that many of the biochemical events observed in explant cultures are also observed in this *in vivo* model. This has allowed us to couple both models in tests of therapeutic compounds and to use the *in vitro* model to test mechanisms that are likely at play in the more relevant *in vivo* model. In characterization of the *in vivo* model we found that injection of Fn-fs into rabbit knee joints caused loss of up to 50% of the knee cartilage PG within 7 days (98). More recent work shows that this degree of matrix damage occurred within 2 days of injection and that MMP-3 levels plateaued by 2 days. The Fn-fs also temporarily suppressed PG synthesis and exposed the NITEGE neopeptide of aggrecan, just as in the *in vitro* model (see above) (Homandberg and Williams, in preparation). PG synthesis, although initially suppressed, slowly increased to "supernormal" levels, also suggesting a "superanabolic" response. This anabolic response led to restoration of PG within 2 weeks in adolescent rabbits, while in skeletally mature rabbits, PG was not restored (99,100). Thus, this model has potential in studies of aging. One of the most striking observations was that the non-injected knee cartilage showed evidence of a systemic effect. Figure 5A shows that injection of Fn-fs into knee joints of adolescent rabbits led to a decrease in steady state PG content, followed by a recovery within 2 weeks. Figure 5B shows that PG synthesis rates were enhanced in the injected knee cartilage much as rates are enhanced after the PG depletion in cultured bovine cartilage explants. Note that the non-injected knee shows a significant increase in PG content by day 12. Thus, by some systemic mechanism, the damage has caused a systemic anabolic effect in the non-injected knee. If a higher concentration of Fn-fs is injected, the non-injected knee shows damage at nearly the same time as the injected knee. Thus, the higher concentration has altered the response in the non-injected knee from anabolic to catabolic, as observed when 1 nM and 100 nM Fn-f concentrations are compared *in vitro* (see above). When the Fn-fs were injected at weekly intervals, the catabolic systemic effect became more obvious. For example, cartilage from the non-injected knee showed a loss of 40% of its PG, suppressed PG synthesis, elevated MMP-3 levels and elevated NITEGE neo-epitope, although about one week after these events occurred in the injected knee (Williams *et al*, in preparation). Since systemic effects are observed in human OA, this model may be useful for identification of the responsible biochemical factors.

The *in vitro* model has been used to compare the metabolism of human ankle and knee cartilages (62). Ankle

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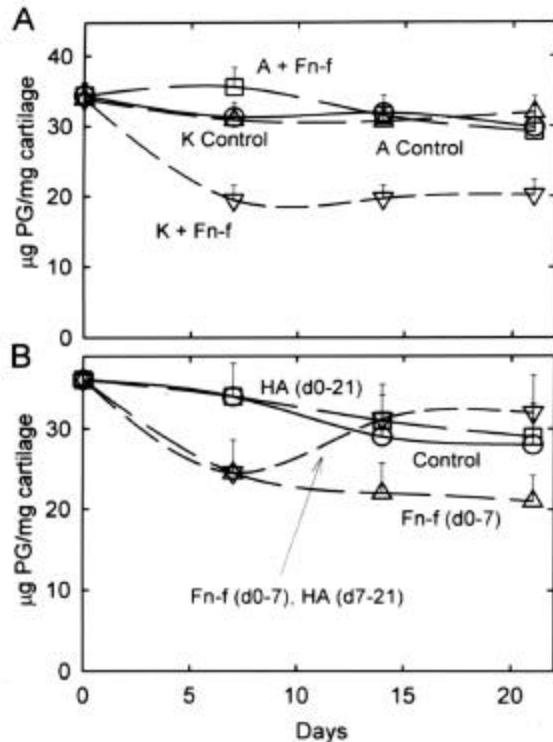


Figure 5. Effect of intra-articular injection of Fn-fs into rabbit knee joints – Panel A; adolescent rabbits were intra-articularly injected with 250 µl of 29/50-kDa mixtures and the injected and non-injected knee cartilage analyzed for PG content at various times. Panel B; knee cartilage from similarly treated rabbits was removed, transferred to explant cultures in DMEM and subjected to labeling with ³⁵S-sulfate to measure PG synthesis. Note that the injected knee shows first a loss of PG, followed by an increase in PG content to above normal levels, while the non-injected knee shows no initial PG depletion but rather a delayed effect of slightly enhanced PG content. The injection also temporally suppresses PG synthesis, which is followed by supernormal rates.

joints are much less susceptible to OA than knee joints according to numerous cadaveric, radiographic and clinical studies. In order to determine whether biochemical differences might account for differences in susceptibility to OA or to mediators of OA-like cartilage damage, the Fn-f model was employed. We found that addition of Fn-fs to cultured human knee cartilage decreased the PG content comparable to that of bovine cartilage. However, ankle cartilage in most cases was not affected as exemplified in **figure 6A**. With some donors, ankle and knee cartilage showed differences in MMP-3 and catabolic cytokine levels. Thus, ankle cartilage is an interesting exception to a generalization of the potency of Fn-fs and this suggests that we cannot assume that the Fn-fs will be active toward every type of cartilage. Nonetheless, the Fn-f system provides some suggestion that at least some of the differences in OA susceptibility between ankle and knee joints may be biochemical in nature and provides a model system for further studies.

Both the *in vitro* and *in vivo* Fn-f models have also been used to test the effects of high molecular weight hyaluronic acid (HA), a chondroprotective agent that has shown mixed clinical results as discussed (101,102). HA added to human knee (103) or bovine cartilage explants *in vitro* (101) partially blocked the effects of Fn-fs and partially promoted restoration of PG in Fn-f damaged cartilage. Figure 6B illustrates that HA has the ability to restore PG in Fn-f damaged human knee explants. HA has also been shown to be effective in decreasing the matrix damaging activities of Fn-fs injected into rabbit knee joints (102). Thus, the *in vivo* model has provided a more relevant test system, while the *in vitro* model will allow us to investigate the mechanism for this reparative activity of HA.

3.12. The Fn-fs may act through the $\alpha_5\beta_1$ RGDS-dependent Fn receptor

Preliminary evidence suggests that the $\alpha_5\beta_1$ RGDS-dependent Fn receptor integrin is at least partly responsible for Fn-f activities. This is the major Fn receptor in chondrocytes (104) and is involved in matrix interactions and proliferation of chondrocytes (105). Antibodies to this receptor as well as RGDS peptides block adhesion of chondrocytes to Fn (106). Interestingly, this receptor has been shown to be a chondrocyte mechanoreceptor (107) and a mechanotransducer in bone (108). The structure and biological activity of integrins has been reviewed (109).

This implication of the Fn receptor in the Fn-f mechanism is based on our observations that antibodies to $\alpha_5\beta_1$ Fn receptor cause both PG synthesis suppression (Purple and Homandberg, manuscript in preparation) and MMP-3 induction, as do the Fn-fs (Bewsey and Homandberg, unpublished). Also, RFDS and GRADSPK synthetic peptide analogs of the RGDS sequence block the action of the Fn-fs (93). The $\alpha_5\beta_1$ receptor has also been implicated in the up-regulation of MMPs in synovial fibroblasts (43) and in chondrocytes (110).

However, the available data do not rule out the involvement of other receptors. There is precedent for cooperative signaling between integrin receptors as reviewed (111). For example, ligation of the $\alpha_5\beta_1$ receptor is required for internalization of vitronectin by $\alpha_v\beta_3$ (112). Also, both the $\alpha_5\beta_1$ and $\alpha_4\beta_1$ receptors are required for Fn-f mediated MMP gene induction in fibroblasts (113). For more detailed information on Fn receptors, the reader is also referred to recent reviews (114,115) and a review in this journal, "Fibronectin-Integrin Interactions" by Johansson *et al* (116) (<http://bioscience.org/1997/v2/d/johansson1/htmls/126-146.htm>).

One of the puzzling characteristics of the Fn-f activities is that not only do the larger central Fn-fs have chondrolytic activities but also amino-terminal 29-kDa heparin-binding and 50-kDa gelatin binding Fn-fs (56). The latter two Fn-fs are not known to bind the Fn receptor.

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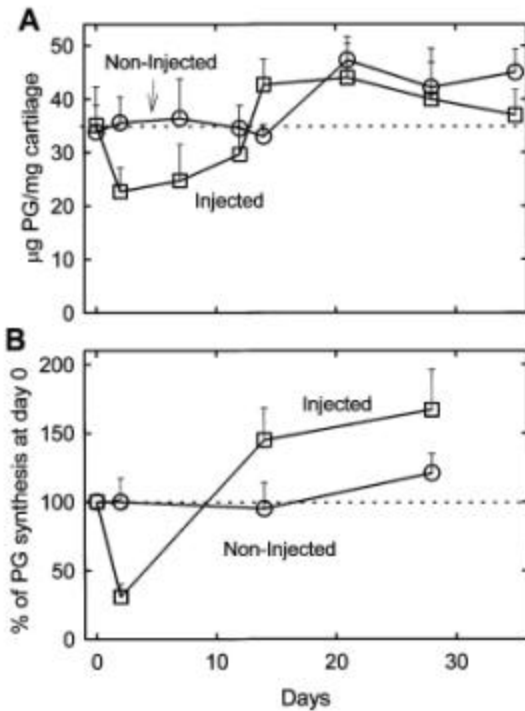


Figure 6. Effect of Fn-fs on human knee and ankle cartilage and use of model to test high molecular weight hyaluronic acid (HA) – Panel A, human knee and ankle cartilage from the same donor were cultured in 10% serum/DMEM with 100 nM Fn-f. Curves shown are knee cartilage with no addition (K Control), ankle cartilage with no addition (A Control) or K with Fn-f addition (K + Fn-f) or ankle cartilage with Fn-f addition (A + Fn-f). Note that ankle cartilage was unaffected by Fn-fs. This effect was confirmed with matched ankle and knee cartilages from at least 7 different donors. Panel B, human knee cartilage was cultured in 10% serum/DMEM with no addition (Control) or with adjustment of cultures to 1 mg/ml HA or with adjustment to 100 nM 29-kDa Fn-f from days 0-7 or with adjustment to Fn-f from days 0-7, followed by adjustment from days 7-21. Note that HA has very effectively restored PG in cartilage previously treated with the Fn-f.

However, more recent observations suggest that the amino-terminus of Fn is indirectly involved in receptor interaction. For example, amino-terminal Fn-fs and antibodies to them inhibit pericellular matrix assembly (117) and block interaction of receptor with receptor antibodies (118). Binding of cells to the amino-terminal 29-kDa segment is required for formation of a Fn matrix, the matrix is required for increased binding of this segment and this segment only binds when the $\alpha_5\beta_1$ integrin is present (119). Others have also shown that this amino-terminal segment helps mediate binding of Fn and receptor (120). Our own preliminary work shows that when the 29-kDa, 50-kDa and 140-kDa Fn-fs are added to chondrocyte monolayer cultures and the cells exposed to chemical crosslinkers, the α_5 subunit of the Fn receptor can be isolated in covalent linkage with each of the Fn-fs. This suggests that the Fn-fs bind at or near the Fn receptor (unpublished). We are

presently investigating whether other types of alpha subunits in cartilage, contained within other types of Fn receptors, become crosslinked to Fn-fs.

These observations fit into a model proposed by others in which the amino-terminal domains might bind Fn fibrils (120). In this model, the fibrils are initially formed through domain to domain interactions involving the interaction of the domains of the most potent chondrolytic Fn-f, the 29-kDa Fn-f, with domains of Fn having the type III homology structures found in the 140-kDa cell-binding Fn-f. We reported years ago that the 29-kDa amino-terminal Fn-f interacted with the 140-kDa Fn-f in polymerization reactions (60,121,122). Subsequently, others have confirmed the interactions of the amino-terminus with more central or C-terminal type III homology domains (123-125) and shown the requirement of these Fn domains for cell interaction (126).

In this model, as additional molecules of Fn bind the fibrils, the amino-terminal domains of each Fn molecule bind the fibrils, which induces a conformational change that allows interaction of the carboxyl-terminal regions of each Fn molecule with the Fn receptor. We hypothesize that each Fn-f might alter Fn/Fn receptor function by binding either soluble Fn prior to incorporation of Fn into fibrils or might bind fibrillar Fn and block incorporation of additional soluble Fn molecules. This would weaken and alter the structure of the fibrils and likely alter receptor signaling. Amino-terminal Fn-fs might bind only Fn fibrils or Fn molecules while the 140-kDa Fn-f with the RGDS receptor-binding domain might also bind directly to the receptor and block Fn assembly. Alternately, the Fn-fs may bind other matrix components near Fn fibrils or near the receptor and this also might weaken the fibrils. The Fn-fs might alter or weaken fibril structure because they lack a full complement of interaction sites for Fn fibrils and once they bind they may weaken or alter fibril growth. From this discussion, it might be predicted that mixtures of Fn-fs, representing the entire polypeptide might be inactive. However, we have not found this to be true, likely because the interaction would not be of high enough affinity to mimic native Fn. This model would certainly predict that native Fn would be inactive in Fn-f activities, which is consistent with observations made in both cartilage explants (56) and in chondrocyte monolayer cultures (64), the latter largely devoid of matrix that would block Fn interaction with chondrocytes. It is also possible that the Fn-fs may simply, because of their smaller size and more exposed structure, have different activities than native Fn and bind to non-receptor matrix components at sites Fn does not bind and indirectly perturb the Fn receptor.

We cannot, however, exclude the possibility that putative receptors for the amino-terminal Fn-fs exist on chondrocyte cell surfaces near the Fn receptor and that these are also involved in Fn fibril formation. For example, the amino-terminal 29-kDa Fn-f has been shown to bind a 67-kDa-membrane protein isolated from rat peritoneal macrophages (127) and a 66-kDa membrane protein from chick myoblasts (128). Furthermore, the 29-kDa Fn-f has been crosslinked to a cell membrane protein on fibroblasts

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to form a 150-kDa protein complex (129). Other candidates for Fn-f binding cell surface proteins have been reviewed (130).

3.13. Could the Fn-fs be altering Fn function by altering communication between the matrix and the Fn receptor?

As pointed out by Schmidt *et al* (131), the cytoskeleton may serve as a structure where mechanical signals can switch into chemical signaling pathways. It is known that integrins such as the Fn receptor act as a transmembrane link among the extracellular matrix, cytoskeletal proteins and actin microfilaments. This may allow ligands, such as Fn, to mediate attachment of the matrix to the cytoskeletal assembly, to regulate cell shape and internal cellular architecture (132) and to transmit signals to the cytosol. Schmidt *et al* (131) have also shown that integrins sense physical forces that control gene expression and consequently activate the mitogen-activated kinase (MAP) pathway. If this linkage between the integrins and ligands is perturbed, for example, by binding of Fn-fs to Fn fibrils or to Fn receptors, it is reasonable that altered signal transduction may occur. For example, when fibroblasts are incubated with RGDS peptides that bind the Fn receptor, the co-localization of the Fn receptors with extracellular Fn fibers is lost (133) and alpha-actinin and vinculin are progressively lost from focal contacts, followed by dissolution of focal contacts (134,135). When rabbit synovial fibroblasts are plated onto a surface containing a 120-kDa Fn-f, focal contacts are decreased and collagenase is induced. However, plating of cells onto surfaces coated with other Fn-fs that allow extensive focal contact formation does not greatly enhance collagenase (113). Furthermore, when Fn-fs are added to fibroblasts, the cytoskeleton reorganizes (136). Perhaps the strongest suggestion that the effect of Fn-fs may be associated with changes in the cytoskeleton comes from observations that cytochalasins B and D, which alter cytoskeletal structure, also induce collagenase and stromelysin (137) and enhance mRNA for c-fos (138), a transcription factor involved in up-regulation of MMPs.

We have hypothesized that the Fn-fs may alter communication between Fn and the Fn receptor(s) and reducing receptor occupancy by Fn could do this. This possibility is intriguing based on studies of other types of cells. For example, in the absence of attachment of HT29 colon carcinoma cells to Fn, integrin $\alpha_5\beta_1$ expression activates a pathway leading to decreased cellular proliferation while ligation of receptor with Fn reverses this signal (139). Furthermore, disruption of binding of Fn to the $\alpha_5\beta_1$ integrin stimulates cyclin dependent kinases and DNA synthesis and activation of MAP kinases (140). Reducing the receptor density may be another means of blocking Fn function. For example, when attachment of substrate to the $\alpha_5\beta_1$ integrins is blocked, the integrins become both internalized and degraded (141). Therefore, we could hypothesize that the Fn-fs destabilize Fn fibrils or block Fn interaction with the Fn receptor, which in turn, decreases receptor density, which leads to weakened focal contacts and activation or de-repression of catabolic pathways.

3.14. Other similar systems may exist and may provide redundancy

While the activities of the Fn-fs may play a role in cartilage matrix degradation, it is likely that other matrix component fragments share this property. It has recently been shown that collagen type II peptides as well as synthetic peptides containing the amino-terminal teleopeptide sequence of type II collagen inhibit collagen synthesis, deplete cartilage matrix PG and enhance gelatinases in cultured articular cartilage (142). This is consistent with another report that collagen peptides stimulate collagenase production by synovial cells exposed to collagen fragments (143). It is interesting that collagen peptides also down-regulate collagen type II message and synthesis in isolated chondrocytes (144) and also activate alveolar macrophages (145). Preliminary work suggests the involvement of the anchorin CII receptor in cartilage damage (Mollenhauer, personal communication).

It has also been shown that treatment of isolated cultured chondrocytes with a hyaluronic acid (HA) hexasaccharide causes loss of PG, suppression of PG synthesis, decreases in degree of aggrecan aggregation and enhances gelatinase activity (146). Anti-sense oligonucleotides to CD44 have been shown to cause some of the effects of the hexasaccharides, consistent with the possibility that hexasaccharides may enhance chondrolysis by down-regulation of the CD44 receptor (146). This HA receptor appears to play a major role in chondrocyte matrix assembly at the cell surface (147,148), as well as in matrix catabolism (149). It has also been shown that Fn-fs up-regulate CD44 (150). Ironically, since the anti-sense data might be interpreted to suggest that reduction of CD44 levels leads to enhanced matrix catabolism, these data might collectively suggest that the Fn-fs would offset the catabolic activity of naturally occurring HA fragments.

Recently, a 16-residue synthetic link protein peptide, derived from the amino-terminus of link protein, a component of PG aggregates, was found to stimulate PG synthesis in human articular cartilage (151). Since link protein can be cleaved near the amino-terminus by enzymes such as MMP-3, such amino-terminal peptides could enhance reparative processes during matrix damage. However, it would be interesting, as with the Fn-fs, if higher concentrations of link peptide were chondrolytic. This additional property would allow link protein fragments to amplify cartilage repair after moderate damage but cause much more extensive tissue remodeling or clearance after severe damage.

4. Perspectives

This review has described the potential of the Fn-f system to be involved in the complicated pathways of cartilage homeostasis in health and disease. These Fn-fs may signal through the respective receptors the health of the surrounding tissue and should be considered catalysts or mediators of both directions of homeostasis, catabolism and anabolism. Thus, the Fn-fs may act continuously to maintain normal cartilage metabolism as well and thus, completely blocking their activities may not be prudent during therapeutic intervention in OA. However, since Fn-

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fs might act upstream of other common pathways, such as that of cytokines, they may also represent an effective target for control of catabolic cytokine pathways. Since other fragment systems and pathways, including those of collagen and hyaluronic acid may intersect, there may be common targets for prevention of their catabolic activities. There are many questions to be addressed of such fragment systems. Because of the potentially ubiquitous nature of these systems, elucidation of their activities in these cartilage homeostasis pathways will be necessary to fully understand cartilage damage and repair.

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