

## Fibronectin fragments induce the expression of stromelysin-1 mRNA and protein in bovine chondrocytes in monolayer culture

Kelly E. Bewsey, Catherine Wen, Chris Purple, Gene A. Homandberg \*

*Department of Biochemistry, Rush Medical College at Rush-Presbyterian-St. Luke's Medical Center, 1653 West Congress Parkway, Chicago, IL 60612-3864, USA*

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### Abstract

Addition of proteolytically generated fibronectin fragments (Fn-f) to cultured cartilage tissue causes greatly enhanced release of metalloproteinases (MMPs), such as pro-stromelysin-1 (proSln-1), and suppression of proteoglycan (PG) synthesis, through release of catabolic cytokines, while native fibronectin is ineffective. We have investigated whether enhanced release of proSln-1 was due to up-regulation of pro-Sln-1 mRNA. We report that the addition of a 29-kDa (amino-terminal heparin-binding Fn-f) or a 140-kDa (central cell-binding Fn-f) to bovine chondrocytes in monolayer culture causes a dose dependent increase in the expression of pro-Sln-1 mRNA and the greatly enhanced release of pro-Sln-1 protein into the culture media. Up to 700 nM pro-Sln-1 was found in the conditioned media and metabolic labeling showed that it constituted a major portion of newly synthesized protein. A potential activator of pro-Sln-1, urokinase (u-PA), was released at elevated levels in the presence of the Fn-f while other activators, tissue plasminogen activator (t-PA) and plasmin activities were not detected. Addition of these activators to conditioned media did not allow conversion of pro-Sln-1 to active Sln-1. However, aminophenyl mercuric acid activated pro-Sln-1 to a 48-kDa Sln-1 form capable of degrading PG when added to cartilage suspensions. Gelatinase A mRNA was also enhanced, suggesting that the Fn-f may induce MMPs in general. However, the major regulator of Sln-1 activity, tissue inhibitor of MMPs form 1 (TIMP-1), was not induced at the gene level. Thus, a major effect of Fn-f on chondrocytes is to up-regulate pro-Sln-1 expression at the gene level, resulting in pro-Sln-1 as a major protein product.

**Keywords:** Fibronectin; Fibronectin fragment; Metalloproteinase; Cytokine; Cartilage

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### 1. Introduction

Specific proteolytic fragments of the extracellular matrix protein, fibronectin (Fn-f), including an amino-terminal 29-kDa, a 50-kDa gelatin-binding and a 140-kDa cell-binding Fn-f, very potently cause chondrocytic chondrolysis in vitro [1]. This is observed as a very rapid decrease in proteoglycan (PG) content of cultured cartilage during the first week in culture [2] and is associated with a marked elevation of metalloproteinases (MMPs) and temporary suppression of PG synthesis [1,3]. This observation is relevant to osteoarthritis and rheumatoid arthritis, since

high concentrations of Fn-f of 29-kDa to 200-kDa are found in synovial fluids of patients with osteoarthritis and rheumatoid arthritis [4–8]. The relevance is also supported by our demonstration that injection of Fn-f into rabbit knee joints also leads to severe cartilage PG depletion within a few days [9].

The mode of action involves binding of Fn-f to the superficial surface of cartilage, as well as localization to the pericellular matrices surrounding chondrocytes [10]. The cellular targets may include a fibronectin (Fn) receptor, based on the observations that antibodies to the  $\alpha_5\beta_1$  integrin receptor suppress PG synthesis and release of stromelysin-1 (Sln-1) as do the Fn-f (unpublished) and analog peptides corresponding to the binding sequence in Fn for this receptor block activities of the Fn-f [10]. Subsequent up-regulation of specific genes and de novo synthesis of specific proteins appear to be involved and required, since the activities of the Fn-f are blocked by actinomycin D, cycloheximide and deoxyglucose [1]. Part

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Abbreviations: Fn, fibronectin; Fn-f, fibronectin fragments; pro-Sln-1, pro-stromelysin-1; pro-Sln-1, stromelysin-1; PG, proteoglycan; AMPA, aminophenylmercuric acid; TIMP-1, tissue inhibitor of metalloproteinases, form 1; DMEM, Dulbecco's modified Eagle's medium; DMB, dimethylmethylene blue; t-PA, tissue plasminogen activator; u-PA, urinary plasminogen activator

\* Corresponding author. Fax: +1 312 9423053.

of the gene up-regulation appears to involve cytokines, which are known to induce proSln-1 and suppress PG synthesis [12,13], since greatly elevated levels of TNF- $\alpha$ , IL-1 and IL-6 are found in conditioned media of Fn-f treated cartilage cultures ([14]; Homandberg et al., data not shown) and neutralizing antibodies to these cytokines suppress Fn-f mediated Sln-1 release and reverse PG synthesis suppression [14].

Since we have found that antibodies to Sln-1 (MMP-3) suppress Fn-f mediated PG degradation [15], we have deduced that Sln-1 plays a major role in Fn-f mediated cartilage chondrolysis. However, there is no evidence that the Fn-f enhance gene expression of pro-Sln-1 as opposed to merely enhancing release of stored forms from cartilage tissue. Further, there is no information as to whether other MMPs are induced or whether a potential regulator of MMPs, such as TIMP-1, the tissue inhibitor of MMPs, is also induced or whether potential activators of MMPs, such as urinary plasminogen activator (u-PA), tissue plasminogen activator (t-PA) or plasmin, were also up-regulated. Since our earlier work has focused only on protein and activity assays of MMPs [15], the data presented here show the first characterization of Fn-f induced release of Sln-1 from a bovine chondrocyte monolayer system at both the protein and mRNA level and provide evidence that Fn-f up-regulate gene expression of MMPs.

## 2. Materials and methods

All common laboratory chemicals were from Sigma (St. Louis, MO, USA), except where otherwise noted. Recombinant human pro-Sln-1 and the polyclonal anti-sera to human pro-Sln-1 were gifts from Dr. Michael Lark (Merck, Sharpe and Dohme, Rahway, NJ, USA). The 1.8-kb cDNA probe for rabbit pro-Sln-1 was a generous gift from Dr. Constance Brinckerhoff (Dartmouth Medical School, Dartmouth, MA, USA). A 3.9-kb genomic DNA clone for human TIMP-1, designated TIMP-3.9X (ATCC #59666) and a 1.117-kb cDNA clone for human type IV collagenase (gelatinase A), designated pH3a (ATCC #79064), were purchased from ATCC (Rockville, MD, USA).

### 2.1. Isolation of Fn-f

The Fn-f studied were the 140-kDa central cell-binding Fn-f and the 29-kDa amino-terminal heparin-binding Fn-f and were generated and treated for cell culturing as described [2,16].

### 2.2. Isolation of chondrocytes for monolayer cultures

Articular cartilage was removed aseptically from the metacarpophalangeal joints of 18–20-month-old bovines. Chondrocytes were isolated using a modification of the technique of Kuettner et al. [17] for preparation of a high

density chondrocyte culture system. Briefly, the cartilage tissue was subjected to a 90-min pronase (Calbiochem, San Diego, CA, USA) digestion followed by an 18-h collagenase P (Boehringer Mannheim, Indianapolis, IN) digestion in DMEM/5% FBS. The resulting cell suspension was washed repeatedly, cell counted and the cells were plated at 21 million cells/100 mm dish or 0.75 million cells/well in a 24-well plate in DMEM/10% FBS. The media were changed every other day and serum-free cultures were started on day 3 by washing with 8 to 10 volumes of DMEM overnight.

### 2.3. Casein and gelatin zymography

Conditioned media from cultures was collected, stored at 9°C without freezing and then dialyzed against 20 mM NaCl. The solution, without concentration, was then mixed 3:1 with Laemmli sample buffer [18] and without prior boiling, the samples were loaded onto a 10% acrylamide/0.1% gelatin or 10% acrylamide/0.1% casein gel and zymography performed as described [19].

### 2.4. Western blot analysis

Serum-free conditioned media was prepared for electrophoresis and blotting onto nitrocellulose as described [15]. The blotted filters were then incubated with a 1:250 dilution of polyclonal rabbit anti-sera to human pro-Sln-1, washed, incubated with  $^{125}$ I-protein G and exposed to X-ray film as described [15].

### 2.5. Pro-Sln-1 ELISA

Serum free conditioned media (0.5 ml vol) were collected and assayed as described [15].

### 2.6. Metabolic labeling with [ $^{35}$ S]methionine and cysteine

Serum free cultures were treated with 20  $\mu$ Ci of Tran $^{35}$ S-label (ICN, Irvine, CA, USA) at the same time as the addition of the Fn-f on days 1, 3 and 5 of the culture. After another 24 h of incubation, the conditioned media were collected, dialyzed against 20 mM NaCl, concentrated 20-fold on a Speed-O-Vac concentrator without application of heat and subjected to SDS-PAGE on 10% gels. The gels were then treated with Enhance (Dupont/NEN, Boston, MA, USA) for fluorography according to the protocol supplied by the company.

### 2.7. Assays for u-PA, t-PA and plasmin like activity

Phenol red deficient DMEM (Sigma) was used for cultures to be assayed in order to avoid interference with the chromogenic assays. Cultures were established and adjusted to 10 nM or 0.1  $\mu$ M 29-kDa Fn-f and media collected at 1, 3 and 5 days after the addition of the Fn-f.

To 1 ml volumes of the conditioned media, 10  $\mu$ l aliquots of a 10 mg/ml solution of S-2444 (pyro-Glu-Gly-Arg-paranitroanilide), or 10  $\mu$ l of Chromazym t-PA (prepared as directed by supplier: Boehringer-Mannheim, Indianapolis, IN, USA) or 10  $\mu$ l of a 10 mg/ml solution of S-2251 (H-D-Val-Leu-Lys-paranitroanilide) were added, respectively, to detect the presence of u-PA, t-PA or plasmin-like activities as described [15,20]. The solutions were incubated for 2 weeks at 37°C. For controls, media from cultures to which no Fn-f were added were also assayed. For additional controls, incubations of substrate without added media were assayed and the amount of hydrolysis subtracted from all other assays. Standard curves were established using known amounts of each proteinase. The limit of detection was about 0.1 ng/ml with a linear range from 0.1 ng/ml to 100 ng/ml for each proteinase.

### 2.8. Quantification of Sln-1 mediated PG release from metabolically inactive bovine cartilage

To prepare cartilage tissue substrate for testing proteolytic activity, test cartilage was first rendered metabolically inactive by incubation of cartilage with 20  $\mu$ M deoxyglucose, 20  $\mu$ M actinomycin D and 40  $\mu$ g/ml cycloheximide in DMEM for 48 h at 22°C, followed by three freeze/thaw cycles. Serum-free media from chondrocytes treated with 10 nM 29-kDa Fn-f in DMEM for 24 h were then added to the treated bovine articular cartilage slices in a final volume of 1 ml DMEM at 37°C. Aliquots of the supernatant were then collected 1 and 3 days later and analyzed for PG content using the DMB assay described by Chandrasekhar et al. [21]. The  $\mu$ g of PG in the media were then related to the mass of the cartilage in the

1 ml incubation. The final quantification was based on a mean and SD of at least three cartilage samples.

### 2.8.1. Northern blot analysis

Total RNA was isolated from control and Fn-f treated serum-free DMEM cultures on days 1, 3 and 5 after the addition of various concentrations of the 29-kDa Fn-f or 140-kDa Fn-f or BSA using a modification of the guanidine isothiocyanate/acid phenol extraction method of Chomzynski and Sacchi [22]. Total RNA (30  $\mu$ g) from each group was subjected to denaturing agarose gel electrophoresis and transferred to a charged nylon membrane [23] (GeneScreen Plus, Dupont/NEN). Radioactively labeled probes were prepared with [ $^{32}$ P]dCTP utilizing the random primed DNA labeling system and protocol supplied by the manufacturer (Boehringer-Mannheim), resulting in a final specific activity of about 6000 Ci/mmol (Amersham, Arlington Heights, IL, USA). Membranes were then hybridized at 43°C overnight, washed stringently and exposed to X-ray film [23] (X-Omat, Kodak, Rochester, NY, USA). The bands on the X-ray film were quantified by laser densitometry using an LKB model 2202 Ultrascan laser gel densitometer controlled by a Hoefer Scientific Instruments data system GS-365-W interface card. Peak areas were calculated using Hoefer data system software.

## 3. Results

### 3.1. A 57-kDa proteinase with caseinolytic activity was detected in 29-kDa Fn-f treated cultures

We attempted to determine whether Sln-1, as well as other proteinases, were enhanced in cultures of bovine

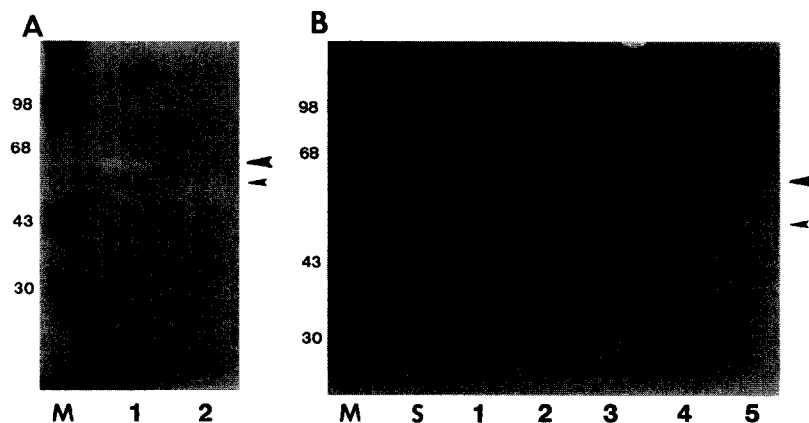


Fig. 1. Identification of proteinase activity in bovine chondrocyte cultures incubated with the 29-kDa Fn-f as shown by casein zymography. Conditioned media from serum free cultures were collected 24 hours after the addition of the 29-kDa Fn-f, dialyzed, concentrated 10 $\times$  and subjected to zymography using a 10  $\mu$ l volume of concentrate. Panel A shows a band of caseinolytic activity which was decreased in size after activation with APMA. Lane M contains molecular weight standards. The 68-kDa albumin standard does not stain on zymograms. Lane 1 is 10 nM 29-kDa Fn-f conditioned media collected from cultures at day 1; lane 2 is the same media incubated with 0.2 mM APMA. The position of the 57-kDa pro-Sln-1 band is denoted by the large arrow and the 48-kDa activation product is denoted by a small arrow on the right hand side of the gel. Panel B shows the effects on increasing concentrations of the 29-kDa Fn-f. M denotes molecular weight standards and S denotes 200 ng of recombinant human pro-Sln-1. Note that the pro-Sln-1 standard which has been determined to be 57 kDa on SDS acrylamide gels migrates with a size similar to that of 68-kDa albumin. Lane 1 is control conditioned media, lanes 2 to 5 are conditioned media concentrates from cultures adjusted to 1, 10, 100 nM and 1  $\mu$ M 29-kDa Fn-f, respectively. The 57-kDa pro-Sln-1 band is denoted by the large arrow and the 48-kDa active Sln-1 form is denoted by the small arrow on the right-hand side of the gel.

chondrocytes, by a highly potent amino-terminal thrombin-generated 29-kDa Fn-f as described [1]. Serum-free conditions were used to avoid addition of serum-derived proteinases to the cultures. Chondrocyte cultures in DMEM were adjusted to 10 nM 29-kDa Fn-f for 24 h. The media were then recovered and subjected to zymography on casein gels. Since SDS can activate zymogens [24], both zymogens and active proteinases could be visualized.

Fig. 1A shows a casein zymogram of conditioned media from these cultures and of conditioned media subsequently adjusted to 0.2 mM aminophenyl mercuric acid (APMA) for 8 h at 22°C, in order to activate any zymogens present. The Fn-f treated media (lane 1) contained a 57-kDa proteinase with a faint 48-kDa band. Treatment of the same media with APMA converted the proteinase (marked with a large arrow) into a smaller form (small arrow), suggesting that the larger proteinase was a zymogen. The 20-kDa band near the bottom of the gel is likely a small Sln-1 form which was also observed in Fn-f treated cartilage cultures [15].

Fig. 1B shows a casein zymogram of media from cultures treated with various concentrations of the 29-kDa Fn-f. The M lane corresponds to size standards. The S lane corresponds to a 57-kDa human pro-Sln-1 standard and in this particular gel shows light bands of 57, 48 and 30 kDa. The expected position of the 57-kDa and 48-kDa forms are marked with a large and small arrow, respectively. The control media in lane 1 shows that proteinases were not enhanced in cultures not treated with the Fn-f. However, very light bands could be observed in cultures treated with 1 nM 29-kDa Fn-f (lane 2). At 10 nM Fn-f (lane 3) very obvious proteinase bands are observed which co-migrated with the 57-kDa pro-Sln-1 and 48-kDa active Sln-1 forms. With 100 (lane 4) and 1  $\mu$ M Fn-f (lane 5), the relative amount of the 57-kDa zymogen decreased and the amount of active 48-kDa increased. Since Sln-1 is the major chondrocyte derived proteinase active on casein, the size of the detected forms strongly suggested that these bands were due to pro-Sln-1 and Sln-1. Gelatin zymography (data not shown) showed that the Fn-f from 1 nM to 1  $\mu$ M caused the additional appearance of bands of about 72 kDa and 98 kDa, likely due to gelatinases A and B, respectively, which are known to be synthesized by chondrocytes [25]. The 57-kDa bands were much lighter in this gelatin gel than those of the 72-kDa and 98-kDa gelatinases and there was a pattern consistent with conversion of 57-kDa bands to 48-kDa bands as the Fn-f concentration was increased.

### 3.2. The 57-kDa proteinase reacted with antibodies to pro-Sln-1 as shown by Western blot analysis

Western blotting was used to confirm that the 57-kDa proteinase was pro-Sln-1. Serum-free media for cultures with both the 29-kDa Fn-f and a 140-kDa Fn-f, which contains the receptor-binding domain of Fn which is also active in cartilage chondrolysis [1], were studied.

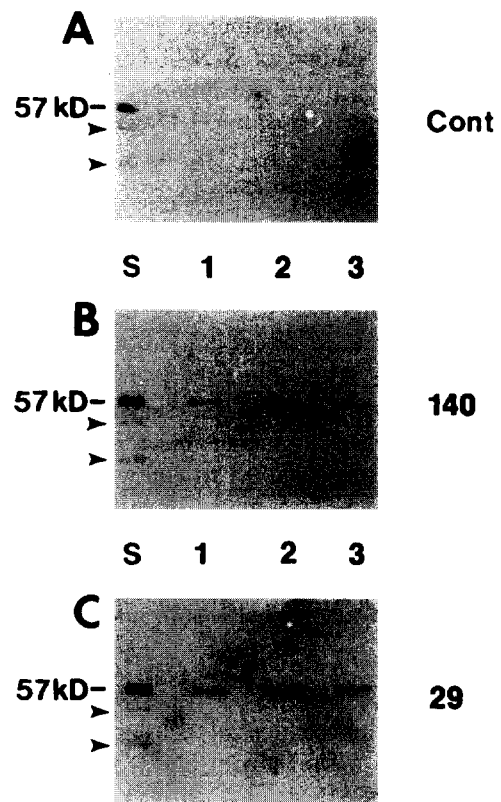


Fig. 2. Analysis of conditioned media by Western blotting with anti-sera to human pro-Sln-1. Chondrocyte cultures in serum-free DMEM were adjusted to 100 nM 140-kDa Fn-f or 100 nM 29-kDa Fn-f and at days 1, 2 and 3 the media were collected, dialyzed, concentrated and subjected to Western blotting with human pro-Sln-1 anti-sera, followed by reaction with  $^{125}$ I-protein G for subsequent fluorography. Panels A, B and C show media from control, 100 nM 140-kDa Fn-f and 100 nM 29-kDa Fn-f treated cultures, respectively. S corresponds to a human pro-Sln-1 standard (0.1  $\mu$ g).

Fig. 2A is a blot from control cultures without added Fn-f. Lane S shows a pro-Sln-1 standard of 57 kDa which contains smaller forms of 43 kDa and 30 kDa, denoted by the arrows. These forms are not present in control samples at days 1, 2 and 3. Fig. 2B shows a blot of media from cultures adjusted to 100 nM 140-kDa Fn-f for days 1, 2 and 3. Strongly reactive bands are observed at days 1, 2 and 3 and a peak of pro-Sln-1 is suggested at day 2. Fig. 2C shows a blot of cultures adjusted to 100 nM 29-kDa Fn-f for days 1, 2 and 3. The intensities of the bands are greater than in the 140-kDa Fn-f treated cultures and a peak at day 2 is also suggested. The lack of detection of 48-kDa forms on the Western blot, in contrast to the casein zymogram in Fig. 1, could be due to the greater sensitivity of the casein zymogram to the small Sln-1 forms. It is also possible that this 48-kDa form detected on zymograms, but not detected by Western blotting, is not a Sln-1 form but rather another type of proteinase. However, since the 48-kDa proteinase was not detected on dye stained gels as will be shown, this form is not present at significant levels.

### 3.3. The release of pro-Sln-1 into conditioned media was dependent on Fn-f concentration as shown by ELISA

An ELISA method [15] was used to quantify the relative increase in pro-Sln-1 protein released into the media of Fn-f cultured cartilage, however since only human pro-Sln-1 was available as a standard, only fold increases and not absolute amounts of pro-Sln-1 are reported. Table 1 shows that BSA (100 nM) treated tissue releases pro-Sln-1 at control levels or lower. However, 1 nM 140-kDa Fn-f treatment enhanced release to 8-fold over control levels by day 5, while 10 nM Fn-f enhanced to this level earlier, by day 3. However, 100 nM 140-kDa Fn-f was half as effective as 10 nM and caused an even earlier peak, at day 1. The 1  $\mu$ M concentration actually suppressed release to one-third of control levels.

The 29-kDa Fn-f also caused about the same concentration dependent relative increases in release which appeared to peak earlier with increasing concentration of Fn-f. Table 1 shows that 1 nM Fn-f only doubled release over control levels with a peak at day 5, while 10 nM caused a 12-fold increase with a peak at day 3. This 12-fold increase can be compared with the 8-fold effect of the 140-kDa Fn-f. A concentration of 100 nM 29-kDa Fn-f caused a 3.6-fold increase with an apparent peak at day 3. Thus, 1  $\mu$ M 29-kDa Fn-f was not as suppressive as 1  $\mu$ M 140-kDa Fn-f. Collectively, these data suggest that the 29-kDa Fn-f is more effective than the 140-kDa Fn-f and that for both, increasing the concentration from 1 to 100 nM causes the peak of release to occur earlier, that the maximal effect of either Fn-f on protein release occurred at 10 nM in cell culture and that 1  $\mu$ M of either Fn-f is either suppressive or sub-optimal. The suppression may be due to suppression of general protein synthesis by higher concentrations of Fn-f as reported [2,3,11].

### 3.4. The 57-kDa pro-Sln-1 form was the major metabolically labeled product released into the media following stimulation with the Fn-f

Chondrocyte cultures in DMEM containing Fn-f were metabolically labeled with  $^{35}$ S methionine/cysteine to determine whether pro-Sln-1 represented a major de novo synthesized product. A concentration of 100 nM was tested here since this concentration appeared to be optimal for pro-Sln-1 release. Fig. 3 shows a fluorogram of the conditioned media. At day 1, the control cultures (Fig. 3A) show a 57-kDa pro-Sln-1 band (position denoted by arrow) with some higher molecular mass bands of 68 kDa and upward (immediately above the arrow). At days 3 and 5, proteins of too great a mass to enter the gel were labeled. However, at day 1, the media of the 29-kDa Fn-f treated cultures (Fig. 3B) showed much higher levels of the 57-kDa protein, with the amounts decreasing by day 3. This trend is consistent with the data shown in Table 1 for cultures with the 100 nM 29-kDa Fn-f. Note that the 29-kDa Fn-f has enhanced synthesis by day 1 of higher molecular mass material, which could include complexes of pro-Sln-1. Nonetheless, the major de novo synthesized protein entering the gel appears to correspond to the size of pro-Sln-1.

### 3.5. The 57-kDa pro-Sln-1 protein was the major protein found in conditioned media

To confirm the identity of the major de novo synthesized protein as pro-Sln-1, bovine chondrocyte cultures in DMEM containing 100 nM 29-kDa Fn-f were cultured for 3 days and media collected. The resultant protein concentration as determined by the Bio-Rad assay of the concentrate was about 840  $\mu$ g/ml. Fig. 4A shows a gelatin zymogram of the concentrates. Gelatin was used instead of

Table 1  
Effect of Fn-f on Pro-Sln-1 release from bovine chondrocytes in DMEM

Condition	Fold increase compared to control values at respective days		
	Day 1	Day 3	Day 5
Control	1.0 ( $\pm$ ) 0.31 <sup>a</sup>	1.0 ( $\pm$ ) 0.36	1.0 ( $\pm$ ) 0.08
BSA (100 nM)	1.1 ( $\pm$ ) 0.25	0.7 ( $\pm$ ) 0.31	0.3 ( $\pm$ ) 0.08
1 nM 140-kDa	2.0 ( $\pm$ ) 0.19	5.3 ( $\pm$ ) 0.09 (s)	8.0 ( $\pm$ ) 0.1(s)
10 nM 140-kDa	3.7 ( $\pm$ ) 0.16 (s)	8.5 ( $\pm$ ) 0.3 (s)	4.8 ( $\pm$ ) 0.1(s)
100 nM 140-kDa	4.2 ( $\pm$ ) 0.9 (s)	3.0 ( $\pm$ ) 0.66	1.0 ( $\pm$ ) 0.19
1 $\mu$ M 140-kDa	0.5 ( $\pm$ ) 0.31	0.3 ( $\pm$ ) 0.47	0.3 ( $\pm$ ) 0.09
1 nM 29-kDa	1.7 ( $\pm$ ) 0.07	2.0 ( $\pm$ ) 0.35	6.1 ( $\pm$ ) 0.1(s)
10 nM 29-kDa	2.6 ( $\pm$ ) 0.06 (s)	11.5 ( $\pm$ ) 0.25 (s)	4.7 ( $\pm$ ) 0.2(s)
100 nM 29-kDa	3.6 ( $\pm$ ) 0.2 (s)	3.1 ( $\pm$ ) 0.09(s)	2.1 ( $\pm$ ) 0.3
1 $\mu$ M 29-kDa	0.4 ( $\pm$ ) 0.12	1.7 ( $\pm$ ) 0.36	0.4 ( $\pm$ ) 0.6

Chondrocyte cultures in DMEM were treated with various concentrations of the 140-kDa Fn-f or with various concentrations of the 29-kDa Fn-f or with 100 nM BSA. Conditioned media were collected at days 1, 3 and 5 after the addition of the Fn-f. Media (0.5 ml) were dialyzed against 20 mM NaCl, concentrated 20-fold and brought up to 200  $\mu$ l with 0.1 M sodium bicarbonate buffer (pH 9.5). The samples were then loaded into a 96-well plate and treated according to the published protocol [15]. Assays were performed in triplicate on all samples

<sup>a</sup> The mean and SD value in parentheses are based on  $n = 3$ . s indicates significance, based on a  $P$  value of  $< 0.05$  using the Student's  $t$ -test, as compared to the same day controls.

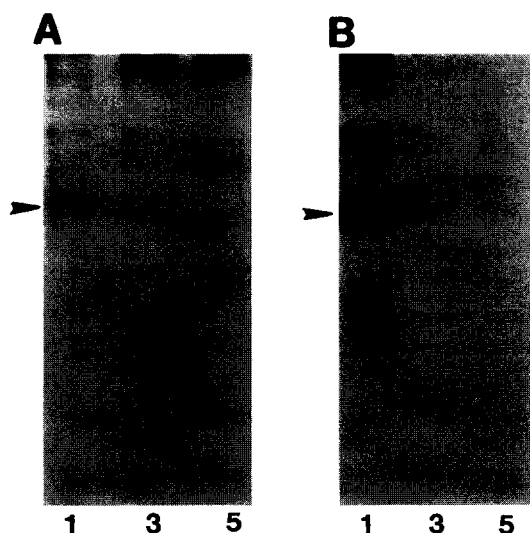


Fig. 3. Fluorogram of conditioned media from cultures exposed to Fn-f and [ $^{35}$ S]met/cys-Bovine chondrocyte cultures in DMEM were incubated with [ $^{35}$ S]Translabel (70% methionine/30% cysteine) and without (A) or with (B) 100 nM 29-kDa Fn-f for 24 h at days 1, 3 and 5 after the addition of the Fn-f. The x-axis shows days in culture. Therefore, for example, the day 3 time-point corresponds to protein labeled between days 3 and 4 in culture with the Fn-f. The media were dialyzed, concentrated 20-fold, and subjected to electrophoresis on a 10% SDS-PAGE gel. The gel was then treated with Enhance and subjected to fluorography. The presence of a band corresponding to 57 kDa is denoted by a large arrow and the presence of a band corresponding to 48 kDa is denoted by a small arrow.

casein, in order to show the presence of other gelatinases in the conditioned media and to illustrate that the 57-kDa caseinase identified in earlier data as pro-Sln-1 was rela-

tively inactive as a gelatinase. Lane S shows molecular weight standards and lanes 1, 2 and 3, the effect of increasing amounts of concentrated media. All the lanes show the presence of the 72-kDa and 98-kDa gelatinases, while lane 3 with the highest volume of media, shows the additional presence of a light 57-kDa proteinase (denoted by arrow) and some diffuse banding at 60–65 kDa. Comparison with Fig. 1 confirms that the 57-kDa pro-Sln-1 was more active on casein than on gelatin and that the 72-kDa and 98-kDa gelatinases detected in Fig. 4A were not very active as caseinases or general proteinases.

Similar samples were subjected to electrophoresis and the gel stained with Coomassie blue. Fig. 4B shows in lane 1, a concentrate from Fn-f treated cultures. An intense band at about 57 kDa and a very light band at about 70 kDa are also observed. Lanes 2 and 3 shows a non-reduced sample and a reduced sample from control cultures, respectively. Lane 4 shows the effect of subjecting the Fn-f treated media to disulfide reduction in the presence of 0.1 M dithiothreitol. Note that the apparent molecular mass has now increased to about 72 kDa, likely due to the effect of reduction on increasing the radius of the denatured protein. It should be noted that the assignment of pro-Sln-1 as 57-kDa has been based on a non-reduced molecular mass as seen on a zymogram for all the data presented herein.

Another gel was subjected to Western blot analysis. Fig. 4C shows that the reduced protein reacted with anti-sera to pro-Sln-1. Activated 48-kDa Sln-1 was not detected on this stained gel or on the Western blot. Therefore, these data collectively show that pro-Sln-1 was the major protein made by chondrocytes in serum-free cultures stimulated with Fn-f. Because pro-Sln-1 is the major protein and the

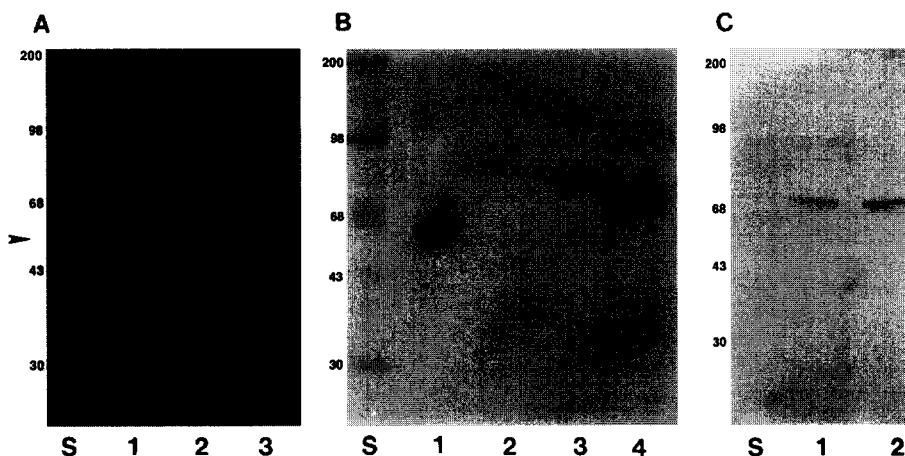


Fig. 4. Characterization of protein found in conditioned media by zymography, SDS-PAGE and Western blot analysis. Bovine chondrocyte cultures in DMEM were adjusted to 100 nM 29-kDa Fn-f. After 3 days, conditioned media were dialyzed against 20 mM NaCl, 50 mM Tris buffer, pH 7.4, and concentrated 20 $\times$  and subjected to analysis. Panel A is a zymogram. Lanes 1 to 3 are conditioned media containing 0.4, 1.2 and 4  $\mu$ g of protein, respectively, or 1, 3 or 10  $\mu$ l of concentrated media. The position of 48 kDa is marked by an arrow on the left hand side. Panel B is a 10% SDS-PAGE gel stained with Coomassie. Lane 1 is a 4  $\mu$ g quantity of protein from the 20 $\times$  concentrated Fn-f conditioned media and lane 4 is the same quantity subjected to reduction with 0.1 M dithiothreitol. Lanes 2 and 3 are the same volumes as in lanes 1 and 4 of unreduced and reduced media from control cultures. Panel C is a Western blot of 20 $\times$  concentrated conditioned media reacted with anti-sera to human pro-Sln-1. The lanes are each of 4  $\mu$ g of protein from the 20 $\times$  concentrate of Fn-f conditioned media. Mol wt. standards are denoted by S in the left lane.

protein assay data showed a protein concentration of 840  $\mu\text{g}/\text{ml}$  for the  $20\times$  concentrate, the concentration of pro-Sln-1 in the conditioned media after 3 days can be estimated as about 700 nM.

*3.6. The 57-kDa pro-Sln-1 form from Fn-f treated bovine chondrocytes was not activatable to the active 48-kDa form with human u-PA, t-PA and plasmin*

Potential activators of MMP zymogens [26–29] were assayed in Fn-f conditioned media. Bovine chondrocyte cultures were incubated in phenol red-deficient DMEM containing 100 nM 29-kDa Fn-f in order to decrease interference in the chromogenic assay. The assays did not show detectable levels of plasmin like activity (S-2251 cleavage) or t-PA like activity in either control or Fn-f conditioned media. However, u-PA like activity (S-2444 cleavage) was detected. Control samples showed levels less than 1 ng/ml ( $\pm$ ) 0.2 ( $n=3$ ) by day 1, 3 or 5 in culture, while the 100 nM 29-kDa Fn-f treated cultures showed maximal release of 103 ng/ml ( $\pm$ ) 11 ( $n=3$ ) at day 1 and levels about 20% lower by days 3 and 5. Thus, release of t-PA or plasmin was not apparently enhanced by the Fn-f to detectable levels, however the Fn-f enhanced release of u-PA like activity by 100-fold.

We then investigated whether u-PA, t-PA or plasmin at concentrations of 1, 10 or 100 ng/ml could activate bovine pro-Sln-1 in Fn-f conditioned media. The conditioned media were incubated with each activator for periods of 1, 3 and 7 days at 22°C in DMEM. Casein

Table 2

Effect of 29-kDa Fn-f conditioned media on PG release from metabolically inactive cartilage

Condition	$\mu\text{g}$ PG/mg cartilage ( $\pm$ ) S.D. ( $P$ value) <sup>a</sup>
Cartilage suspension	1.4 ( $\pm$ ) 0.3
Fn-f solution	1.4 ( $\pm$ ) 0.2
Media from controls at day 1	1.5 ( $\pm$ ) 0.4
Media from controls at day 1 + APMA	1.6 ( $\pm$ ) 0.3
Media from Fn-f treatment at day 1	1.4 ( $\pm$ ) 0.5
Media from Fn-f treatment at day 1 + APMA	4.1 ( $\pm$ ) 0.9 ( $P=0.005$ )

Test cartilage was first rendered metabolically inactive to prevent up-regulation of Sln-1 protein as described in Section 2.

Conditioned media from bovine chondrocytes incubated with 100 nM 29-kDa Fn-f in DMEM for 24 h were then added to the treated bovine articular cartilage slices in a final volume of 1 ml DMEM at 37°C and aliquots of the supernatant were then collected 1 and 3 days later and analyzed for PG content using the DMB assay. The  $\mu\text{g}$  of PG were then related to the mass of the cartilage in the 1 ml incubation

<sup>a</sup>  $P$  values were based on one sided Student's  $t$ -test with  $n=3$  and where a  $P < 0.05$  was considered significant. Values for Fn-f media with or without APMA were compared to control media without APMA. Only those values found to be significantly different are shown with a numerical  $P$  value.

zymography was performed in order to test for decreases in mass of the treated pro-Sln-1. Neither decreases in mass of the pro-Sln-1 nor decreases in intensities of the 57-kDa band occurred at any of the concentrations for any of the time periods tested (data not shown).

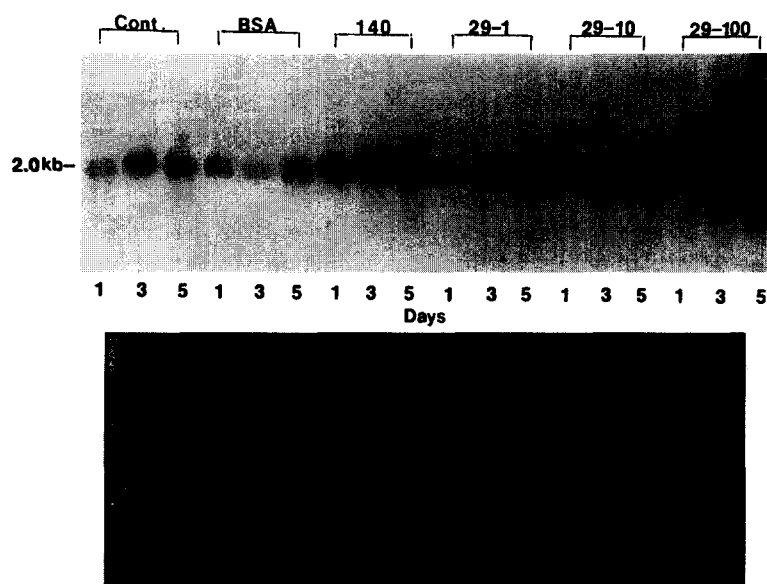


Fig. 5. Northern blot for pro-Sln-1 mRNA for cultures exposed to various concentrations of Fn-f for 1, 3 and 5 days. Northern blot of total RNA was performed on cultures at 1, 3 and 5 days after addition of the Fn-f. Cultures to which nothing was added or which were adjusted to 100 nM BSA were used as controls. Total RNA was isolated at days indicated and an aliquot of 25  $\mu\text{g}$  of total RNA was loaded in each lane and subjected to electrophoresis on a denaturing agarose gel. The gel was then blotted onto nylon and hybridized to [ $^{32}\text{P}$ ]dCTP-labeled cDNA for rabbit pro-Sln-1, washed stringently and exposed to X-ray film overnight. From left to right are control cultures (no treatment), cultures adjusted to 1  $\mu\text{M}$  BSA, or 100 nM 140-kDa Fn-f, or 1 nM 29-kDa Fn-f, or 10 nM 29-kDa Fn-f or 100 nM 29-kDa Fn-f. Days are at the bottom of the figure. The pro-Sln-1 mRNA is denoted with the 2.0-kb marker. The bottom shows the gel stained with ethidium bromide prior to the blotting and shows the even loading of the total RNA.

### 3.7. The pro-Sln-1 form could be activated with APMA to a form capable of degrading and releasing PG when added to cartilage suspensions

The ability of the pro-Sln-1 to be activated to a form capable of degrading PG from cartilage made metabolically inactive was investigated as described in Section 2. Table 2 shows that the passive release of PG from a cartilage suspension in DMEM occurred at the level of 1.4  $\mu$ g PG/mg cartilage. Addition of 100 nM Fn-f to the inactive cartilage did not enhance release of PG, suggesting that the tissue was indeed inactive and that the Fn-f solution did not contain contaminating proteinases. Media from tissue not cultured with Fn-f which were assayed both with and without prior activation with AMPA did not enhance release of PG. Media from Fn-f treated cultures that was not treated with APMA did not show enhanced activity; however, addition of APMA greatly increased release of PG. Therefore, the pro-Sln-1 in the Fn-f conditioned media was activatable to an active form. Other experiments shown earlier (Fig. 1) confirmed that this form was 48 kDa.

### 3.8. The enhanced release of the pro-Sln-1 form was due to a time- and dose-dependent increase in mRNA for pro-Sln-1 in bovine chondrocytes as shown by Northern blot analysis

Total RNA was isolated from chondrocytes at various times after addition of the 29-kDa and 140-kDa Fn-f, Northern blotting performed and blots probed with radioactively labeled rabbit pro-Sln-1 cDNA as described in Methods. Initial slot blot analysis of total RNA showed expression of mRNA for pro-Sln-1 as early as 4 h after the addition of the Fn-f (data not shown). Total RNA was then subjected to Northern blot analysis as shown in Fig. 5 (upper) which shows that control cultures and 100 nM BSA treated cultures express pro-Sln-1 mRNA at measurable levels as observed as a 1.8–2.0 kb band that strongly hybridized with the radioactively labeled probe. The band is the same size as that reported previously by Fini et al. [30] for pro-Sln-1 mRNA. Treatment with 100 nM of the 140-kDa Fn-f greatly enhanced levels by days 1, 3 or 5. Treatment with 1, 10 and 100 nM 29-kDa Fn-f caused progressively faster increases in mRNA. However, 100 nM of either Fn-f caused a greater induction than lower con-

Table 3

Peak integration of bands from stromelysin-1 Northern blot

Condition	Fold increase compared to control values at respective days		
	Day 1	Day 3	Day 5
100 nM BSA	1.8	0.3	1.1
100 nM 140-kDa	4.2	2.0	1.8
1 nM 29-kDa	1.1	1.0	3.6
10 nM 29-kDa	2.8	4.4	2.5
100 nM 29-kDa	5.3	2.1	2.4

Lanes from the Northern blot in Fig. 5 were scanned using an LKB model 2202 Ultrascan laser gel densitometer controlled by a Hoefer Scientific Instruments data system GS-365-W interface card. Peak areas were calculated using Hoefer data system software. A membrane with bands of various intensities was used to verify that the bands from the Northern blot fell within the linear range of quantitation.

centrations, in contrast to the protein ELISA data in Table 1. This difference might be due to proteolytic processing of pro-Sln-1 to forms incapable of reacting to anti-sera. Note that the effect of the 100 nM 29-kDa Fn-f appears to be more potent than 100 nM 140-kDa Fn-f as also suggested by the protein data in Table 1. Fig. 5 (bottom) shows the gel stained with ethidium bromide prior to the blotting and demonstrates the even loading of the total RNA.

The fold increases were quantified by laser densitometry as summarized in Table 3. For 100 nM of either Fn-f, the peak was at day 1 and the timing of the peak was delayed as the concentration was decreased, consistent with the protein data shown in Table 1. The magnitude of these maximal fold increases in mRNA of 4- to 5-fold can be compared with the fold increases in protein of 8- to 11.5-fold shown in Table 1.

### 3.9. The 29-kDa and 140-kDa Fn-f also up-regulated the gelatinase A gene in a dose and time dependent fashion as shown by Northern blot analysis

In order to determine whether there was a more general effect on MMPs, the blot used in Fig. 5 was stripped and re-probed with a pH3a probe for human gelatinase A obtained from ATCC (#79064). This probe hybridizes to the mRNA coding for the amino-terminal portion of human gelatinase A and has been used to identify the mRNA for gelatinase A as a 3.1 kb band [31]. Fig. 6 shows that while the control and BSA treated cultures showed rela-



Fig. 6. Northern blot for gelatinase A mRNA for cultures exposed to various concentrations of Fn-f for 1, 3 and 5 days - The blot is the same as used in Fig. 5 after stripping the pro-Sln-1 probe by boiling the blot in 0.1  $\times$  SSC/1% SDS for 30 min. The lanes are the same as shown in Fig. 5 with experimental groups on top and days on the bottom. The gelatinase A mRNA is shown as a 3.1-kb band.



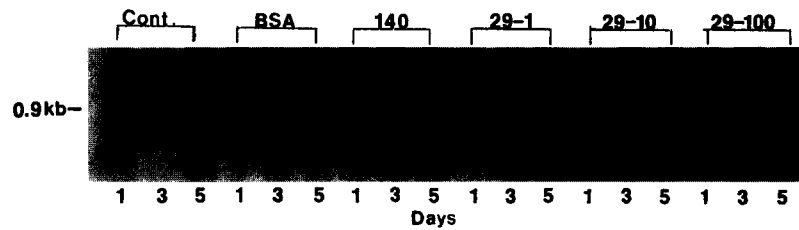


Fig. 7. Northern blot for TIMP-1 mRNA for cultures exposed to various concentrations of Fn-f for 1, 3 and 5 days. The Northern blot in Fig. 6 was stripped of probe by boiling in 0.1% SSC/1% SDS for 30 min, and checked to ensure total removal of the probe. The blot was then probed with  $^{32}$ P-labeled cDNA for human TIMP-1, washed stringently and exposed to X-ray film. The lanes are the same as shown in Fig. 6 with experimental groups on top and days on the bottom. The TIMP-1 mRNA is denoted by the 0.9-kb marker.

tively low mRNA levels at days 1, 3 or 5, treatment with 100 nM 140-kDa or 1 to 100 nM 29-kDa Fn-f greatly up-regulated the gelatinase A mRNA.

### 3.10. TIMP-1 mRNA expression was not altered by the addition of the Fn-f

The same blot as used in Figs. 5 and 6 was stripped and re-probed with a cDNA probe for human TIMP-1 (designated TIMP-3.9X by ATCC) which has been described [32]. Fig. 7 shows that while control and BSA treated cells did express mRNA for TIMP-1, the addition of the 140-kDa or 29-kDa Fn-f did not greatly up-regulate the TIMP-1 mRNA which appears as a 0.9-kb band, the same size as reported elsewhere [32].

## 4. Discussion

This work is novel in that the data suggest that in Fn-f-mediated cartilage chondrolysis as caused by greatly enhanced MMP activities, the enhancement of MMP activities likely occurs by greatly enhanced gene expression. This conclusion was supported by several observations. Analysis of conditioned media from Fn-f treated chondrocyte cultures by zymography showed the dose dependent release of a 57-kDa proteinase which was more active on casein than on gelatin. Western blot analysis confirmed that the band was pro-Sln-1. ELISA analysis confirmed that pro-Sln-1 was released in a dose dependent fashion, while labeling experiments showed that pro-Sln-1 represented a major protein synthesized in chondrocytes incubated with Fn-f. Quantification showed that pro-Sln-1 was released at very high levels of about 700 nM with a three day culture incubation.

In studies of the effects of varying concentrations of Fn-f on the kinetics of pro-Sln-1 expression, it was observed by analysis of protein and mRNA levels for pro-Sln-1 that with lower concentrations of Fn-f, the peaks of release were delayed but the same amount was eventually released. In reported studies of bovine cartilage tissue, as the Fn-f concentration is decreased, a lag period occurs before damage is apparent and the period becomes longer

as the concentration is decreased but the same amount of damage eventually occurs [2]. This may be due to a set time required for amplification of catabolic cytokines to a minimal threshold concentration required for initiation of damage. Another possibility is that the Fn-f must slowly concentrate in the matrix and reach a threshold tissue concentration before they are effective.

The 29-kDa Fn-f has been shown to be more effective on cartilage tissue than the 140-kDa Fn-f [1] and was shown here to be more effective on induction of pro-Sln-1 mRNA and protein in chondrocyte cultures as well. Since the interaction of the larger 140-kDa Fn-f with chondrocytes in monolayer culture should not have been hampered as might occur with chondrocytes in cartilage tissue, the lesser activity of the 140-kDa Fn-f may be due to differences in interaction with the chondrocyte cell surfaces. We do not yet know the mechanism for either Fn-f but we have proposed that the 140-kDa Fn-f may block the binding of native cartilage Fn to its receptor and remove a stabilizing effect on signal transduction. The 29-kDa Fn-f may do so indirectly based on observations that amino-terminal Fn-f co-localize with  $\alpha_5\beta_1$  integrins in focal adhesions [33] and amino-terminal Fn-f block binding to the Fn receptor of antibodies to the  $\alpha_5\beta_1$  Fn receptor [34].

Northern blot analysis showed that the pro-Sln-1 transcription was up-regulated in a fashion in which the peak of expression occurred earlier as the concentration of Fn-f was increased, as also shown with the protein data. With 100 nM Fn-f, the maximum was at day 1, followed by a decline. This early and short term induction may be related to the observation that in cartilage tissue, cytokine expression which induces pro-Sln-1, is also short term [14]. The delayed induction by 1 nM Fn-f is similar to the protein data and, as discussed above, may be due to the requirement for a threshold accumulated concentration of cytokine or Fn-f.

Gelatinase A mRNA was also enhanced, although the MMP protein induced to the highest levels was pro-Sln-1, based on metabolic labeling and SDS gel and Western blot analysis of the culture media. TIMP-1 mRNA was not induced, suggesting that the Fn-f do not induce a general up-regulation of mRNA synthesis and that TIMP-1 induc-

tion cannot counter the effects of induced MMPs in this system. Of the potential proteolytic activators studied, the Fn-f only enhanced release of u-PA, although none of the activators appeared to activate pro-Sln-1. Preliminary data also suggest that t-PA and u-PA mRNA levels are not elevated in Fn-f treated chondrocytes (Homandberg, unpublished). Thus, at present we have no data to support the role of these activators in activation of pro-Sln-1 in this system. Nonetheless, these studies collectively show that the ability of Fn-f to enhance cartilage chondrolysis occurs through gene induction of MMPs.

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## References

- [1] Homandberg, G.A., Meyers, R. and Xie, D.L. (1992) *J. Biol. Chem.* 267, 3597–3604.
- [2] Homandberg, G.A. and Hui, F. (1994) *Arch. Biochem. Biophys.* 311, 213–218.
- [3] Xie, D.L., Hui, F. and Homandberg, G.A. (1993) *Arch. Biochem. Biophys.* 307, 110–118.
- [4] Clemmensen, I. and Bach Andersen, R. (1982) *Arthritis Rheum.* 25, 25–31.
- [5] Carsons, S., Lavietes, B.B., Diamond, H.S. and Kinney, S.G. (1985) *Arthritis Rheum.* 28, 601–612.
- [6] Dutu, A., Vlaicu-Rus, V., Bolosiu, H.D., Parasca, I. and Cristea, A. (1986) *Med. Int.* 24, 61–68.
- [7] Griffiths, A.M., Herbert, K.E., Perrett, D. and Scott, D.L. (1989) *Clin. Chim. Acta* 184, 133–146.
- [8] Xie, D.L., Meyers, R. and Homandberg, G.A. (1992) *J. Rheumatol.* 19, 1448–1452.
- [9] Homandberg, G.A., Meyers, R. and Williams, J. (1993) *J. Rheumatol.* 20, 1378–1382.
- [10] Xie, D.L. and Homandberg, G.A. (1993) *Biochim. Biophys. Acta* 1182, 189–196.
- [11] Homandberg, G.A. and Hui, F. (1994) *Arch. Biochem. Biophys.* 310, 40–48.
- [12] Goldring, M.B., Birkhead, J., Sandell, L.J., Simura, T. and Krane, S.M. (1988) *J. Clin. Invest.* 82, 2026–2037.
- [13] Ito, A., Goshawaki, H., Sato, T., Mori, Y., Yamashita, K., Hayakawa, T. and Nagase, H. (1988) *FEBS Lett.* 234, 326–330.
- [14] Homandberg, G.A. and Hui, F. (1994) *Trans. Orthop. Res. Soc.* 19, 464a.
- [15] Xie, D.L., Hui, F., Meyers, R. and Homandberg, G.A. (1994) *Arch. Biochem. Biophys.* 311, 205–212.
- [16] Homandberg, G.A. and Erickson, J. (1986) *Biochemistry* 25, 6917–6925.
- [17] Kuettner, K.E., Memoli, V.A., Pauli, B.U., Wrobel, N.C., Thonar, E.J.-M.A. and Daniel, J.C. (1982) *J. Cell Biol.* 93, 751–757.
- [18] Laemmli, U.K. (1970) *Nature (London)* 22, 680–685.
- [19] Chin, J., Murphy, G. and Werb, Z. (1985) *J. Biol. Chem.* 260, 12367–12376.
- [20] Homandberg, G.A. and Wai, T. (1990) *Biochim. Biophys. Acta* 1038, 209–215.
- [21] Chandrasekhar, A., Esterman, M.A. and Hoffman, H.A. (1987) *Anal. Biochem.* 161, 103–108.
- [22] Chomzynski, P. and Sacchhi, N. (1987) *Anal. Biochem.* 162, 156–159.
- [23] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) in *Molecular Cloning, A Laboratory Manual* (Ford, N., Nolan, C., Ferguson, M., eds.), Second ed., sections 7.3–8.2., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
- [24] Nagase, H., Engchild, J.J., Suzuki, K. and Salveson, G. (1990) *Biochemistry* 29, 5783–5789.
- [25] Woessner, J.F. (1991) *FASEB J.* 5, 2145–2154.
- [26] Werb, Z., Mainardi, C.L., Vater, C.A. and Harris, E.D., Jr. (1977) *N. Engl. J. Med.* 296, 1017–1023.
- [27] Collier, S. and Ghosh, P. (1988) *J. Rheumatol.* 15, 1129–1137.
- [28] Mochan, E. and Uhl, J. (1984) *J. Rheumatol.* 11, 123–128.
- [29] Werb, Z. (1989) Proteinases and matrix degradation. In *Textbook of Rheumatology* (Kelly, W.N., Harris, E.D., Jr., Ruddy, S. and Sledge, C.B., eds.), 3rd ed., pp. 300–321, Saunders, Philadelphia.
- [30] Fini, M.T., Matsubara, M., Kublin, C., Tessier, M.T., Cintron, C. and Fini, M.E. (1993) *J. Cell Sci.* 104, 1001.
- [31] Levy, A.T., Cioce, V., Sobel, M.E., Garbisa, S., Grigioni, W.F., Liotta, L.A., Stetler-Stevenson, W.G. (1991) *Cancer Res.* 51, 439–444.
- [32] Gasson, J.C., Golde, D.W., Kaufman, S.E., Westbrock, C.H., Hewick, R.M., Kaufman, R.J., Wang, G.G., Temple, P.A., Leary, A.C., Brown, E.L., Orr, E.C. and Clark, S.C. (1985) *Nature* 315, 768–771.
- [33] Dzamba, B.J., Bultmann, H., Akiyama, S.K. and Peters, D.M. (1994) *J. Biol. Chem.* 269, 19646–19652.
- [34] Fogerty, F.J., Akiyama, S.K., Yamada, K.M. and Mosher, D.F. (1990) *J. Cell. Biol.* 111, 699–708.