The Fibronectin Extra Domain A Activates Matrix Metalloproteinase Gene Expression by an Interleukin-1-dependent Mechanism*  
(Received for publication, April 5, 1999, and in revised form, July 22, 1999)  
Shigeki Saito†, Noboru Yamaji, Kunio Yasunaga, Tetsu Saito, Shun-ichiro Matsumoto, Masao Katoh, Seiji Kobayashi, and Yasuhiro Masuho  
From the Institute for Drug Discovery Research, Yamanouchi Pharmaceutical Co., Ltd., 21 Miyukigaoka, Tsukuba, Ibaraki 305-8585, Japan  

The extra-domain-A (EDA), present in fibronectin (FN) molecules arising from alternatively spliced transcripts, appears only during specific biological and pathogenic processes. However, its function is poorly understood. To define the physiologic role of this domain in joint connective tissue, the biological effects on rabbit cartilage explants, chondrocytes, and synovial cells were studied. A recombinant EDA protein (rEDA) increased proteoglycan release (3.6-fold) in cartilage explant cultures and markedly induced production of matrix metalloproteinase (MMP)-1 in chondrocytes. In addition, rEDA induced MMP-1, MMP-3, and MMP-9 in synovial cells. These effects were elicited only by rEDA, while its neighboring type III repeats, III₁₁ or III₁₂, scarcely had any such effects. Interestingly, reorganization of F-actin stress fibers accompanied MMP-1 expression in synovial cells treated with rEDA, suggesting alteration of cellular phenotype. Subsequent Northern blotting revealed expression of pro-inflammatory cytokines, including interleukin (IL)-1α and IL-1β, was induced by rEDA prior to MMP-1 expression. Delayed MMP-1 expression suggests that rEDA-induced IL-1α promote MMP-1 expression in an autocrine manner. This hypothesis is supported by the reduction of EDA-induced MMP-1 production by IL-1 receptor antagonist. The effect of EDA on MMP-1 production was reduced by connection with an adjacent type III repeat on either the NH₂ or COOH side of EDA and was abolished by connection on both sides of EDA, suggesting that exposure of either the NH₂ or COOH terminus of EDA domain by proteolytic cleavage releases the inducing activity. In agreement with these results, full-length cellular FN did not induce MMP-1 production. Furthermore, a 160-kDa EDA-positive FN fragment, which was purified from human placental tissue and corresponds to the region from NH₂ terminus through the EDA, induced MMP-1 production. Taken together, these results suggest that the EDA in FN fragments triggers alterations of cell physiology and plays a role in matrix degradation in joint connective tissue.

Fibronectin (FN)† is a multifunctional glycoprotein abundant in plasma and widely distributed in the extracellular matrix (1). It is a dimer of subunits cross-linked by disulfide bonds. Each FN monomer is comprised of three types of repeating units designated type I, II, and III (2). Some of these repeats bind to cell surface and extracellular matrix components such as integrins, collagens, heparin, and fibrin. Several of these binding activities have been assigned to the motif sequences in FN, including the Arg-Gly-Asp (RGD) motif in the type III₁₀ domain (3), the Pro-His-Ser-Arg-Asn (PHSRN) motif in the type III₇ domain (4), and the CS-1 sequence in the III-CS region (5). Consequently, this multifunctional glycoprotein mediates a variety of cellular functions including cell adhesion, cell migration, and cell differentiation.

FN molecules have multiple isoforms generated from a single gene by alternative splicing of combinations of 3 exons: extra domain-A (EDA), extra domain-B (EDB), and III-CS. Both EDA and EDB exons are type III repeating units (6). Plasma fibronectin (pFN), produced by hepatocytes and abundant in plasma, lacks both the EDA and EDB domains. However, cellular FNs (cFNs), many of which are insoluble and incorporated into the pericellular matrix, contain the EDA and EDB segments in various combinations. The EDA domain is present in FN molecules produced during embryonic development (7). However, its presence in adults is minimal except in some disease states such as rheumatoid arthritis (8), wound healing (9), epithelial fibrosis (10), and vascular intimal proliferation (11). Thus, the highly regulated splicing of the EDA domain into transcripts suggests significantly different FN functions. It was reported that pFN and cFN differ in structural and physical properties such as glycosylation (12) and solubility (13) as well as presence of EDA domain. However, the biological functions the EDA domain confers upon these molecules are still poorly understood (7).

In this study, the biological functions of the FN EDA domain were studied in synovial cells and chondrocytes, which are integral components of joint connective tissue metabolic regulation. The results show induction of MMPs and pro-inflammatory cytokines occurs in response to exposure to the EDA domain. This observation, that a distinct domain of an extracellular matrix protein can trigger gene expression, has profound implications on biological and pathologic processes.  

EXPERIMENTAL PROCEDURES  
Preparation of Recombinant FN Type III Repeat Proteins—cDNAs encoding individual human fibronectin type III repeats were amplified in lar endothelial cell growth factor; IL-1RA, interleukin-1 receptor antagonist; FBS, fetal bovine serum; ELISA, enzyme-linked immunosorbent assay; RT-PCR, reverse transcription-polymerase chain reaction; DMEM, Dulbecco’s modified Eagle’s medium; mAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis; G3PDH, glyceraldehyde-3-phosphate dehydrogenase.
by reverse transcription-polymerase chain reaction (RT-PCR) of human liver mRNA (CLONTech, Palo Alto, CA), using Takara RNA PCR kit (AMV) version 2.1 (Takara, Kyoto, Japan) according to the manufacturer's instructions. The PCR profile consisted of 40 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min. After the final cycle, the reaction was extended at 72°C for 7 min. The primers for EDA (from Afn1600 through Thr1609) are the sense primer EDA-s, 5'-CCATATGACATGTGCGCCCTAAAGGACT-3' and the antisense primer EDA-a, 5'-AGCGGCCGTCTGGACTGTTCAATCAGGGG-3'. The primers for III1 as depicted in Fig. 9a, are the sense primer III1-s, 5'-CATATTGAAATTGACAACTCACA-3' and the antisense primer III1-a, 5'-AGCGGCCGTCTGTTCTGCTGCTGCAAAC-3'. The primers for III2, from Ala1690 through Thr1779 are the sense primer III2-s, 5'-CCATATGCTTTCTGCAACACTGTA-3' and the antisense primer III2-a, 5'-AGCGGCCGCTTGGTGGCAACGCT-3'.

EDA-induced Expression of MMP and IL-1

Gelatin Zymography—The culture supernatants of rabbit synovial cells were applied without reduction to a 10% polyacrylamide slab gel impregnated with 1 mg/ml gelatin. Gel electrophoresis was performed at 4°C. After electrophoresis, the gel was incubated in 2.5% (v/v) Triton X-100 for 1 h and then for 18 h at 37°C in 50 mM Tris-HCl, pH 7.5, containing 200 mM NaCl, 10 mM CaCl₂, 10 mM ZnCl₂, and 0.02% Brij-35. The gel was then stained with Coomassie Brilliant Blue.

Northern Blotting—Northern blotting was performed as described previously (17). Total RNA was isolated from rabbit synovial cells using an ISOGEN kit (WAKO, Osaka, Japan), according to the manufacturer's instructions. Total RNA (20 μg) was separated on agarose gels, transferred by Hybond-N nylon membrane (Amersham Pharmacia Biotech) and hybridized to 32P-labeled cDNA probes. Probes for rabbit MMP-1, rabbit MMP-3, rabbit MMP-2, rabbit MMP-9, and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) were prepared using a BcaBest Labelling Kit (Takara, Kyoto, Japan), as described previously (19). The probe for rabbit vascular endothelial cell growth factor (VEGF) was prepared from cDNA fragments obtained according to the method of Maniscalco et al. (19). Other probes were generated from cDNA fragments corresponding to nucleotide 6–496 for rabbit interleukin-1α (IL-1α) (GenBank accession number X02852) and nucleotide 227–778 for rabbit IL-1β (GenBank accession number M26295).

Cytostology—Rabbit synovial cells were cultured using a Lab-Tek Chamber Slide system (Nunc, Naperville, IL) in DMEM containing 10% FBS. Subconfluent synovial cells were treated with different reagents for 48 h. Slides were washed with phosphate-buffered saline, fixed with 3.7% paraformaldehyde for 20 min, permeabilized with ice-cold acetone, and stained with fluorescein-phalloidin (Molecular Probes, Eugene, OR) and propidium iodide (WAKO, Osaka, Japan). The coverslips were then mounted using a SlowFade-Light Antifade kit (Molecular Probes) and examined with confocal laser scanning microscopy (Olympus, Tokyo, Japan).

Preparation and Characterization of FN Proteins and an FN Protein Fragment—pFN was purified from human plasma as described previously (17). cFN, obtained from Upstate Biotech (Lake Placid, NY), was treated with the Kurimover II (Kurita, Tokyo, Japan) column according to the manufacturer's instructions to remove endotoxin. The EDA-positive FN protein fragment was prepared from human placenta. Placental tissue was homogenized and extracted in 4 M urea in 20 mM Tris-Cl (pH 7.5) containing 1 mM phenylmethylsulfonyl fluoride according to the method of Laine et al. (20). The extract was diluted 20-fold with 20 mM Tris-Cl (pH 7.5), 1 mM phenylmethylsulfonyl fluoride and applied to a Gelatin-Sepharose CL-4B column. The gelatin-Sepharose column was washed with 20 mM Tris-Cl (pH 7.5), 0.15 mM NaCl (TBS), 1 mM phenylmethylsulfonyl fluoride, then eluted with 1.5 M KBr-TBS, followed by 8x u.TBS. The EDA-positive FN fragment was found in fractions eluted with 8 M urea by Western blotting using rabbit anti-EDA polyclonal antibodies, which were raised against rEDA. These fractions were dialyzed against 10 mM phosphate buffer (pH 7.0) and further purified by hydroxyapatite chromatography (HAT-10, BioLogic system, Bio-Rad) with a 10–500 mM linear gradient of phosphate buffer.
RESULTS

Cartilage Catabolism Is Enhanced by Recombinant EDA Protein—The recombinant human FN type III repeat proteins, illustrated in Fig. 1A, were expressed in E. coli as 6×His fusion proteins. Each protein was then purified on a TALON Metal Affinity Resin column and gave a single band on SDS-PAGE (Fig. 1B). The purified recombinant protein encoding the EDA domain (rEDA) was added to rabbit cartilage explant cultures to determine whether the EDA domain affects cartilage catabolism. rEDA increased proteoglycan release 3.6 times more than the unstimulated control level (Fig. 2). However, its adjacent type III repeat, III11, did not significantly increase proteoglycan release. This result suggests that rEDA facilitates cartilage catabolism, possibly by inducing or modulating protease expression.

rEDA Induces Expression of MMPs—Rabbit chondrocyte and synovial cell monolayers were treated for 48 h with rEDA and other recombinant type III repeats. MMP-1 levels in the culture supernatants were then assessed by ELISA to determine whether rEDA affects protease levels or not. In chondrocyte cultures, Fig. 3A shows MMP-1 levels markedly increased to more than 1.5 mg/ml after treatment with rEDA concentrations ranging from 30 to 300 nM. In contrast, MMP-1 levels were negligible in unstimulated control cultures (Fig. 3A). This induction was specific for rEDA, since treatment with either of its adjacent type III repeats III11 or III12 did not increase MMP-1 levels. Similar increased MMP-1 levels due to rEDA treatment were observed in synovial cell cultures (Fig. 3B). In addition to ELISA experiments, Western blotting analysis detected pro-MMP-1 (55 and 57 kDa) and pro-MMP-3 (53 kDa) in the culture supernatants of synovial cells treated with rEDA, but not in cell culture supernatants of unstimulated cultures or cultures treated with III11 or III12 (Fig. 4, A and B). These data are consistent with previous reports that MMP-1 and MMP-3 proenzymes were detected in stimulated synovial cell cultures (21) and chondrocyte cultures (22). In addition, gelatin zymography showed that gelatinase activity corresponding to pro-MMP-9
increased slightly in synovial cell cultures after treatment with rEDA, but was negligible in unstimulated control cultures (Fig. 4C). Another gelatinase activity corresponding to pro-MMP-2, which is constitutively expressed (22), was not affected by rEDA treatment (Fig. 4C). Thus, it is unlikely that the increased production of MMP-1, -3, and -9 by rEDA is due to a general induction of protein synthesis. Northern blotting analysis of MMP transcripts in rabbit synovial cells was performed to determine whether rEDA induces transcription of MMPs. As shown in Fig. 5, rEDA treatment increased MMP-1 mRNA level. However, neither III11 nor III12 protein treatment significantly increased MMP-1 mRNA level. These observations are in good agreement with the protein results. rEDA also induced transcription of MMP-3 and MMP-9 mRNA. Any change was scarcely observed in the level of MMP-2 mRNA, but its doublet signals are consistent with a previous report (18). Thus, results from protein and mRNA experiments show that FN EDA domain treatment promotes expression of MMPs at the transcription level.

rEDA Induces Cytoskeletal Reorganization—Because rabbit synovial cell MMP genes responded differently to treatment with rEDA or other recombinant FN type III repeats, cell morphology was examined to determine whether rEDA causes any changes to cell shape or cytoskeletal structure. F-actin microfilaments in synovial cell monolayers were stained with fluorescein-labeled phalloidin after treatment with rEDA for 48 h (Fig. 6). In untreated cultures, cell morphology was fibrous, with F-actin stress fibers aligned parallel to the long axis of the cell (Fig. 6, A and D). This is consistent with the fibroblast-like shape of synovial cell monolayers previously reported (23). In contrast, cells treated with rEDA showed a marked change to a more rounded morphology, with partial disassembly of F-actin stress fibers (Fig. 6, B and E). No morphologic change or change in F-actin fibers was observed in cells treated with III11 protein (Fig. 5, C and F). Thus, rEDA appears to cause reorganization of F-actin stress fibers, leading to morphologic change of synovial cells.

rEDA Induces Pro-inflammatory Cytokine Gene Expression—Since morphologic change in synovial cells was induced by rEDA, an examination of other genes possibly induced by rEDA was undertaken. Cytokine gene expression was analyzed by Northern blotting of mRNA from synovial cells treated with rEDA. IL-1α and IL-1β genes were induced by rEDA, but not by its adjacent type III repeats III11 or III12, as measured by increases in mRNA levels (Fig. 7A). Transcripts of these cytokines were detected as early as 1 or 3 h after treatment, with expression peaking at 8 h (Fig. 7B). In contrast, the VEGF gene, whose product has been reported in the synovium of rheumatoid arthritis patients with arthritis but not healthy individuals (24), was expressed in unstimulated synovial cells but was not induced by rEDA. Thus, rEDA induces IL-1 gene expression in addition to MMP gene expression.

IL-1 Mediates rEDA-induced MMP-1 Expression—A North-
treated with rEDA. A, total RNA (20 μg) from rabbit synovial cells unstimulated or treated with 300 nM rEDA, III11 or III12 for 16 h was blotted and then hybridized to 32P-labeled cDNAs encoding IL-1α, IL-1β, VEGF, and G3PDH. B, time course of rEDA-induced gene expression. Total RNA (20 μg) from rabbit synovial cells treated with rEDA (300 nM) for 0, 1, 3, 8, or 16 h was blotted and then hybridized to 32P-labeled cDNAs encoding IL-1α, IL-1β, VEGF, MMP-1, and G3PDH. The results show the 160-kDa EDA-positive FN fragment was recognized by FN9-1, FN4C-4, and FN12-8, but not by FNH3-8 and FN8-12 (Fig. 10D), whereas pFN was recognized by all of these mAbs (Fig. 10C). In addition, protein sequencing found the NH2-terminal sequence of this fragment was blocked, just like an intact FN molecule. Thus, the epitope mapping suggests this fragment contains the NH2 terminus through the sequence of the induced IL-1α and IL-1β. The addition of 100 ng/ml IL-1RA resulted in inhibition of rEDA-induced MMP-1 production by 4.3-fold (Fig. 8). These results suggest that EDA-induced MMP-1 expression is mediated, at least in part, by IL-1 cytokines.

**Structures Surrounding the EDA Domain Required for MMP-1 Inducing Activity**—To examine the effects of surrounding structures on MMP-1 inducing activity of EDA, recombinant FN fragment proteins with several permutations of type III repeats consisting of III11, EDA, and III12, were generated as depicted in Fig. 9A. Neither III11-EDA-III12 nor III11-III12 induced MMP-1 production. In contrast, III11-EDA and EDA-III12 could induce MMP-1 production although they were less potent than rEDA (Fig. 9B). These results may suggest that exposure of either the NH2 or COOH terminus of EDA domain releases the inducing activity. In agreement with these results, cFN did not promote MMP-1 expression, even at concentrations up to 300 nM (Fig. 9B). pFN, which does not have an EDA domain, did not induce MMP-1 expression from the basal level either (Fig. 9B), which is consistent with the report of Arner et al. (22).

**EDA Domain Activities in FN Fragments Purified from Placental Tissue**—Purification of FN fragments from human placenta was performed to explore the existence of FN fragments containing the EDA domain in vivo. One candidate FN fragment was positive in Western blotting using anti-rEDA antibodies and migrated to 160 kDa in SDS-PAGE analysis under nonreducing conditions (Fig. 10B). The smaller apparent molecular weight than intact FN suggests the absence of several domains. In order to determine which domain was included in this fragment, ELISA was performed using several anti-FN mAbs that recognize the particular FN regions as shown in Fig.

**Discussion**

The present study demonstrated that treatment with rEDA protein facilitates cartilage catabolism and markedly induces expression of MMP in chondrocytes and synovial cells. The induction of MMP-1 was specific for rEDA among adjacent FN type III proteins tested. The physical properties of the cartilage matrix, which distribute loads over bone surfaces and provide a low-friction surface over which bones can move, are regulated through the biosynthesis and degradation of extracellular matrix by chondrocytes and synovial cells (25). In both healthy and pathologic conditions, the biological functions of synovial cells and chondrocytes are regulated through interactions with the extracellular matrix molecules, including FN, collagen, and other glycoproteins, and various mediators such as cytokines (22, 26). Indeed, cellular interaction with structural components of the extracellular matrix influences expression of many proteases, including MMP-1 induction by type I and III collagens (27), urokinase-type plasminogen activator induction by laminin (28), and MMP-2 induction by vitronectin (29). However, this study is the first demonstration that MMP expression of synovial cells and chondrocytes is regulated by the FN EDA domain. Induction of MMP, which is implicated in biological processes such as inflammation (30) and angiogenesis (31), may result in matrix degradation and remodeling of extracellular matrix structure, since the MMP family degrades extracellular matrix proteins (32). Thus, the finding that rEDA induces MMPs may indicate a role for the EDA domain in regulating functions of chondrocytes and synovial cells in joint connective tissue.

rEDA treatment causes cell rounding with accompanying
F-actin stress fiber reorganization of synovial cells in addition to induction of MMPs. Recently, it has been reported that anti-integrin \( \alpha_5\beta_1 \) antibody triggers cytoskeletal reorganization by a small GTP-binding protein Rac1-dependent mechanism, resulting in induction of MMP-1 (26). Thus, modulation of the extracellular matrix by the EDA domain might influence cytoskeletal structure, leading to MMP-1 expression. Furthermore, regulation of cell shape by the extracellular matrix triggers diverse signaling pathways and expression of many genes (33–35). As the data indicate, rEDA triggers gene expression of pro-inflammatory cytokines, including IL-1\( \alpha \) and IL-1\( \beta \). Expression of IL-1 genes increased as early as 1 or 3 h after rEDA treatment, while MMP-1 gene expression required 8 h. Delay of MMP-1 gene expression suggests that it is a secondary event in response to rEDA treatment. IL-1 may subsequently induce MMP-1 expression, supported by the finding that IL-1 (14) induces MMP-1, -3, and -9, similar to induction by rEDA. This hypothesis is strongly supported by the partial reduction of MMP-1 production by IL-1RA. Taken together, these results indicate that modulation of the extracellular matrix by rEDA and subsequent cytoskeletal reorganization activate an autocrine loop of IL-1 (36), leading to expression of MMP genes.

The examination of the effects that flanking sequences may have on EDA, using recombinant FN fragments encoding several permutations of type III repeats consisting of III\(_{11}\), EDA, and III\(_{12}\), shows connection with an adjacent type III repeat on either the NH\(_2\) or COOH side of EDA (III\(_{11}\)-EDA, EDA-III\(_{12}\)) reduced the potency of EDA to induce MMP-1 production. Moreover, the flanking sequences on both sides of EDA (III\(_{11}\)-EDA-III\(_{12}\)) abolished the induction ability, which is in good agreement with the failure to detect the potency in full-length cFN. It is known that cleavage of extracellular matrix proteins can mediate cellular responses to changes in the microenvironment (37), such as cleavage of laminin-5 that induces cell migration (38) and cleavage of plasminogen that generates angiostatin (39). Therefore, these results suggest that the activity of EDA to induce MMP-1 is masked by adjacent type III repeats and disclosed by removal of them. The failure to detect MMP-1-inducing potency in cFN is not consistent with the report that the effect of EDA domain was detected in cFN on activation of lipocytes (40). However, the recent study showed that cFN did not affect \( \alpha \)-smooth muscle actin expression in fibroblasts, whereas rEDA protein modulated expression of \( \alpha \)-smooth muscle actin and type I collagen during myofibroblas-
tic phenotype induction by transforming growth factor-β1 (41). Inability of exogenous cFN to affect α-smooth muscle actin expression might be consistent with the present study with synovial fibroblasts. The effect of the EDA domain on fibroblasts might be regulated by a slightly different mechanism than lipocyte activation.

According to the results of permutated FN fragments, the existence of potent FN fragments containing EDA was preliminarily explored in vivo to suggest their biologic relevance. A 160-kDa EDA-positive FN fragment was found, which induced
MMP-1 production. The COOH terminus of the 160-kDa FN fragment was missing just posterior to the EDA domain as determined by mAb epitope mapping. Therefore, the exposure of COOH terminus of EDA domain by proteolytic cleavage would give the 160-kDa EDA-positive FN fragment this potency. In synovial fluids of patients with arthritis, EDA-positive FN proteins were detected as fragments weighing 220, 200, 180, 170, 110, and 100 kDa (42). Expression of FN containing the EDA domain is increased in cartilage and synovium of patients with arthritis (8, 42). In addition, proteases including MMPs increase in synovial fluids of patients with arthritis (43).

In summary, treatment with rEDA protein facilitated cartilage catabolism and markedly induced expression of MMP in chondrocytes and synovial cells. In addition, rEDA induced cytoskeletal reorganization and expression of IL-1 in synovial cells, leading to subsequent expression of MMPs containing EDA domain, leading to further deterioration of pathogenesis, by induction of proinflammatory cytokines and subsequent MMPs.

Acknowledgments—We thank Steven E. Johnson for editing the manuscript. We also thank Dr. Jun Takasaki for helpful discussions.

REFERENCES

1. Hynes, R. O. (1990) Fibronectin. Springer-Verlag, New York
2. Petersen, T. E., Thogersen, H. C., Skorstengaard, K., Vibe Pedersen, K., Sahl, P., Sottrup Jensen, L., and Magnusson, S. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 137–141
3. Fierschbacher, M. D., and Rusolaiti, E. (1984) Nature 309, 30–33
4. Aota, S., Nomizu, M., and Yamada, K. M. (1994) J. Biol. Chem. 269, 24756–24761
5. Humphries, M. J., Komoriya, A., Akiyama, S. K., Olden, K., and Yamada, K. M. (1987) J. Biol. Chem. 262, 6886–6892
6. Bennett, V. D., Pallante, K. M., and Adams, S. L. (1991) J. Biol. Chem. 266, 5918–5924
7. Fiersch-Constant, C. (1995) Exp. Cell Res. 221, 261–271
8. Hino, K., Shiozawa, S., Kuroki, Y., Ishikawa, H., Shiozawa, K., Sekiguchi, K., Hirano, H., Sakashita, E., Miyashita, K., and Chihara, K. (1995) Arthritis Rheum. 38, 678–683
9. Fiersch-Constant, C., Van de Water, L., Dvorak, H. F., and Hynes, R. O. (1989) J. Cell Biol. 109, 903–914