

INVESTIGATIVE REPORT

Matrix Metalloproteinases-2 and -9 are Secreted from Human FibroblastsTAKASHI KOBAYASHI¹, SHUNJI HATTORI² and HIROSHI SHINKAI¹¹Department of Dermatology/Clinical Biology of Extracellular Matrix (F7), Chiba University School of Medicine, Chiba, Japan and ²Nippi Research Institute of Biomatrix, Tokyo, Japan

To investigate the pathophysiological role of fibroblasts in tissue metabolism, we analysed gelatin-degrading enzymes secreted from skin cells in culture. Using a gelatin-zymography method, matrix metalloproteinase (MMP)-2 was constitutively secreted from fibroblasts, whereas both MMP-2 and MMP-9 were secreted from keratinocytes. MMP-9 expression by fibroblasts was induced by the addition of transforming growth factor (TGF)- β 1 or tumour necrosis factor (TNF)- α to the cultures. Moreover, TGF- β 1, TNF- α , or epidermal growth factor augmented the secretion of MMP-2 from fibroblasts. These results indicate that MMP-9 together with MMP-2 secreted from fibroblasts could play important roles in tissue metabolism, including cytokine-induced inflammation. Key words: MMP-2; MMP-9; epidermal growth factor; transforming growth factor- β 1; tumour necrosis factor- α ; fibroblast.

(Accepted December 16, 2002.)

Acta Derm Venereol 2003; 83: 105–107.

Takashi Kobayashi, Department of Dermatology/Clinical Biology of Extracellular Matrix (F7), Chiba University School of Medicine, 1-8-1, Inohana, Chuo-ku, Chiba 260-8677, Japan. E-mail: tkobayas@derma01.m.chiba-u.ac.jp

More than 20 species of matrix metalloproteinases (MMPs) have been reported to play important roles in the metabolism of the extracellular matrix, including pathological situations (1). Among them, MMP-2 and MMP-9, also called gelatinases A and B, are reported to cleave a wide variety of substrates, although their primary substrates are considered to be gelatin (1, 2). Gelatin-zymography is useful not only for identifying gelatinolytic enzymes, but also for quantitating them (3–6). Here, we report the secretion of these two kinds of gelatinases from cultured human fibroblasts and keratinocytes using the gelatin-zymography method.

MATERIALS AND METHODS

Human fibroblasts were derived from outgrowths of normal human dermis and were split equally into each well of 24 well plates in Dulbecco's modified Eagle's medium with 10% fetal calf serum. Human keratinocytes were cultured using

keratinocyte-serum free medium (SFM) (Life Technologies, Gaithersburg, MD) containing 0.09 mM Ca²⁺ without added serum, as previously reported (4–6). Conditioned media were harvested for 48 h after the cells reached subconfluence. For fibroblasts, the conditioned medium from each well was collected for 48 h after changing into SFM. For experiments concerning the effects of cytokines, human epidermal growth factor (EGF) (Sigma, St. Louis, MO, USA), tumour growth factor (TGF)- β 1 (Genzyme, Cambridge, MA, USA), or tumour necrosis factor (TNF)- α (Sigma) was added to the medium without serum for 48 h. No significant morphological changes were observed in any of these fibroblast growth conditions and there were no significant differences concerning the proliferation of fibroblasts as determined using a cell counting kit-8 (Dojindo, Kumamoto, Japan).

Gelatin-zymography analyses were performed as previously reported (4–6). Aliquots of conditioned media (without reduction or boiling) were separated by SDS-polyacrylamide gel electrophoresis on 7.5% gels containing 0.5% gelatin. After electrophoresis, the gels were washed with 2.5% Triton X-100 for 1 h. Gelatinolytic zymography reactions were then induced by incubating the gels in buffer (0.05 M Tris-HCl, pH 7.4, 0.15 M NaCl, 5 mM CaCl₂ and 0.02% NaN₃) at 35°C for appropriate periods of time (usually 5 h, or 10 h for more sensitive detection such as MMP-9 from fibroblasts). The gels were stained with 0.1% amide black B-10 and then destained with 10% acetic acid and 30% methanol. The intensity of the bands was read using NIH image 1.55 and the content of MMP-2 was calculated from the measurement of known amounts of the purified reagent using a gelatin Sepharose column (Pharmacia, Uppsala, Sweden) and a concanavalin A Sepharose column (Pharmacia) as previously reported (4, 6).

RESULTS

Gelatin-zymography revealed that human fibroblasts without added cytokines secreted only MMP-2 (a 72 kDa band). In contrast, keratinocytes secreted MMP-9 (a 92 kDa band) as well as MMP-2 (Fig. 1A). These results are in accordance with previous reports (1–9).

The quantitation of MMP-2 activity by gelatin-zymography is shown in Fig. 1B. The secretion of MMP-2 was augmented in a dose-dependent manner by TGF- β 1 (about 1.5-fold), by TNF- α (1.5-fold), or by EGF (approximately 2-fold) (Fig. 2). In addition, not only MMP-2 but also MMP-9 was secreted from fibroblasts following the addition of TGF- β 1 or TNF- α . EGF apparently had no effect on MMP-9 secretion by fibroblasts (Fig. 2).

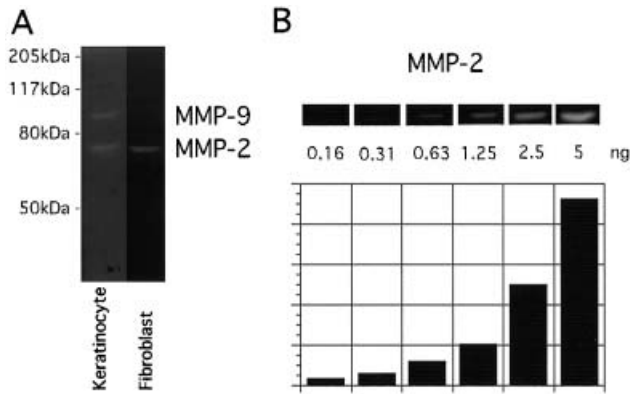


Fig. 1. Gelatin-zymography showing a comparison of conditioned media obtained from fibroblasts or from keratinocytes (A) and the standard curve obtained from known amounts of MMP-2 (B). Keratinocytes secreted MMP-2 (the 72 kDa band) and MMP-9 (the 92 kDa band). Fibroblasts secreted only MMP-2 under normal conditions.

DISCUSSION

Not only gelatin, but also other common substrates have been reported for MMP-2 and MMP-9, although the enzymes are distinctive gene products and have their own gene regulatory elements (1, 2). For instance, three TPA-responsive elements (TREs), NF-κB and our recently identified KRE-M9 element are present in the MMP-9 promoter, but have not been identified as MMP-2 gene regulatory elements (9–12). The specific secretion of MMP-9 from keratinocytes, but not from fibroblasts in the absence of cytokines, might be explained by the existence of these three TREs, as has been reported for the specific human laminin A3 gene expression by keratinocytes (13). In this respect, a remote 5'-flanking region in the murine MMP-9 promoter has also been reported to be responsible for tissue specific MMP-9 expression (14).

In a previous report, the secretion of MMP-2 from fibroblasts was augmented by treatment with TGF-β1, while secretion of MMP-9 was induced by TNF-α and

synergistically stimulated by both cytokines (7, 9). Although our results are basically in accordance with this, the secretion of MMP-2 was also shown to be induced by TNF-α and EGF.

MMP-9 from polymorphonuclear leucocytes has been reported to play a role in blister formation (15–17), and in tumour invasion and metastasis (18, 19). In addition, MMP-9 from keratinocytes reportedly works in wound healing (20–22), and in apoptosis after UVB irradiation (23, 24). EGF, which is known to work in wound healing and to induce MMP-9 expression by keratinocytes (20), augmented MMP-2 expression without MMP-9 expression by fibroblasts. Induction of MMP-9 expression in fibroblasts, as shown here by TGF-β1, also known for its role in wound healing (7, 21), could play a similar role in wound healing, as was previously proposed in the case of MMP-2 induction (7). These results indicate that MMP-2 secreted from fibroblasts has at least some effect on wound healing through the distinctive signal transduction mechanism(s) of TGF-β1 and EGF.

In contrast, TNF-α, which is known as an inflammatory cytokine as well as an apoptotic one (25), reportedly induces MMP-9 expression by fibroblasts through TRE and NF-κB (8, 9). In keratinocytes, the same mechanism of MMP-9 induction after UVB irradiation was reported (23). In this relation, TGF-β1 has been reported to induce apoptosis of keratinocytes (24), and a synergistic effect of TGF-β1 and TNF-α on the TRE and NF-κB elements in the case of MMP-9 induction has been reported (9). We recently reported that MMP-9 expression by keratinocytes is induced through the KRE-M9 element, which is probably common to involucrin expression by high Ca²⁺ induction in the model of keratinization, a kind of programmed cell death (11). In addition to MMP-9, the expression of MMP-2 induced in fibroblasts by TNF-α and/or by TGF-β1 (as shown here) may also suggest their roles in inflammation or apoptosis, although

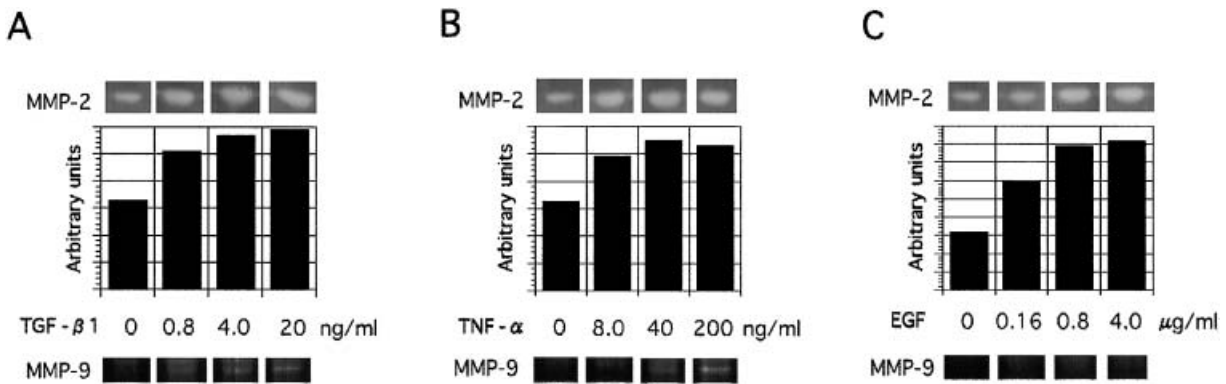


Fig. 2. Gelatin-zymography patterns of MMP-2 (top panel) and MMP-9 (lower-panel) from fibroblasts 48 h after the addition of transforming growth factor (TGF)-β1 (A), tumor necrosis factor (TNF)-α (B) or epidermal growth factor (EGF) (C), at the concentrations noted. The bar-graphs indicate MMP-2 activity as measured by gelscanning. TGF-β1 and TNF-α augmented MMP-2 secretion and induced MMP-9, while EGF induced MMP-2 secretion without inducing MMP-9.

the specific responsive element involved needs to be elucidated in the case of MMP-2 induction by TNF- α .

Taken together, secretion of MMP-2 and/or MMP-9 from fibroblasts seems to work on tissue metabolism in harmonized ways via regulation by cytokines, although the specific substrates for these two enzymes *in vivo* remain to be elucidated.

ACKNOWLEDGEMENT

This work was supported by a Grant-in-Aid for Scientific Research (C) from the Japan Society for the Promotion of Science, Japan.

REFERENCES

- McCawley LJ, Matrisian LM. Matrix metalloproteinases: they're not just for matrix anymore! *Curr Opin Cell Biol* 2001; 13: 534–540.
- Wilhelm SM, Collier IE, Marmer BL, Eisen AZ, Grant GA, Goldberg GI. SV40-transformed human lung fibroblasts secrete a 92-kDa type IV collagenase which is identical to that secreted by normal human macrophages. *J Biol Chem* 1989; 264: 17213–17221.
- Kleiner DE, Stetler-Stevenson WG. Quantitative zymography: detection of picogram quantities of gelatinases. *Anal Biochem* 1994; 218: 325–329.
- Kobayashi T, Hattori S, Nagai Y, Tajima S, Nishikawa T. Differential regulation of MMP-2 and MMP-9 gelatinases in cultured human keratinocytes. *Dermatology* 1998; 197: 1–5.
- Kobayashi T, Hattori S, Nagai Y, Tajima S. Differential regulation of the secretions matrix metalloproteinase-9 and tissue inhibitor of metalloproteinases-1 from human keratinocytes in culture. *IUBMB Life* 2000; 50: 221–226.
- Danno K, Mori N, Toda K, Kobayashi T, Utani A. Near-infrared irradiation stimulates cutaneous wound repair: laboratory experiments on possible mechanisms. *Photodermatol Photoimmunol Photomed* 2001; 17: 261–265.
- Overall CM, Wrana JL, Sodek J. Independent regulation of collagenase, 72-kDa progelatinase, and metalloendoproteinase inhibitor expression in human fibroblasts by transforming growth factor- β . *J Biol Chem* 1989; 264: 1860–1869.
- Segain JP, Harb J, Gregoire M, Meflah K, Menanteau J. Induction of fibroblast gelatinase B expression by direct contact with cell lines derived from primary tumor but not from metastases. *Cancer Res* 1996; 56: 5506–5512.
- Han YP, Tuan TL, Hughes M, Wu H, Garner WL. Transforming growth factor- β - and tumor necrosis factor- α -mediated induction and proteolytic activation of MMP-9 in human skin. *J Biol Chem* 2001; 276: 22341–22350.
- Sato H, Seiki M. Regulatory mechanism of 92 kDa type IV collagenase gene expression which is associated with invasiveness of tumor cells. *Oncogene* 1993; 8: 395–405.
- Kobayashi T, Kishimoto J, Ge Y, Jin W, Hudson DL, Ouahes N, et al. A novel mechanism of matrix metalloproteinase-9 gene expression implies a role for keratinization. *EMBO Rep* 2001; 2: 604–608.
- Qin H, Sun Y, Benveniste EN. The transcription factors Sp1, Sp3, and AP-2 are required for constitutive matrix metalloproteinase-2 gene expression in astrogloma cells. *J Biol Chem* 1999; 274: 29130–29137.
- Violle T, Djabari Z, Ortonne JP, Aberdam D. DNA conformation driven by AP-1 triggers cell-specific expression via a strong epithelial enhancer. *EMBO Rep* 2000; 1: 328–333.
- Munaut C, Salonurmi T, Kontusaari S, Reponen P, Morita T, Foidart JM, et al. Murine matrix metalloproteinase 9 gene. 5'-upstream region contains cis-acting elements for expression in osteoclasts and migrating keratinocytes in transgenic mice. *J Biol Chem* 1999; 274: 5588–5596.
- Liu Z, Shipley JM, Vu TH, Zhou X, Diaz LA, Werb Z, et al. Gelatinase B-deficient mice are resistant to experimental bullous pemphigoid. *J Exp Med* 1998; 188: 475–482.
- Kobayashi T, Sakurao K, Iwamoto M, Kurihara S. A case of anaphylactoid purpura with multiple blister formation: possible pathophysiological role of gelatinase (MMP-9). *Dermatology* 1998; 197: 62–64.
- Verraes S, Hornebeck W, Polette M, Borradori L, Bernard P. Respective contribution of neutrophil elastase and matrix metalloproteinase 9 in the degradation of BP180 (type XVII collagen) in human bullous pemphigoid. *J Invest Dermatol* 2001; 117: 1091–1096.
- Stähle-Bäckdahl M, Parks WC. 92-kd gelatinase is actively expressed by eosinophils and stored by neutrophils in squamous cell carcinoma. *Am J Pathol* 1993; 142: 995–1000.
- Coussens LM, Tinkle CL, Hanahan D, Werb Z. MMP-9 supplied by bone marrow-derived cells contributes to skin carcinogenesis. *Cell* 2000; 103: 481–490.
- McCawley LJ, O'Brien P, Hudson LG. Epidermal growth factor (EGF)- and scatter factor/hepatocyte growth factor (SF/HGF)-mediated keratinocyte migration is coincident with induction of matrix metalloproteinase (MMP)-9. *J Cell Physiol* 1998; 176: 255–265.
- Salo T, Lyons JG, Rahemtulla F, Birkedal-Hansen H, Larjava H. Transforming growth factor- β 1 up-regulates type IV collagenase expression in cultured human keratinocytes. *J Biol Chem* 1991; 266: 11436–11441.
- Mohan R, Rinehart WB, Bargagna-Mohan P, Fini ME. Gelatinase B/lacZ transgenic mice, a model for mapping gelatinase B expression during developmental and injury-related tissue remodeling. *J Biol Chem* 1998; 273: 25903–25914.
- Fisher GJ, Wang ZQ, Datta SC, Varani J, Kang S, Voorhees JJ. Pathophysiology of premature skin aging induced by ultraviolet light. *N Engl J Med* 1997; 337: 1419–1428.
- Benassi L, Ottani D, Fantini F, Marconi A, Chiodino C, Giannetti A, et al. 1,25-dihydroxyvitamin D₃, transforming growth factor β 1, calcium, and ultraviolet B radiation induce apoptosis in cultured human keratinocytes. *J Invest Dermatol* 1997; 109: 276–282.
- Reinhard C, Shamon B, Shyamala V, Williams LT. Tumor necrosis factor α -induced activation of c-jun N-terminal kinase is mediated by TRAF2. *EMBO J* 1997; 16: 1080–1092.