A novel mechanism of matrix metalloproteinase-9 gene expression implies a role for keratinization

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To investigate the pathophysiological role of matrix metalloproteinase (MMP)-9 in the skin, we analyzed MMP-9 expression from human keratinocytes in culture. MMP-9 and the terminal differentiation marker involucrin were co-localized in the same keratinocytes with a high concentration of Ca^{2+} , a potent stimulator of differentiation. We identified the novel KRE-M9 element, further downstream to the previously reported TPA responsive element in the MMP-9 promoter, and both of these two elements were shown to be important for MMP-9 transcription and Ca^{2+} induction. The concomitant upregulation of MMP-9 and involucrin transcripts was probably due to the very similar gene regulatory elements, KRE-M9 and KRE-4, in their respective promoters. These results indicate a novel mechanism of transcriptional regulation for MMP-9 in the process of keratinization, implying the probable association of apoptosis and differentiation of keratinocytes in epidermal skin tissue.

INTRODUCTION

Matrix metalloproteinase (MMP)-9, also known as gelatinase B (Birkedal-Hansen, 1995; Mohan *et al.*, 1998; Vu *et al.*, 1998; Liu *et al.*, 2000), is considered to play important roles in the tissue metabolism, such as tumor invasion and metastasis (Wilhelm *et al.*, 1989; Sato and Seiki, 1993; Ståhle-Bäckdahl and Parks, 1993; Kobayashi *et al.*, 1996), wound healing (Salo *et al.*, 1991; Mohan *et al.*, 1998), blister formation (Liu *et al.*, 2000) and apoptosis (Vu *et al.*, 1998). Other than gelatin, numerous substrates have been investigated *in vitro* for MMP-9 and MMP-2 (Birkedal-Hansen, 1995), although the substrate of these two enzymes *in vivo* has not been well elucidated. MMP-2 has close substrate specificity to MMP-9 and is also known as gelatinase A,

but is in fact a distinctive gene product (Birkedal-Hansen, 1995). MMP-9 is produced by a variety of epithelial tissues including keratinocytes, polymorphonuclear leukocytes and tumor cell lines (Birkedal-Hansen, 1995). Among several species of tissue inhibitor of metalloproteinases (TIMP), which are also known to be important substances for considering the mechanism of tissue metabolism *in vivo*, TIMP-1 is reportedly the relatively specific inhibitor for MMP-9 (Bertaux *et al.*, 1991; Birkedal-Hansen, 1995).

The epidermis, mainly composed of keratinocytes, is a stratifying squamous epithelia that is a biologically important tissue surrounding the whole body (Watt and Green, 1982; Fuchs, 1990; LaPres and Hudson, 1996; Lopez-Bayghen *et al.*, 1996). Keratinocytes in the proliferative basal cell layer express the envelope precursor protein, involucrin, and switch in keratin expression from K5/K14 to K1/K10 as they differentiate upward (Fuchs, 1990). Involucrin is a well characterized differentiation marker in epidermal keratinocytes (Watt and Green, 1982; Fuchs, 1990; LaPres and Hudson, 1996; Lopez-Bayghen *et al.*, 1996; Ng *et al.*, 2000) and the specific transcriptional element KRE-4 in its promoter has been identified (LaPres and Hudson, 1996). Keratinocytes undergo terminal differentiation into a cornified envelope and reach the outermost stratum corneum (Fuchs, 1990). This differentiating process of keratinization is also considered to be a kind of programmed cell death (Polakowska *et al.*, 1994).

UVB irradiation reportedly induces MMP-9 expression from the epidermis (Fisher *et al.*, 1997) and this process is thought to cause apoptosis of keratinocytes (Benassi *et al.*, 1997). We previously described the possible association between MMP-9 expression from human keratinocytes and their differentiation immunohistochemically using pathological specimens (Kobayashi

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et al., 1996). The aim of our current work is to further elucidate this association by investigating the gene regulatory mechanism.

RESULTS AND DISCUSSION

Simultaneous induction of MMP-9 and involucrin expressions

Following a double fluorescent labeling experiment, confocal laser microscopy revealed that MMP-9 positive cells after high Ca^{2+} stimulation were also involucrin-immunoreactive positive cells (Figure 1A). In addition, differentiated cells changed keratin expression from K14 to K10, which is shown to be co-localized with involucrin immunoreactivity (Figure 1A). Thus, these MMP-9 positive cells appear to undergo a Ca^{2+} -induced differentiation process. The induction of involucrin in differentiated keratinocytes was previously found to result from an increase in the involucrin mRNA level caused by transcriptional activation (LaPres and Hudson, 1996). *In situ* hybridization showed that, unlike MMP-2 mRNA, MMP-9 mRNA was increased in cultured keratinocytes after stimulation with a high Ca^{2+} concentration (Figure 1B), indicating that induction of MMP-9 immunoreactivity resulted from the transcriptional activation of the MMP-9 gene.

Transcriptional regulation by the KRE-M9 element in the MMP-9 promoter

The potential AP-2 binding sequence, the KRE-4 element of 10 bp (5'-GCCTGTCAGG-3'; -1686 to -1677) in the human involucrin promoter, was reported to have a characteristic sequence different from that of the AP-2 element (LaPres and Hudson, 1996; Lopez-Bayghen *et al.*, 1996). Interestingly, we identified a very similar sequence having only one base substitution for the KRE-4 element (5'-GCCTGTCAAG-3'; -66 to -57) in the human MMP-9 promoter (Sato and Seiki, 1993). We tentatively named this characteristic sequence the KRE-M9 element.

In the human MMP-9 promoter (Sato and Seiki, 1993), the KRE-M9 element (-66 to -57) is localized downstream close to the TPA responsive element (TRE; -79 to -73), where AP-1 proteins can bind (Figure 2). This TRE is reportedly important for MMP-9 transcriptional activity in the CAT reporter assay using tumor cell lines in culture (Sato and Seiki, 1993). However, our luciferase assays of keratinocytes in culture using the DNA fragment from the human MMP-9 promoter/enhancer region showed that some transcriptional activity and Ca^{2+} induction occurred using the KRE-M9 element, despite the absence of the TRE, although the promoter activity was partially lost and the ratio of Ca^{2+} induction was reduced. On the other hand, with the absence of the KRE-M9 element or mutation of KRE-M9 element for a 4-base substitution, promoter activity was completely lost (Figure 2). More interestingly, the mutations of KRE-M9 element for 2-base and 4-base substitutions with the presence of TRE caused inductions of MMP-9 transcriptional activities by several times (Figure 2). These results indicate the importance of the KRE-M9 element as well as that of TRE in MMP-9 transcription. In this respect, the requirement of an AP-1 site in the Ca^{2+} response region of the involucrin promoter in keratinocytes has also been reported (Ng *et al.*, 2000).

Fig. 1. Immunofluorescence and *in situ* hybridization patterns using human keratinocytes in culture. (A) After stimulation with a high Ca^{2+} concentration, MMP-9, K10 and K14 immunoreactivity were detected by FITC (green images in the left three panels), and involucrin immunoreactivity was detected by rhodamine (red images in the right three panels). The center three panels show the combined images produced by both the FITC and rhodamine dyes. MMP-9 and involucrin were colocalized in the exact same keratinocytes, and K10 and involucrin were colocalized, indicating a differentiated stage of individual keratinocytes. (B) Without additive Ca^{2+} in the left three panels, low signals of MMP-9 mRNA (anti-sense) and MMP-2 mRNA (anti-sense) were observed. With the addition of Ca^{2+} , a significantly high signal of MMP-9 mRNA (anti-sense) was observed in comparison with that of MMP-2 mRNA (anti-sense). No signals were observed in negative controls (MMP-9 sense). Scale bars indicate 100 μm .

Specificity of KRE-M9 and KRE-4 elements distinct from AP-2 element

In the gel shift assay experiment, the labeled AP-2 oligonucleotide was shifted together with AP-2 protein, which was competed by the unlabeled AP-2 oligonucleotide, but by neither the KRE-4 nor the KRE-M9 oligonucleotide (Figure 3A). On the other hand, the labeled KRE-4 and KRE-M9 oligonucleotides were shifted together with the nuclear extract from the keratinocytes, and

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Fig. 2. (A and B) Luciferase assay patterns. Each column on the right shows promoter activity. Cells were grown on tissue culture plates and aliquots of DNA were transfected using polybrene and dimethylsulfoxide. After 3 h, the concentration of Ca^{2+} was raised for the differentiation of keratinocytes. After 24 h, luciferase activities were measured. Each value was standardized according to the protein concentration. S2 and S4 represent the mutations of each of the 2- and 4-substituted sequences for KRE-M9, respectively. (C) MMP-9 sequence from -90 to +30 containing promoter region. TRE, KRE-M9, TATA box and the first methionine-coding site are underlined.

these shifts were competed by the unlabeled KRE-4 or KRE-M9 oligonucleotides, but not by the AP-2 oligonucleotide (Figure 3B). These data indicate that the KRE-4 and KRE-M9 oligonucleotides probably bind to the protein in the nuclear extract in the same manner, which differs from that of AP-2 protein.

The gel shifts of the labeled KRE-4 and KRE-M9 oligonucleotides were not competed by either the 2- or the 4-base substituted oligonucleotides (Figure 3C). In addition, the labeled oligonucleotide similarly found in the TIMP-1 promoter, which has the 3-base substitution for KRE-M9 in the MMP-9 promoter, failed to shift with the same nuclear extract (Clark *et al.*, 1997) (Figure 3C). These data indicate that this binding of the protein from the nuclear extract of keratinocytes to the KRE-M9 and KRE-4 elements involves highly sequence-specific events.

In Southwestern blotting, labeled KRE-4 and KRE-M9 oligos reacted with protein of the same molecular weight, ~90 kDa, which is similar in size to that of the previously reported KDF-1. However, we are unable to conclude that KRE-4 and KRE-M9 elements bind to exactly the same protein in the same manner until the protein is identified and characterized (Figure 4).

Fig. 3. Gel shift assay patterns. Aliquots of AP-2 extract (A) or the nuclear extract (B and C) were incubated with or without 50-fold excess of unlabeled competitor DNA. Following the subsequent addition of a labeled probe, the reaction was allowed to proceed. Reactions were assessed on a 5% polyacrylamide gel by electrophoresis, the gels dried and autoradiography performed. Arrows and arrowheads indicate the shifted signals and free probes, respectively.

In this study we showed a novel mechanism of transcriptional regulation for MMP-9. This regulation suggests the close association of MMP-9 expression and involucrin expression in keratinocytes. This is the first report to show the importance of the novel KRE-M9 element for MMP-9 transcription using not a cell line, but eventually mortal keratinocytes in culture. Although both the more concrete gene regulatory mechanism for MMP-9 expression and the specific substrate for MMP-9 *in vivo* remain to be clarified, MMP-9 does appear to have some involvement in the differentiation of keratinocytes, the process of keratinization that is biologically thought to be one of the most fundamental nature. The proliferation of keratinocytes by TIMP-1 could reflect inhibition of this role of MMP-9 for differentiation (Bertaux *et al.*, 1991).

Fig. 4. Southwestern blot analysis using the KRE-4 oligo in the involucrin promoter and the KRE-M9 oligo in the MMP-9 promoter. The labeled KRE-4 oligonucleotides (left panel) and KRE-M9 oligonucleotides (right panel) reacted with the nuclear protein in keratinocytes of the same molecular weight, ~90 kDa (arrow).

Speculation

MMP-9 expression from the epidermis was recently reported to be induced by the upregulation of AP-1 and of NF- κ B by UVB irradiation (Fisher *et al.*, 1997). In addition, TGF- β 1, which reportedly induces MMP-9 in keratinocytes (Salo *et al.*, 1991), and Ca²⁺ have been reported to cause apoptosis of keratinocytes (Benassi *et al.*, 1997). Although it remains controversial whether the etiologies of apoptosis and differentiation of keratinocytes share certain common features (Polakowska *et al.*, 1994; Gandarillas *et al.*, 1999), the simultaneous induction of MMP-9 and involucrin upon stimulation with Ca²⁺ could provide such evidence of this association.

METHODS

Culture of human keratinocytes. Newborn human foreskins were treated with dispase (25.0 caseinolytic units/ml) at 4°C for 16 h. After peeling away the epidermis from the dermis with forceps, the former was treated with 0.05% trypsin for 5 min. The isolated cells were then plated and cultured using keratinocyte-SFM medium (Life Technologies, Gaithersburg, MD) containing 0.09 mM Ca²⁺. For the differentiation of keratinocytes, the Ca²⁺ concentration of the medium was raised as described below.

Immunofluorescence. Monoclonal anti-K10 and K14 antibodies (Sigma Chemical Co., St Louis, MO), the monoclonal anti-MMP-9 antibody (Kobayashi *et al.*, 1996) and polyclonal anti-involucrin antibody (Biogenesis, Poole, UK) were used as primary antibodies. FITC-conjugated anti-mouse IgG and anti-mouse IgM antibodies, and rhodamine-labelled anti-rabbit IgG were used as secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA). Human keratinocytes were cultured on Lab-Tek chamber slides (Nalge Nunc International, Naperville, IL) in keratinocyte-SFM with the addition of calcium chloride at a final concentration of 1.5 mM for 24 h. Specimens were imaged using a Leica confocal laser scanning microscope (Leica TCS-NT, Leica

Microsystems, Buffalo, NY) as previously reported (Koch *et al.*, 1999).

In situ hybridization. Human MMP-2 and MMP-9 cDNAs inserted in pBluescript II (Stratagene, La Jolla, CA) were kindly provided by Dr G.I. Goldberg from the Division of Dermatology, Washington University School of Medicine, USA. Human keratinocytes cultured on Lab-Tek chamber slides with or without the addition of calcium chloride for 24 h were prepared as described above, and were fixed in 0.1% paraformaldehyde for 10 s. After being washed in phosphate-buffered saline, these specimens were processed according to the standard protocol of *in situ* hybridization as described elsewhere (Kishimoto *et al.*, 1994; Koch *et al.*, 1999).

Luciferase assay. The 730 bp fragment of the human MMP-9 promoter (−714 to +16) (Sato and Seiki, 1993) was obtained from human genomic DNA (Clontech, Palo Alto, CA) by PCR. Sequences were confirmed by dideoxy sequencing. The fragment was inserted using the *Kpn*I and *Xho*I multiple cloning sites into the pGL3-Basic Vector (Promega). Vectors containing shorter fragments (−165 to +16, −89 to +16, −80 to +16, −73 to +16 and −56 to +16) were prepared in the same manner with *Kpn*I and *Sph*I (747 of 4818 bp apart from the multiple cloning site) restriction enzyme sites. The vectors containing the 2- and 4-substituted sequences, which are the same as those used in the gel shift assay described below, were prepared using the Quick-Change site-directed mutagenesis kit (Stratagene). All plasmid DNA constructs were confirmed by direct DNA sequencing. Subconfluent cells were grown on six-well plastic tissue culture plates and 5 μ g of DNA per well were transfected using 10 μ g/ml polybrene and 27% dimethylsulfoxide as previously reported (Jiang *et al.*, 1991). After 3 h, the concentration of Ca²⁺ was raised to 1.5 mM for the differentiation of keratinocytes. After 24 h, luciferase activities were measured by the Luciferase assay system (Promega). Each value was standardized according to the protein concentration using the BCA protein assay kit (Pierce, Rockford, IL).

Gel shift assay. The nuclear extract was prepared from keratinocytes in keratinocyte-SFM medium without fetal calf serum as reported previously (Dignam *et al.*, 1983). Basal rich keratinocytes were cultured with additional Ca²⁺ (raised to 1.0 mM) for 2 days to allow for the differentiation of keratinocytes. Double-stranded DNA was prepared by annealing complementary strands of the following oligomers: KRE-4 oligo, 5′-GAAATGTGCCTGTCAGGAA-3′ (−1693 to −1675); KRE-M9 oligo, AGCACTTGCTGTCAGGAA-3′ (−73 to −55); 2-base substitution for KRE-M9 oligo, 5′-AGCACTTGTCATGCCAAGGA-3′; 4-base substitution for KRE-M9 oligo, 5′-AGCACTTGAATTCCAAGGA-3′; the oligo including KRE-M9-like sequence (3-base substitution) found in TIMP-1 promoter, 5′-CGGCCATGCCTGTGATCTT-3′ (−821 to −803); and human AP-2 consensus oligo, 5′-GATCGA-ACTGACCGCCCGCGCCCGT-3′ (Promega, Madison, WI) (Sato and Seiki, 1993; LaPres and Hudson, 1996; Lopez-Bayghen *et al.*, 1996; Clark *et al.*, 1997). The gel shift assay was performed as previously reported (Tamai *et al.*, 1994; LaPres and Hudson, 1996) using the gel shift assay system (Promega). Briefly, aliquots of AP-2 extract (1.4 μ g) or 6 μ g of protein of the nuclear extract were incubated at room temperature for 10 min with or without 50-fold excess of unlabeled competitor double-stranded DNA in Gel Shift Binding Buffer (Promega). Following the subsequent addition of a labeled probe (50 000 c.p.m.) by T4 polynucleotide

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kinase (Promega) in the presence of [γ - 32 P]ATP, the reaction was allowed to proceed at room temperature for an additional 20 min. Reactions were assessed on a 5% polyacrylamide gel (acrylamide:bis-acrylamide, 80:1) by electrophoresis, the gels dried and autoradiography performed overnight with an intensifier screen (BioMax MS, Kodak, Rochester, NY).

Southwestern blotting. The nuclear extract was electrophoresed separately in the 7.5% SDS–polyacrylamide gels after reduction with 2-mercaptoethanol. Approximately 20 μ g aliquots of nuclear extract were used for each lane. Proteins were transferred to nitrocellulose filters and Southwestern analysis was performed as previously reported (Tamai et al., 1994). Autoradiography was carried out using an intensifier screen (BioMax TranScreen HE, Kodak).

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