Epigallocatechin Gallate's Protective Effect against MMP7 in Recessive Dystrophic Epidermolysis Bullosa Patients

Sylvie Igondjo-Tchen-Changotade[1], Antoine Assoumou[1], Farida Guéniche[1], Florence Fioretti[1], Sylvie Séguier[1], Yves de Prost[2], Christine Bodemer[2], Gaston Godeau[1] and Karim Senni[1]

The analysis of phenotype–genotype correlations of patients suffering from recessive dystrophic epidermolysis bullosa (RDEB) evidenced intrafamilial and interfamilial phenotype variability occurring for the same mutation of COL7A1; this underscores the role of other genetics environmental factors in the expressivity of the disease. In this work, we checked whether matrilysin 1 (matrix metalloproteinase (MMP)7) could take part in the epidermal detachment in RDEB. Furthermore, we investigated epigallocatechin 3 gallate (EGCG) to determine whether it could inhibit matrilysin activities on collagen type VII and fibrillin 1 known to be associated with the dermo-epidermal junction. In this work, matrilysin 1 was detected in affected and unaffected skins of the three RDEB patients; furthermore, MMP7 was shown to degrade *ex vivo* on healthy normal skin collagen VII and fibrillin 1. Thus, we suspect that MMP7 could take an active part in the epidermal detachment occurring during RDEB. We evidenced that EGCG in *in vitro* as well as in *ex vivo* experiments was a good inhibitor of MMP7 and developed a good protection of collagen type VII and fibrillin 1 susceptible of being degraded by MMP7. We therefore propose that EGCG could be used beneficially in patients suffering from RDEB.

Journal of Investigative Dermatology (2007) **127,** 821–828. doi:10.1038/sj.jid.5700645; published online 30 November 2006

INTRODUCTION

In the most severe form of recessive dystrophic epidermolysis bullosa (RDEB) ''Hallopeau-Siemens'' type, collagen type VII is mostly absent but in some cases rare anchoring fibrils composed of this collagen type at the dermo-epidermal junction are still present (Bruckner-Tuderman *et al.*, 1989). A number of COL7A1 mutations have been reported in RDEB patients and the analysis of phenotype–genotype correlations has shown evidence of interfamilial and intrafamilial phenotypic variabilities occurring for the same mutations (Hovnanian *et al.*, 1997). These results underscore the role of other genetics or environmental factors in expression of the disease.

In a previous work conducted in three RDEB-affected sibling brothers with the same mutation in COL7A1, we evidenced that the amounts of matrix metalloproteinase (MMP)1, MMP2, MMP3, and MMP9 increased particularly in the skin of the more clinically affected patient (Bodemer *et al.*, 2003). We also noted with this patient higher amounts of MMP1 as well as lower amounts of TIMP1 in his affected and unaffected skins compared with the other two affected patients and healthy control donors, suggesting that tissue destruction in the disease process results from an imbalance of MMP with tissue inhibitor of metalloproteinases (Bodemer *et al.*, 2003). In this work, our aim was to determine whether matrilysin 1 (MMP7), could be involved in the epidermal detachment in RDEB patients as this MMP was shown to be present in the culture media of affected and unaffected skins maintained in organ culture. Furthermore, with cryostat section of two healthy human control skins, we investigated whether MMP7 was able *ex vivo* to hydrolyze some extracellular matrix (ECM) components associated with the dermo-epidermal junction that is, collagen VII and fibrillin 1. We investigated as well whether there was a potential inhibitory effect on MMP7 in *ex vivo* experiments using human control skin cryostat sections of epigallocatechin gallate (EGCG) because this polyphenolic compound derived from green tea has been demonstrated in *in vitro* experiments to be able to inhibit matrilysin activity in a non-competitive manner with K_i value of 0.47–1.65 μmol (Oneda *et al.*, 2003).

[1]*Faculté de Chirurgie Dentaire de Montrouge, Université Paris, Montrouge, France and* [2]*Département de dermatologie, Hôpital Necker Enfants Malades, AP-HP Université Paris 5, Paris, France*

Correspondence: Gaston Godeau, Faculté de Chirurgie dentaire de Montrouge, Université Paris, 1, rue Maurice Arnoux, 92120 Montrouge, France. E-mail: godeau_g@yahoo.fr

Abbreviations: ECM, extracellular matrix; EGCG, epigallocatechin gallate; MMP, matrix metalloproteinase; PBS, phosphate-buffered saline; RDEB, recessive dystrophic epidermolysis bullosa

Received 6 January 2006; revised 28 September 2006; accepted 3 October 2006; published online 30 November 2006

RESULTS

Skin biopsies

Immunostaining of matrilysin 1 (MMP7). The MMP7 immunoperoxydase labeling of the skins of the three RDEB patients in affected and non-affected skins (patients M, T, F, Figure S1:

a, b, c, unaffected skins; d, e, f: affected skins) revealed an intense staining of the epidermis in the affected as well as in the unaffected skins of the three RDEB patients compared with control skin for which a discreet labeling was observed at the epidermis level (Figure S1g).

Colocalization of MMP7 and CD68

Double labeled of MMP7 and CD68 for macrophages have been performed on affected and unaffected skin of RDEB patients. As shown both markers are present within the same cells in the dermis of affected skin patient (Figure 1), leading us to suppose that macrophages are involved in the production of MMP7 in RDEB skin patient.

Staining of elastic fibers

After (+) catechin-fuchsin staining, three types of fibers could be distinguished: oxytalan fibers perpendicular to the dermo-epidermal junction, elaunin fibers parallel to the dermo-epidermal junction, and mature elastic fibers in the dermis in the two control donors (see Figure S2a control no 1). With RDEB patients, skin elastic network was considerably disorganized particularly oxytalan fibers were dispersed and rarefied in affected and unaffected skins of the three RDEB patients as illustrated for patient F (see Figure S2: b, unaffected skins; c, affected skin).

Qualitative analysis of MMP7 secreted from skin explants after 72 hours of organ culture. Western blotting analysis

Qualitative analysis: SDS-PAGE and Western blotting of proteins secreted in the conditioned media of skin explants from the three affected and non-affected skins of patients with RDEB were analyzed. In non-affected skin patient, MMP7 in non-reducing conditions was detected as bands with approximate molecular weight of 97 kDa (Figure 2a, line L1). In reducing conditions (Figure 2a, line L2), this band appears as two bands: one at about 66 kDa and the other at 25–30 kDa.

In affected skin of the same patient, in non-reducing conditions, MMP7 is detected as two bands one at 97 kDa and the other at 66 kDa (Figure 2a, line L4). In reducing conditions, three bands are evidenced at 25–30 kDa, at 66 kDa, and a faint band at 97 kDa (Figure 2a, line L3). With controls, no bands for MMP7 were observed (not shown).

To show the specificity of the antibody used for MMP7, rhMMP7 detection was performed after Western blotting and was detected with an apparent molecular weight of about 20 kDa (see Figure 2b).

Quantitative analysis: The amounts of MMP7 were increased in the culture conditioned media for unaffected or affected skins in patients F, M, and T. The increase being particularly clear for patient M. For this patient, the amount of MMP7 expressed from his unaffected skin is about 37,973 a.u., whereas the amount expressed from the affected skin is about 42,136 a.u. as illustrated in Figure 2c.

***In vitro* assay for inhibition of MMP7 activity using EGCG as possible inhibitor**

Matrilysin activities appeared on casein zymograms as bands with an apparent molecular weight of 20 kDa, caseinolytic activities being proportional to the amounts of MMP7 electrophorized (125, 250, 500 ng of MMP7) (not shown).

When rhMMP7 (500 ng) is submitted to an incubation with EGCG, whatever the amount of EGCG the caseinolytic activity of MMP7 is totally abolished (see Figure 3a).

When EGCG is added at 30 μg/ml or 60 μg/ml to the incubation buffer media after electrophoresis, the intensity of

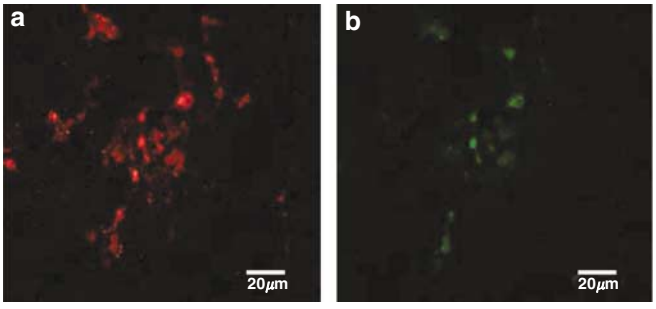

Figure 1. Colocalization of CD 68 and MMP7 in affected skin of RDEB patients. Positive cells for CD68 appeared in (**a**) red and positive cells for MMP7, in (**b**) green . Both markers were shown to be present simultaneously within same cells in the RDEB skin. Bar = 20 μm.

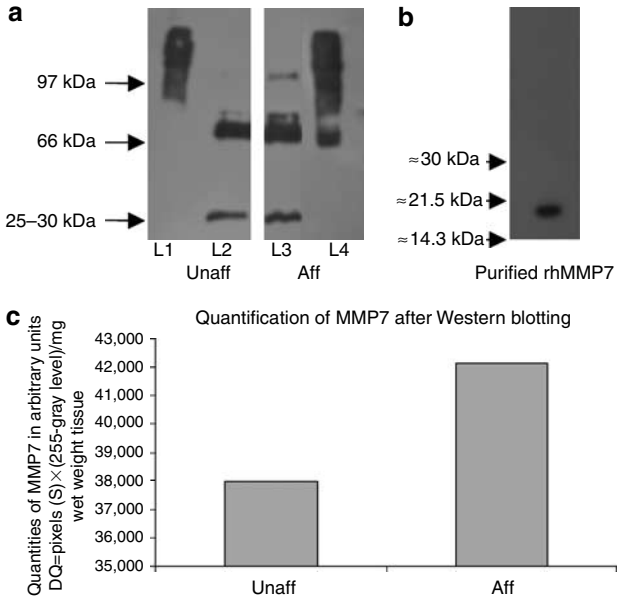

Figure 2. MMP7 detection and quantification in culture media. (**a**) MMP7 detection by Western blotting after 72 hours of culture. MMP7 was detected in the culture media of affected and unaffected skins of RDEB patient M under reducing and non-reducing conditions. In the unaffected skin (line L1, L2), in non-reducing condition (L1), a spot of 90 kDa can be detected, whereas under reducing conditions (L2) two bands appear at 66 and 25–30 kDa. In the affected skin (line L3, L4) under non-reducing condition, two spots at 90 and 66 kDa are observed, whereas under reducing condition three bands at 25, 66 and 97 kDa can be detected. (**b**) rhMMP7 detection by Western blotting. RhMMP7 (1 μg) was detected after electrophoresis to show the specificity of the antibody used. (**c**) Quantification of MMP7 in conditioned media of patient M after Western blotting.

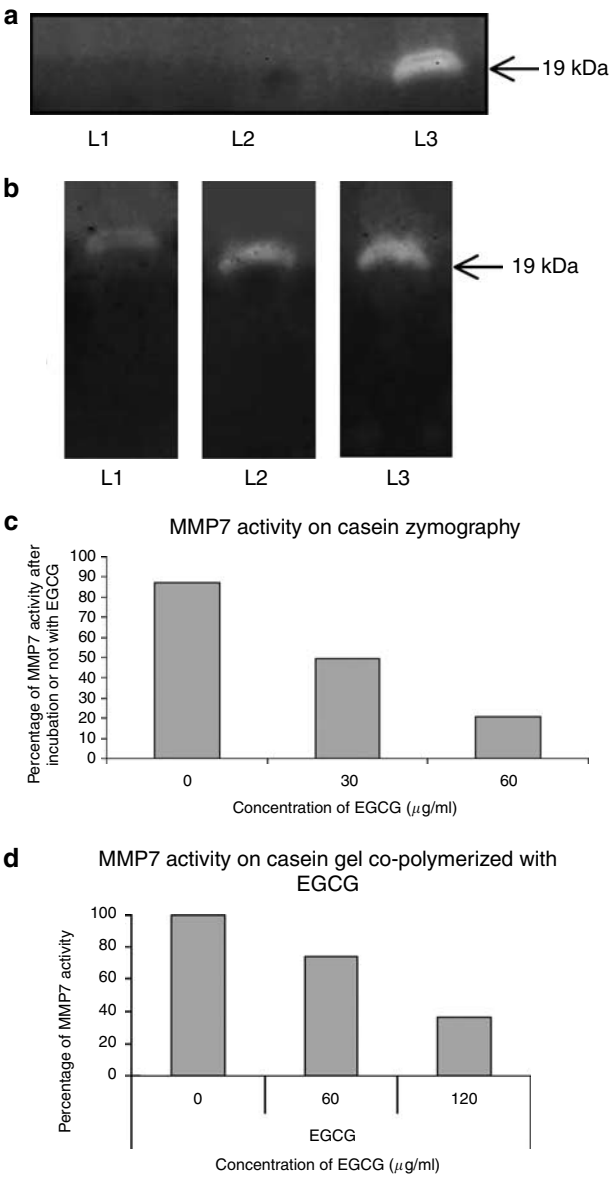

Figure 3. Effect of EGCG on caseinolytic activity of MMP7. (**a**) Incubation of EGCG with rhMMP7 before electrophoretic migration. L1: rhMMP7 (500 ng) was incubated with EGCG (60 μg/ml); L2: rhMMP7 (500 ng) was incubated with EGCG (30 μg/ml); L3: rhMMP7 alone. Preincubation of MMP7 with EGCG induced an inhibition of caseinolytic activity. (**b**) Caseinolytic activity of MMP7 after incubation with EGCG added in the incubation buffer. L1: rhMMP7 (500 ng) was incubated with EGCG (60 μg/ml); L2: rhMMP7 (500 ng) was incubated with EGCG (30 μg/ml); L3: rhMMP7 without EGCG. EGCG in the incubation buffer induce an inhibition of MMP7 caseinolytic activity. (**c**) Quantification of MMP7 activity on casein zymography after addition of EGCG in the incubation buffer. EGCG at 30 or 60 μg/ml induce a dose-dependant inhibition of MMP7 caseinolytic activity. (**d**) Quantification of MMP7 activity after zymography on casein gel co-polymerized with EGCG. EGCG at 60 or 120 μg/ml induce a dose-dependant inhibition of the caseinolytic activity.

the caseinolytic activity is proportional to the amount of EGCG added (see Figure 3b). The inhibition of the caseinolytic activity after image analysis was estimated at 43% with 30 μg/ml of EGCG and 76% with 60 μg/ml of EGCG (see Figure 3c).

When EGCG is co-polymerized with casein gel an inhibition of MMP7 activity of 26 and 64% is observed respectively for EGCG at 60 μg/ml or 120 μg/ml (see Figure 3d).

Enzymatic digestion and immunodetection of collagen type VII and fibrillin 1

When skin sections were incubated with buffer alone, collagen VII (see Figure 5a) was observed as net continuous and linear labeling underlying the dermo-epidermal junction; fibrillin 1 was observed as thin branches of candelabrum perpendicular to the dermo-epidermal basement membrane and also associated with elastic fibers of the dermis (see Figure 4a). When tissue sections were incubated with increasing quantities of MMP7, the skin matrilysin degradation was clearly visible for the higher amount of rhMMP7 (200 ng) concerning collagen VII which after immunodetection was noted thin and punctiform (see Figure 5b). Concerning fibrillin 1 it appeared considerably rarefied and disorganized (see Figure 4b) when compared with tissue section incubated with buffer alone (see Figure 4a).

Skin sections were incubated with EGCG (43 ng), then indirect immunodetection of collagen VII and fibrillin 1 was performed as described in Materials and Methods, and we verified that EGCG alone did not affect the quality and intensity of the immunotection of collagens VII and fibrillin 1 (not shown). On the other hand, when rhMMP7 (200 ng) and EGCG (43 ng) were preincubated during 1 hour at 37°C and were deposited onto a set of control skin tissue sections for a period of 48 hours at 37°C after indirect immunodetection of collagen VII (see Figure 5c) and fibrillin 1 (see Figure 4c), no discernable breakdown was observed for the two ECM components mentioned above, the intensity of the immunostaining being identical to that obtained after incubation with the buffer alone.

When control skin tissue sections were preincubated with the buffer containing 43 ng of EGCG during 1 hour at 37°C then submitted during 48 hours at 37°C to the action of 200 ng of rhMMP7, the quality and intensity of the immunodetection of collagen VII and fibrillin 1 were comparable to those evidenced without MMP7 (not shown).

In the first set of experiments, MMP7 and EGCG were first mixed and incubated for 1 hour then deposited onto the tissue sections, whereas in the second set of experiments the tissue sections were first incubated with EGCG for 1 hour before the addition of MMP7 (not shown). These two sets of experiments gave the same results, illustrating the inhibition effect of EGCG on the actions of human MMP7 toward collagen VII and fibrillin 1.

DISCUSSION

In the present work, we hypothesized that matrilysin (MMP7) could take part in the epidermis detachment which occurs at skin level in patients suffering from RDEB. Therefore, we conducted immunomorphological analysis on affected and non-affected skins from three RDEB patients belonging to the same family and presenting the same mutations in COL7A1.

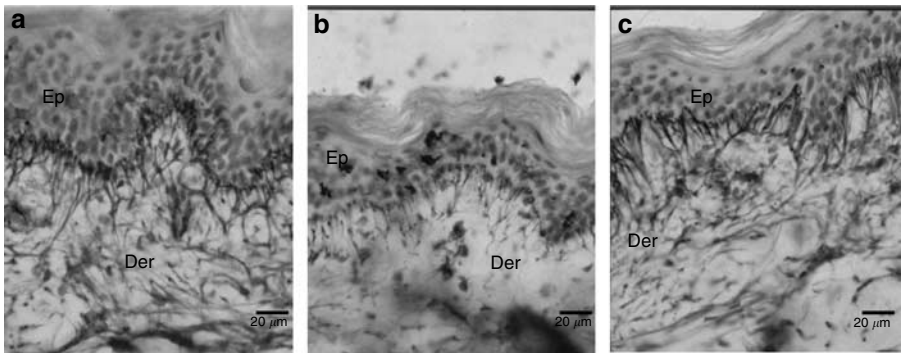

Figure 4. Immunodetection of fibrillin 1. Indirect immunodetection of fibrillin 1 was performed on skin from (**a**) healthy control donor without enzymatic treatment, on skin from healthy control donor (**b**) with enzymatic digestion with rhMMP7, and on skin from healthy control donor (**c**) after incubation with EGCG + rhMMP7. (Ep: epidermis; Der: dermis). Bar = μm. (**a**) Immunodetection of fibrillin 1 on skin from healthy control donor (anti-fibrillin dilution: 1/50). Bar = 20 μm. (**b**) Immunodetection of fibrillin 1 (dilution 1/50) on skin from healthy control donor after incubation at 37°C in a moist chamber with 200 ng of rhMMP7. The immunodetction of fibrillin 1 is clearly altered with rarefaction of fibrillin fibers in the superficial dermis. Bar = 20 μm. (**c**) Immunodetection of fibrillin 1 (dilution 1/50) on skin from healthy control donor treated as follows: rhMMP7 (200 ng) and EGCG 43ng were co-incubated during 1 hour at 37°C in a moist chamber then deposited on to the tissue section during 48 hours at 37°C. Positive fibrillar structures were comparable to those observed in panel a. Bar = 20 μm.

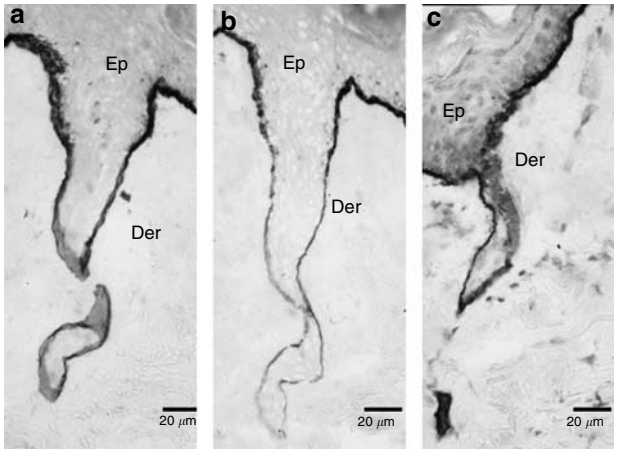

Figure 5. Immunodetection of Collagen type VII. Indirect immunodetection of collagen type VII was performed on skin from healthy control donor without enzymatic treatment (7a), on skin from healthy control donor with enzymatic digestion with rhMMP7 (7b) and on skin from healthy control donor after incubation with EGCG + rhMMP7 (7c). (Ep: epidermis; Der: dermis). (**a**) Immunodetection of collagen type VII (anti-collagen type VII, dilution:1/1,000) on skin from healthy control donor. Bar = 20 μm. (**b**) Immunodetection of collagen type VII (dilution 1/1,000) on skin from healthy control donor after incubation at 37°C in a moist chamber with 200 ng of rhMMP7. The immunodetection of collagen type VII is clearly altered, it appears thin and punctiform. Bar = 20 μm. (**c**) Immunodetection of collagen type VII (dilution 1/1,000) on skin from healthy control donor treated as follows: rhMMP7 (200 ng) and EGCG 43 ng were co-incubated during 1 hour at 37°C in a moist chamber then deposited on to the tissue section during 48 hours at 37°C. Positive structures were comparable to those observed in panel a. Bar = 20 μm.

MMP7 belongs to a family of zinc-dependent enzymes able to degrade a wide range of matrix molecules (Woessner, 1994; Massova *et al.*, 1998). They play a major role in connective tissue remodeling in embryonic development and in wound repair (Woessner, 1991). MMP7 has been shown to degrade *in vitro* the ECM proteins (Imai *et al.*, 1995), gelatins (Type I, II, IV, V) (Woessner, 1994), collagen type IV (Murphy *et al.*, 1991), laminin (Miyazaki *et al.*, 1990), entactin/nidogen (Mayer *et al.*, 1993), and elastin (Murphy *et al.*, 1991). The proteolytic activities of MMP7 have been defined by experiments using activated recombinant enzymes and purified substrates. Using monoclonal antibodies directed against human matrilysin 1 (MMP7), we evidenced that MMP7 was expressed in the outer root sheath of hair follicules and sweat glands in the two human healthy control skins, this result being in accordance with those reported by Karelina *et al.* (1994). In the affected and non-affected skins of the three RDEB patients, MMP7 was immunodetected at the level of the keratinocytes and several cells were also shown positive for MMP7 in the dermis not far from the zone which corresponds to the detachment between the epidermis and the dermis. In the dermis of affected patients, some cells were positive both for MMP7 and CD68, a marker of macrophages.

This expression of matrilysin by human mononuclear phagocytes was also reported by Busiek *et al.* (1995) who in the process of examining various cutaneous and lung inflammatory disorders for matrilysin expression by immunohistochemistry and *in situ* hybridization found that monocytes within blood vessels and extravasated tissue-associated macrophages exhibited matrilysin production. We observed after staining of skin elastic network by (+) catechin fuchsin that mature elastic fibers were considerably diminished and pre-elastic fibers rarefied and disorganized in the affected skin as well as in the unaffected skin of the three RDEB patients when compared with control skins. This observation concerning pre-elastic fibers and RDEB skins is in accordance with the results reported by McGrath *et al.* (1994) who observed in areas of dermal–epidermal separation a number of thin fragmented wisp-like structures labeled for fibrillin. Because matrilysin was shown to develop elastolytic activity (Murphy *et al.*, 1991) *in vitro*, we endeavored to verify using human skin cryostat sections from skin of control healthy

subjects that MMP7 *ex vivo* was able to perform its proteolytic functions against skin elastic network. After indirect immunodetection of fibrillin 1 on both human control skins, a net positivity was observed at the level of the oxytalan fibers (Cotta-Pereira *et al.*, 1976) in the papillary dermis, which corresponds also to observations reported by Sakai *et al.* (1986). When control skins were incubated with active MMP7, MMP7 was substantially revealed to have oxytalan-solubilizing capacity, as demonstrated after indirect immunodetection of fibrillin 1. In our hands, MMP7 was shown to hydrolyze *ex vivo* fibrillin 1, one of the microfibrillar components which belongs to oxytalan fibers and is associated in the deeper part of the skin with elastic fibers, but no appreciable elastic-solubilizing capacity was evidenced at the level of dermal elastic fibers. This result concerning elastolytic activity of MMP7 against elastin seems to contradict the results reported by Filippov *et al.* (2003) who conducted *in vitro* experiments (purified ^{3}H-elastin) with human macrophage progelatinase B (pro-MMP9), prometalloelastase (pro-MMP12), and promatrilysin (pro-MMP7). Nevertheless, we should be cautious about excluding elastolytic potentiality from matrilysin on human skin elastic fibers, the period of incubation being 48 hours only. Longer period of incubation could be necessary to evidence hydrolysis of the skin elastin fibers. Furthermore, we evidenced, when skin sections were overlaid with rhMMP7, that collagen type VII after indirect immunodetection with appropriate mAb was clearly broken down. The enzymatic hydrolysis of these two skin basement membranes-associated components owing to MMP7 raises the possibility of its potential major role in blister formation in RDEB of MMP7.

According to our experiment such an *ex vivo* test provides more information than *in vitro* assays using purified substrates because it closely mimics the *in vivo* situation where the investigated metalloproteinase is faced with fibrillin 1, collagen VII embedded in other matrix macromolecules which may alter its potential enzymatic activities.

In a previous work conducted in organ culture with the skin of these same three RDEB patients, we evidenced an imbalance between collagenase 1 (MMP1) and tissue inhibitor of metalloproteinase TIMP1 (Bodemer *et al.*, 2003). In the present work conducted again with organ culture, we demonstrated that after 3 days of culture the amounts of MMP7 expressed in culture conditioned media were increased in affected and unaffected skins of the three RDEB patients when compared with the two controls donors. After Western blotting, the molecular weight obtained for MMP7 is 97 kDa, suggesting that MMP7 is strongly bond to another component; the component could be a matrix component, but the exact nature of this component remains to be investigated. The tissue inhibitor of MMP7 being TIMP1, and traces of TIMP1 being expressed in affected and unaffected skins in these RDEB patients (Bodemer *et al.*, 2003), matrilysin 1 could be active on its substrates particularly those recovered at the skin basement membrane that we investigated in this work.

Recently polyphenols from green tea have attracted attention because of their skin photoprotective effect by inhibiting UVB-induced expression of matrix degrading MMP such as MMP2, MMP3, MMP7, and MMP9 in hairless mouse skin (Katiyar *et al.*, 2001; Vayalil *et al.*, 2004). So we tried to investigate whether EGCG, which was also demonstrated to inhibit matrilysin enzymatic activity on synthetic substrate, kept its enzymatic inhibitory effect on MMP7 *in vitro* and in *ex vivo* experiments. We evidenced after casein zymography preincubation of rhMMP7 and EGCG before electrophoresis that the caseinolytic activity of matrilysin was totally abolished. After electrophoresis of the rhMMP7 alone, the addition of EGCG in the incubation buffer resulted in a decrease in the caseinolytic activity of MMP7 proportional to the amount of EGCG added. These results are in accordance with those reported by Oneda *et al.* (2003). We demonstrated *in vitro* and *ex vivo* that EGCG is a good inhibitor of MMP7 and offers a good protection of matrix components susceptible of being degraded by this particular MMP. These *ex vivo* studies further emphasized the potential effect of EGCG in preventing particularly collagen VII, and fibrillin degradation as occurred in blister formation during EBDR.

It has long been known that polyphenolic compounds have a high affinity for ECM components rich in PRO such as collagens and elastin (Tixier *et al.*, 1984). We can thus speculate on EGCG being linked on the one hand to the active site of MMP7, and on the other to the matrix component, this ternary complex being involved in the abolition of the hydrolysis of the matrix components by matrilysin 1.

In conclusion, our data provide *in vitro* and *ex vivo* evidences that MMP7 could contribute to the degradation of ECM components belonging to the dermo-epidermal junction and thus participate in blister formation observed in patients suffering from EBDR. Furthermore, we propose that EGCG could be used beneficially to modulate or control the epidermal detachment as occurs during pathological states such as epidermolysis bullosa.

MATERIALS AND METHODS

This study was performed after institutional review board approval of protocol and informed consent according to the Declaration of Helsinki Principles. Three RDEB-affected sibling brothers with the same mutation in COL7A1 and demonstrative intrafamilial phenotypic variability were included in this study. The characteristics of their mutation were described previously (Bruckner-Tuderman *et al.*, 1989; Bodemer *et al.*, 2003). Two healthy control patients in the same age group (control 1, aged 20; control 2 aged 24) were also included in this study.

Skin biopsies

After informed consent was obtained, punch biopsies (punch 4 mm) were performed under lidocaïne anesthesia. For RDEB patients, two samples were taken at the same time: one from a new typical spontaneous blistering and the other from a skin area without any bullous lesions and scarring feature. For control healthy patients (for whom only one skin biopsy was taken), the biopsies were performed at the internal part of the arm. Skin biopsies were divided into two parts for RDEB patients, one for immunostaining histology and the second for organ culture and biochemical studies.

Biochemical studies

The wet weight of skin biopsies was determined, then skin biopsies were maintained for up to 3 days in DMEM containing: 1.5 mmol. per liter Ca^{2+} as recommended previously (Varani, 1998), supplemented with 0.2% lactalbumin hydrolysate (Sigma, France), 2 mmol per liter L-glutamine, 100 U per ml, penicillin, and 100 μg per ml streptomycin (Boehringer, Manheim, Germany), at 37°C in an atmosphere of 95% air, 5%CO_2. Cell viability was evaluated by lactate deshydrogenase determination in conditioned medium.

Western blotting performed on culture conditioned medium after 3 days of culture

Electrophoresis was carried out using a mini protean II system (Biorad, Morne La Coquette, France), 10% polyacrylamide gels (10 cm height, 1.5 mm. thickness) contained buffered solution consisting of 2.5 ml 1.5 M Tris HCl pH8.8; 100 μl SDS 10%, 4 ml polyacrylamide, and 3.3 ml of distilled water; stacking gel contained 4% polyacrylamide in 0.5 M Tris HCl pH 6.8. Gels were polymerized by adding 50 μl of 10% ammonium persulfate and 10 μl of 0.1% TEMED (Biorad, France). Samples were half diluted in 1 M Tris pH6.8 containing 50% glycerol and 0.4% bromophenol blue (non-reducing conditions) or in 1 M Tris pH 6.8 containing 50% glycerol, 0.4% bromophenol blue, and 10% β mercaptho-ethanol (reducing conditions). Samples in reducing conditions were heated for 1 minute at 100°C. Gels were run under Laemmli conditions (40 mA, 1 hour).

To show the specificity of the antibody used for the detection of MMP7 in the conditioned media of RDEB patient, a parallel electrophoresis of rhMMP7(1 μg) was carried out on a 15% polyacrylamide gel (10 cm height, 1.5 mm thickness), buffered solution consisting of 2.5 ml 1.5 M Tris HCl pH 8.8; 100 μl SDS 10%, 4.95 ml polyacrylamide, and 2.4 ml of distilled water; stacking gel contained 4% polyacrylamide in 0.5 M Tris HCl pH 6.8. Gels were polymerized by adding 50 μl of 10% ammonium persulfate and 10 μl of 0.1% TEMED (Biorad, France). Samples were half diluted in 1 M Tris pH 6.8 containing 50% glycerol and 0.4% bromophenol blue (non-reducing conditions) (see Figure 2b).

To assess the molecular weight, a Mr markers, rainbow RPN756 (5 μl) (Amersham, Orsay, France) containing myosin (220 kDa, appears in blue), phosphorylase b (97.3 kDa, brown), bovine serum albumin (66 kDa, red), ovalbumin (46 kDa, yellow) carbonic anhydrase (30 kDa, orange), trypsin inhibitor (21.5 kDa, blue), and lysozyme (14.3 kDa, magenta) in non-reducing conditions were submitted to a parallel electrophoresis.

Gels were transferred for 75 minutes (75 V) on polyvinylidene difluoride membrane (Immobilon MILLIPORE, Bedford, MA). Blots were incubated with anti-MMP7 (clone ID2) (Oncogene, Fontenay s/ Bois, France) mAb diluted 1:500 in phosphate-buffered saline (PBS) for 1 hour at room temperature. Membranes were washed four times during 10 minutes with PBS 0.1% Tween 20 (vol/vol) (PBS/ Tween) and incubated with peroxidase-labeled second antibody diluted 1:1,000 in PBS. They were then washed extensively with PBS/ Tween, treated with Covalight (Ab-Cys, France) for 1 minute and revealed using KODAK BIOMAX MR film (Sigma, St Louis, MA). Multiple exposures were examined to ensure that the results analyzed reflected those produced with linear range of the film.

The quantitative estimation of MMP7 in conditioned media from skin explants is based on tissue wet weight basis, the tissue being weighed before incubation. We demonstrated that this quantitative estimation was strictly correlated with the amount of proteins contained in the skin explants determined with the Coomassie blue method of proteins quantification (Mohammedi *et al.*, 1989).

Enzyme assays

Matrilysin (MMP7) was identified by zymography using 0.1%. SDS (Biorad, France) polyacrylamide gel impregnated with 1 mg per ml casein (Sigma, Saint Quentin, France) (Fernandez-Resa *et al.*, 1995).

Protein standard underwent a parallel electrophoresis to determine the molecular weight of lysis bands.

Enzyme assays with EGCG

Active purified matrilysin (MMP7) was purchased from Calbiochem (France). Casein (1 mg per ml) zymography was used as described above to demonstrate its enzymatic activity on polyacrylamide gel after electrophoresis.

Inhibitory effects of EGCG (from Sigma, France) on the matrilysin casein activity were examined as follows:

1. Buffer (5 μl) containing MMP7 (1 μg) alone, MMP7 (1 μg) and 30 μg/ml EGCG, MMP7 (1 μg) and 60 μg/ml EGCG, were incubated at 37°C in an atmosphere of 95% air, 5% CO_2 during 30 minutes before electrophoresis.
2. MMP7 (1 μg) alone was electrophoresed and 30 μg/ml EGCG or 60 μg/ml EGCG were added to the incubation buffer (Tris-HCl 0.1 M pH 7.4, $CaCl_2$ 30 mM, $ZnCl_2$ 0.1 μM) during 48 hours at 37°C in an atmosphere of 95% air, 5% CO_2 before staining of the electrophoresis gel containing 1 mg/ml of casein for the visualization of the MMP7 enzymatic activity.
3. EGCG (0 μg/ml, 60 μg/ml or 120 μg/ml) was polymerized in casein gel (1 mg/ml) then MMP7 (1 μg) alone was electrophoresed. Caseinolytic activities were observed after 48 hours at 37°C in the incubation buffer.

Quantification of zymogram lysis bands and blots by image analysis

The average surface (*S*) of polyacrylamide gel electrophoresis bands or/and membrane blots was determined semi-automatically by following its contour with a calibrated electronic slide as described previously (Beranger *et al.*, 1994).

For zymographic analysis, matrilysin activities were expressed as gel U = pixels (*S*) × gray level/mg wet weight.

Concerning Western blotting analysis, matrilysin 1 was expressed as dot Q = pixels (*S*) × (255−gray level) per mg of wet weight (Gogly *et al.*, 1998).

Histology

Skin biopsies with or without any bullous lesions from the three RDEB patients were routinely processed for histological investigations. Serial tissue sections, 6 μm thick, prepared with a manual microtome were stained with hematoxylin eosin to assess tissue quality (Ganter and Jolles, 1969).

To analyze elastic fibers, the (+) catechin fuchsin method was used (Godeau *et al.*, 1986).

Immunolocalizations of matrilysin (MMP7) (clone ID2) (Oncogene) were performed on cryostat tissue sections (8 μm thick) from

affected and unaffected skins of the three RDEB patients. Cryostat tissue sections were air dried and fixed with acetone before immunoperoxidase staining. Appropriated primary antibody and secondary antibodies were used according to the manufacturer's recommendations then tissue sections were counterstained with hematoxylin for 1 minute before light microscopic observation.

Double-labeled immunostaining

Cryostat tissue section, (8 μm) thick, from affected and unaffected skins of patients were air dried and rinsed in PBS for 10 minutes. Nonspecific binding was blocked for 30 minutes at 37°C with blocking solution (PBS-Tween 0.05%, 1% BSA, 10% normal horse serum, 10% normal goat serum) and then incubated overnight at room temperature with a mixture of primary antibody rabbit anti-MMP7 (1/25) (Interchim, France) and mouse anti-CD68 (1/25) (clone PG-M1) (Dako, France) diluted in PBS-Tween (0.05%) bovine serum albumin (1%) (Sigma, France). Tissue sections were washed in PBS for 30 minutes and then incubated in the dark with a mixture of secondary antibody: goat anti-rabbit Hilyte Fluor^{TM} 488 conjugated (1/100) (Euromedex, France) and horse anti-mouse biotinylated (1/200) (Vector Laboratories, Biovalley, France) diluted in PBS-T (0.05%) for 2 hours at room temperature. For CD68 immnolocalization, after rinsing three times with PBS, slides were incubated with streptavidin conjugated to fluoroprobes 547 (1/500) (Interchim, France) 1 hour at room temperature.

Enzyme assays with or without EGCG followed by immunolocalizations of fibrilllin-1 and collagen VII

Ex vivo MMP7 assay. Experimental procedures were performed on control healthy human skin cryostat tissue sections (8 μm thick and four sections per condition).

Active MMP7 (rhMMP7 from Calbiochem, France) at different quantities, 25, 50, 100, and 200 ng in appropriate buffer (HEPES 10 mM, NaCl 150 mM, $CaCl_2$ 5 mM) was laid on four serial tissue sections, and one tissue section was overlaid with the buffer alone. The preparations were then incubated in a moist chamber at 37°C for 48 hours; rinsed with the buffer, fixed in absolute ethanol for 1 minute and immunostaining for fibrillin-1 and collagen type VII was performed following the protocol described above for the MMP7 immunostaining.

Ex vivo MMP7 inhibition assay

Active MMP7 rhMMP7 from Calbiochem was used at the concentration of 200 ng in the appropriate buffer.

In a first set of experiments, 200 ng of MMP7 and 43 ng of EGCG in the appropriate buffer were incubated during 1 hour at 37°C, then laid onto tissue sections (controls with the buffer alone and the buffer containing EGCG alone), and placed for 48 hours at 37°C in a humidified chamber. The addition of an excess of cold incubation buffer stopped the reaction then cryostat tissue sections were processed as described above for the immunodetection of collagen VII (cloneLH7.2) (Sigma, St Louis, MA) and fibrillin-1 (clone 26) (Euromedex, France).

In a second set of experiments, cryostat tissue sections were preincubated with 43 ng of EGCG in the appropriate buffer at 37°C in the moist chamber during 1 hour, then tissue sections were overlaid with 200 ng of MMP7 for 48 hours at 37°C before the immunodetection of collagen VII and fibrillin-1.

CONFLICT OF INTEREST

The authors state no conflict of interest.

SUPPLEMENTARY MATERIAL

Figure S1. Immunodetection of MMP7.

Figure S2. Staining of the elastic network.

REFERENCES

Beranger JY, Godeau G, Frances C, Robert L, Hornebeck W (1994) Presence of gelatinase A and metalloelastase type protease at the plasma membrane of human skin fibroblasts. Influence of cytokines and growth factors on cell-associated metalloendopeptidase levels. *Cell Biol Int* 18:715–22

Bodemer C, Tchen SI, Ghomrasseni S, Seguier S, Gaultier F, Fraitag S *et al.* (2003) Skin expression of metalloproteinases and tissue inhibitor of metalloproteinases in sibling patients with recessive dystrophic epidermolysis and intrafamilial phenotypic variation. *J Invest Dermatol* 121:273–9

Bruckner-Tuderman L, Mitsuhashi Y, Schnyder UW, Bruckner P (1989) Anchoring fibrils and type VII collagen are absent from skin in severe recessive dystrophic epidermolysis bullosa. *J Invest Dermatol* 93:3–9

Busiek DF, Baragi V, Nehring LC, Parks WC, Welgus HG (1995) Matrilysin expression by human mononuclear phagocytes and its regulation by cytokines and hormones. *J Immunol* 154:6484–91

Cotta-Pereira G, Guerra Rodrigo F, Bittencourt-Sampaio S (1976) Oxytalan, elaunin, and elastic fibers in the human skin. *J Invest Dermatol* 66:143–8

Fernandez-Resa P, Mira E, Quesada AR (1995) Enhanced detection of casein zymography of matrix metalloproteinases. *Anal Biochem* 224:434–5

Filippov S, Caras I, Murray R, Matrisian LM, Chapman HA Jr, Shapiro S *et al.* (2003) Matrilysin-dependent elastolysis by human macrophages. *J Exp Med* 198:925–35

Ganter P, et Jolles G (1969) Méthodes histologiques de base. In: *Histochimie normale et pathologique*. (Ganter P and Jolles G, eds), Paris: Gauthier-Villard 5–108

Godeau G, Gonnord G, Jolivet O, Schoevaert D, Pompidou A, Lagrue G *et al.* (1986) A selective histochemical method for the quantitative estimation of elastic fibers by computerized morphometric analysis. Effect of colchicin treatment. *Anal Quant Cytol Histol* 8:321–5

Gogly B, Groult N, Hornebeck W, Godeau G, Pellat B (1998) Collagen zymography as a sensitive and specific technique for the determination of subpicogram levels of interstitial collagenase. *Anal Biochem* 255: 211–6

Hovnanian A, Rochat A, Bodemer C, Petit E, Rivers CA, Prost C *et al.* (1997) Characterization of 18 new mutations in COL7A1 in recessive dystrophic epidermolysis bullosa provides evidence for distinct molecular mechanisms underlying defective anchoring fibril formation (1997). *Am J Hum Genet* 61:599–610

Imai K, Yokohama Y, Nakanishi I, Ohuchi E, Fujii Y, Nakai N *et al.* (1995) Matrix metalloproteinase 7 (matrilysin) from human rectal carcinoma cells. Activation of the precursor, interaction with other matrix metalloproteinases and enzymic properties. *J Biol Chem.* 270:6691–7

Karelina TV, Goldberg GI, Eisen AZ (1994) Matrilysin (PUMP) correlates with dermal invasion during appendageal development and cutaneous neoplasia. *J Invest Dermatol* 103:482–7

Katiyar SK, Afaq F, Perez A, Mukhtar H (2001) Green tea polyphenol (-)-epigallocatechin-3-gallate treatment of human skin inhibits ultraviolet radiation-induced oxidative stress. *Carcinogenesis* 22:287–94

Massova I, Kotra LP, Fridman R, Mobashery S (1998) Matrix metalloproteinases: structures, evolution, and diversification. *FASEB J* 12:1075–95

Mayer U, Mann K, Timpl R, Murphy G (1993) Sites of nidogen cleavage by proteases involved in tissue homeostasis and remodelling. *Eur J Biochem* 217:877–84

McGrath JA, Sakai LY, Eady RA (1994) Fibrillin immunoreactivity is associated with normal or fragmented elastic microfibrils at the dermal-epidermal junction in recessive dystrophic epidermolysis bullosa. *Br J Dermatol* 131:465–71

Miyazaki K, Hattori Y, Umenishi F, Yasumitsu H, Umeda M (1990) Purification and characterization of extracellular matrix-degrading metalloproteinase, matrin (pump-1), secreted from human rectal carcinoma cell line. *Cancer Res* 50:7758–64

Mohammedi H, Mamouzi S, Allal C, Ghaffor M, Rabhi H, Abbadi MC (1989) Rapid and sensitive micromethod for protein determination by the Coomassie-blue technique. *Arch Inst Pasteur Alger* 57: 151–62

Murphy G, Cockett MI, Ward RV, Docherty AJ (1991) Matrix metalloproteinase degradation of elastin, type IV collagen and proteoglycan. A quantitative comparison of the activities of 95 kDa and 72 kDa gelatinases, stromelysins-1 and -2 and punctuated metalloproteinase (PUMP). *Biochem J.* 277(Part 1):277–9

Oneda H, Shiihara M, Inouye K (2003) Inhibitory effects of green tea catechins on the activity of human matrix metalloproteinase 7 (matrilysin). *J Biochem (Tokyo)* 133:571–6

Sakai LY, Keene DR, Engvall E (1986) Fibrillin, a new 350-kD glycoprotein, is a component of extracellular microfibrils. *J Cell Biol* 103(Part 1): 2499–509

Tixier JM, Godeau G, Robert AM, Hornebeck W (1984) Evidence by *in vivo* and *in vitro* studies that binding of pycnogenols to elastin affects its rate of degradation by elastases. *Biochem Pharmacol* 33:3933–9

Varani J (1998) Preservation of human skin structure and function in organ culture. *Histol Histopathol* 13:775–83

Vayalil PK, Mittal A, Hara Y, Elmets CA, Katiyar SK (2004) Green tea polyphenols prevent ultraviolet light-induced oxidative damage and matrix metalloproteinases expression in mouse skin. *J Invest Dermatol* 122:1480–7

Woessner JF Jr (1991) Matrix metalloproteinases and their inhibitors in connective tissue remodelling. *FASEB J* 5:2145–54

Woessner JF Jr (1994) The family of matrix metalloproteinases. *Ann NY Acad Sci* 732:11–21