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SHORT COMMUNICATION

Metalloproteinase 9 is the outer executioner of desmoglein 3 in apoptotic keratinocytes

N Cirillo, F Femiano, F Gombos, A Lanza

Regional Center on Craniofacial Malformations-MRI, Department of Odontostomatology, 1st School of Medicine and Surgery, II University of Naples, Naples, Italy

OBJECTIVE: To investigate the specific matrix metalloproteinases (MMPs) targeting desmoglein 3 (Dsg3) in apoptotic keratinocytes.
METHOD: Inhibitor studies on cultured keratinocytes and Western blot analysis.
RESULTS: Blocking of MMP-9 activity strongly reduces shedding of Dsg3 from cell surface. MMP-2 has a less relevant role in the cleavage of Dsg3 while other MMPs, such as MMP-1, -3, and -8, do not target Dsg3.
CONCLUSION: Apoptic keratinocytes impair the extracellular domain of cell surface Dsg3 by MMP-9 activity. The discovery of a specific targeting of Dsg3 could be useful to understand the pathophysiology of diseases in which Dsg3 is affected.
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Keywords: matrix metalloproteinase; desmoglein 3; apoptosis; staurosporine; keratinocytes

Introduction

Abnormalities in the apoptotic machinery can cause diseases such as cancer (Strasser *et al*, 1990), promote autoimmunity (Watanabe-Fukunaga *et al*, 1992) and mucocutaneous disorders (Qin *et al*, 2002; Pelacho *et al*, 2004). To realize those morphological changes typical of cell death and to release apoptotic cells from a tissue, cell–cell contacts need to be disrupted. In epithelia, cadherin complexes provide the major intercellular adhesive forces (for review, see Garrod *et al*, 2002). While caspases cleave cadherins intracellularly, the main executors of the proteolytic events taking place in the extracellular substance are matrix metalloproteinases (MMPs) (Steinhusen *et al*, 2001).

Correspondence: Dr Nicola Cirillo, Department of Odontostomatology, Second University of Naples, Via Luigi de Crecchio, 7 80138 Naples, Italy. E-mail: nicola.cirillo@unina2.it
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Matrix metalloproteinases are a family of zinc-containing endopeptidases that are either secreted or expressed at the cell surface of a number of cell types; MMPs are produced as zymogens, with a propeptide segment removed extracellularly by proteases such as plasmin, and show wide proteolytic activity and overlapping specificities. Given this complexity, it is not surprising that multiple roles for MMPs have been proposed, including regulation of cell migration, proliferation and death (Johnson *et al*, 2005). Targets identified as MMP substrates include desmogleins (Weiske *et al*, 2001), type I integral membrane glycoproteins belonging to the cadherin family of calcium-dependent cell adhesion molecules. The human desmoglein family includes four isoforms (Dsg1–4), which are expressed in a cell-type and differentiation-specific manner (King *et al*, 1997; Kljuic *et al*, 2003). Dsg2 is the most widespread desmoglein, found in all desmosome-assembling tissues, including the stratum basale of epidermis and oral mucosa (Schafer *et al*, 1994). As the basal keratinocytes exit the cell cycle and enter the strata spinosum and granulosum, desmosomes become enriched in Dsg3 and Dsg1, respectively (Arnemann *et al*, 1993; Mahoney *et al*, 2006). In particular, Dsg3 is found in the basal and spinous layers of the epidermis and gradually diminishes as the cells become more differentiated, whereas in the oral mucosa Dsg3 is detected at high levels in all cell layers (Mahoney *et al*, 1999). Dsg3 is also the main desmocaderin expressed *in vitro* in keratinocyte monolayers (Koch *et al*, 1992; Denning *et al*, 1998). There is increasing evidence that disruption of Dsg3 adhesion results in alterations of tissue architecture with subsequent epithelial blister formation, as shown by acquired and inherited diseases (Amagai *et al*, 1991; Allen *et al*, 1996; Koch *et al*, 1997). During apoptosis, Dsg3 has been reported to be targeted by caspase-3 intracellularly and by metalloproteinases extracellularly, in a manner that was neither cell type-specific nor apoptotic stimulus-dependent (Weiske *et al*, 2001).

The aim of this study was to investigate the specific MMP(s) responsible for the cleavage and shedding of Dsg3 during programmed keratinocyte cell suicide.

Apoptosis was triggered by staurosporine (STS), a chemical which showed several advantages as a programmed cell death inducer (Jacobson *et al*, 1996), including its ability to trigger apoptosis in a broad spectrum of cell types. For our experiments, we used HaCaT cells, a nontumorigenic human keratinocyte cell line which exhibits normal differentiation (Boukamp *et al*, 1988) and is capable of endogenously expressing high levels of Dsg3.

Materials and methods

Antibodies and reagents

The 5H10 monoclonal antibody against extracellular domains of Dsg3, anti-Dsg3 H-145 rabbit polyclonal antibodies raised against the cytoplasmic domain of Dsg3, rabbit anti-Dsg2 H-145 antibodies against an internal region of Dsg2, H-290 rabbit antibodies against C-terminal residues 760–1046 of Dsg1, were from Santa Cruz Biotecnology (Santa Cruz, CA, USA). Fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG antibodies were obtained from DAKO (Dako, Glostrup, Denmark A/S, Denmark). Alkaline phospotase (AP)-conjugated anti-mouse antibodies and WesternBreeze chemiluminescent immunodetection kit were purchased from Invitrogen (Carlsbad, CA, USA). MMP inhibitors were obtained from Calbiochem (Darmstadt, Germany); staurosporine (Hoechst 33342), and reagents for cell cultures and protein extraction were purchased from Sigma (St Louis, MO, USA). All reagents used for reverse transcriptase-polymerase chain reaction (RT-PCR) were obtained from Promega (Promega Corporation, Madison, WI, USA).

Cell cultures and treatments

HaCaT cells, a human immortalized keratinocyte cell line (Boukamp *et al*, 1988), were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, penicillin (50 U ml^{-1}), streptomycin (50 $\mu g\ ml^{-1}$) and fungizone (2.5 $\mu g\ ml^{-1}$) in an atmosphere humidified with 5% CO_2. At the time of the experiment, cells were seeded and grown to confluence on six-well dishes. Apoptosis was induced by addition of 800 nM staurosporine in DMSO. For inhibitor studies, cells were pre-incubated for 30 min with MMP inhibitors as follows: MMP inhibitor I at 8 μM (resulting in the inhibition MMP-1 and MMP-8), 80 μM (inhibiting MMP-1, -8 and -9) and 200 μM (inhibiting MMP-1, -8, -9 and -3); MMP-8 inhibitor I at 10 nM; MMP-3 inhibitor II at 200 nM; MMP-2/MMP-9 inhibitor IV at 40 nM (inhibiting only MMP-2) and 1 μM (that inhibits both MMP-2 and MMP-9), according to the manufacturer's data.

Assessment of apoptosis

Apoptosis was evaluated by nuclear staining with Hoechst 33342 (5 $\mu g\ ml^{-1}$) on 3% formaldehyde-fixed cells using a Zeiss Axiophot microscope (Carl Zeiss Inc., Thornwood, NY, USA) at 400× magnification. Nuclear morphology was examined with an excitation wavelength of 355–425 nm and fluorescence images were acquired with an Evolution VF fast digital camera (MediaCybernetics, UK). Detachment from substrate was estimated by counting adherent cells at different time points after treatment with staurosporine.

Western blot analysis

After the addition of phemylmethylsulphanefluoride (PMSF) 1 mM, keratinocyte-depleted cell culture medium (2 ml for each well) was concentrated 100-fold in a vacuum stove and stored as culture supernatant. Protein concentration of samples was determined using Bradford colorimetric assay. Equal amounts of proteins were mixed with 4X sodium dodecyl sulphate (SDS) sample buffer, heated at 95°C in Laemmli sample buffer for 5 min and then separated by 10% SDS-polyacrylamide gel electrophoresis. Western blot analysis was carried out after SDS-PAGE gels were transferred onto polyvinyldiene fluoride (PVDF) membranes for 1 h at 350 mA. Blocked membranes were incubated for 2 h with 5H10 anti-desmoglein 3 mouse monoclonal antibody (diluted 1:100) and successively for 1 h with anti-mouse alkaline phosphatase-conjugated IgG as secondary antibody. Bound antibodies were detected by WesternBreeze chemiluminescent immunodetection system.

Immunofluorescence microscopy

One-day confluent cells were fixed and permeabilized in paraformaldehyde solution [3% paraformaldehyde in phosphate-buffered saline (PBS) containing 0.1% Triton X-100] for 20 min at room temperature. Samples were then washed three times in PBS containing 2% bovine serum albumin (BSA) to block non-specific sites, incubated with primary antibody (1:10) for 1 h at room temperature, washed in BSA/PBS, and exposed to specie-specific antibodies (1:100) conjugated to FITC. Specimens were examined as reported above.

RT-PCR

Total cellular RNA was extracted using Trizol according to the manufacturer's instruction, treated with DNAase I and quantified at $OD_{260\ nm}$. cDNA synthesis was performed with 1.5 unit of AMV reverse transcriptase and oligo(dT) primers and then used as template for PCR. The specific pairs of primers are reported in Table 1. Comparable quantities of cDNA were ensured by amplifying a 299-bp fragment of β-actin and semi-quantitative evaluations of the respective mRNA expression levels was performed by densitometry with

Table 1

	Sequence 5′ to 3′
Dsg1	Forward TCAAGTTCGCAGCAGCCTGT
	Reverse TGTTCGGTTCATCTGCGTCA
Dsg2	Forward CAGTAGCTTCCCAGTTCC
	Reverse CTGTAAGCTTCATGAAAAATCAG
Dsg3	Forward ACCGAATCTCTGAAGTGGGAA
	Reverse GCACCACTCACAACCAGAC GAT
β-actin	Forward GCGAGAAGATGACCCAGATCATGTT
	Reverse GCTTCTCCTTAATGTCACGCACGAT

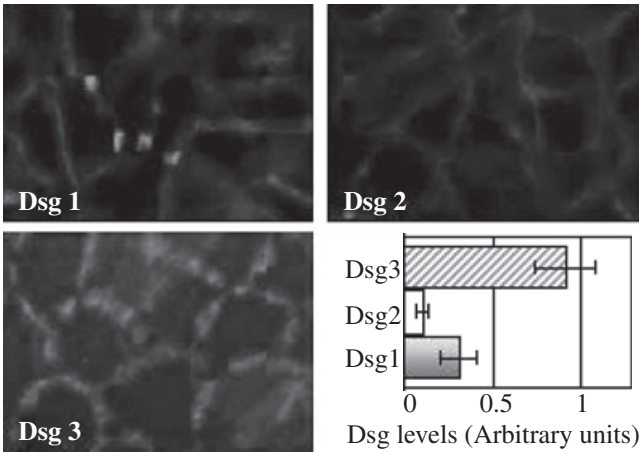

Figure 1 Expression pattern of desmogleins. As revealed by immunofluorescence, desmoglein isoforms 1, 2 and 3 were found on keratinocyte surface. Dsg1–3 mRNA expression was measured by RT-PCR. mRNA levels were compared with β-actin mRNA level (=1) used as control. Values are the mean ($\pm$s.d.) of three independent experiments

the Molecular Analysis Software (Bio-Rad, Richmond, CA, USA).

Results

Expression pattern of desmogleins

Keratinocytes were seeded on six-well plates and grown to confluence. One day after reaching confluence, cultured monolayers expressed Dsg3 and low rates of Dsg1 and Dsg2 (assessed by immunofluorescence and RT-PCR; Figure 1). This *in vitro* pattern of expression resembles that observed *in vivo* in the spinous layer of mucous membranes (Mahoney *et al*, 1999). Indeed, at the selected stage of differentiation, HaCaT cells synthesized high levels of Dsg3 and were useful for our purposes.

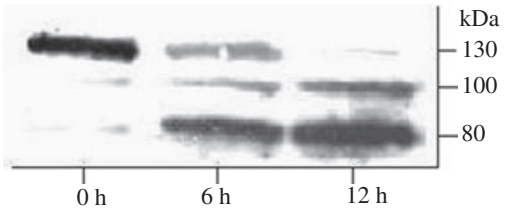

Figure 3 Proteolytic cleavage of Dsg3 during apoptosis. Using the 5H10 monoclonal antibody, we demonstrated the appearance of two proteolytic fragments of about 80 and 100 kDa formed after 6 and 12 h of treatment. The figure is representative of three independent experiments

Induction of apoptosis and processing of Dsg3

One-day confluent cells were treated with 800 nM staurosporine. Changes in morphology, detachment from the substrate, DNA fragmentation and nuclear condensation demonstrated that HaCaT cells responded to the apoptotic stimulus (Figure 2).

Appearance of fragmentation products concomitantly with decline of full-length Dsg3 from cell lysates gave evidence of proteolytic events against Dsg3 that occurred during apoptosis: distinct cleavage products of Dsg3 were generated with apparent molecular masses of 100 and 80 kDa (Figure 3). The presence of a 75-kDa fragment of Dsg3 in culture supernatants (Figure 4a) indicated that Dsg3 was proteolytically cleaved extracellularly in apoptotic pathways, as reported previously (Weiske *et al*, 2001).

Inhibitor studies showed that gelatinases are involved in Dsg3 cleavage

To investigate the role of specific MMPs involved in the shedding of Dsg3 from keratinocytes, we exposed cells to different concentrations of MMP inhibitors before the addition of staurosporine. MMPI-1 (80 and 200 μM) inhibited the formation of the 75-kDa fragment of Dsg3,

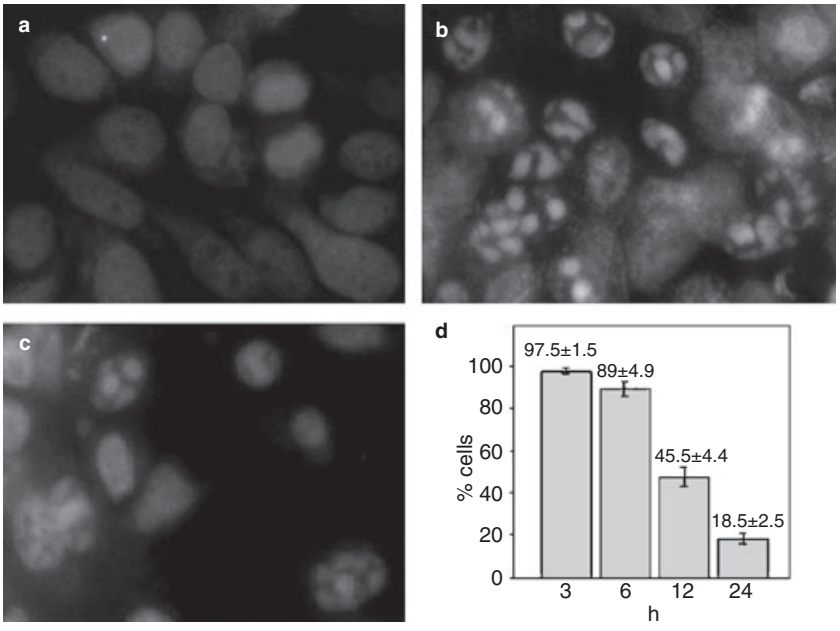

Figure 2 Cell apoptosis and detachment from the substrate. Apoptosis was induced by treating cells with 800 nM staurosporine and nuclei were stained with Hoechst 33342. (**a**) Non-treated cells exhibited an homogeneous nuclear staining. (**b**, **c**) Nuclear condensation, a well established morphological marker of apoptosis, was found after exposure to staurosporine at 6 and 12 h. (**d**) Percentage of cells attached to substrate (Petri dishes) after treatment at selected time points. Values are the mean ($\pm$s.d.) of three independent experiments

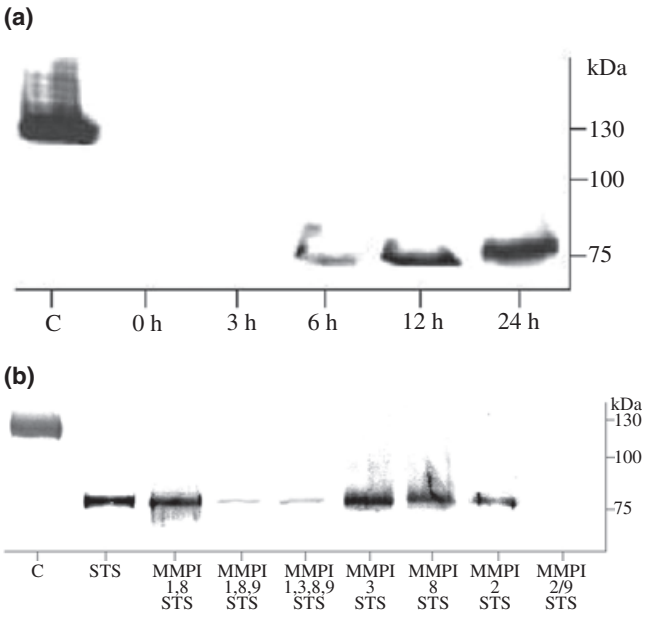

Figure 4 Extracellular cleavage of Dsg3 is blocked by MMP-9 inhibitors. (**a**) Shedding of Dsg3 extracellular domain revealed by imunoblotting of the 100-fold-concentrated culture supernatant with the 5H10 monoclonal antibody. (**b**) Cells pre-treated with MMPs inhibitors at concentrations inhibiting MMP-9 showed a strong reduction of the 75-kDa extracellular domain of Dsg3 in culture supernatants (lanes 4, 5 and 9). Inhibition of MMP-2 determined a reduced shedding of Dsg3 if compared with STS control (lane 8). When MMP-9 and MMP-2 were not inhibited, Dsg3 shedding appeared comparable with STS control (lanes 3, 6 and 7). Cell culture supernatants were harvested 12 h after induction of apoptosis. Figures are representative of three independent experiments

whereas MMPI-1 at lower concentrations or specific MMP3 and MMP8 inhibitors did not (Figure 4b, lanes 3–7). These findings provide evidence that MMPI-1 reduces the cleavage of Dsg3 via inhibition of MMP-9. However, MMP-2 inhibition reduced the formation of the 75-kDa fragment (Figure 4b, lane 8), suggesting a role for MMP-2 in the apoptotic cleavage of Dsg3. Consistently, inhibition of both MMP-2 and MMP-9 completely abolished the shedding of Dsg3 (Figure 4b, lane 9). These data demonstrate that gelatinase activity is essential for the cleavage of Dsg3 in apoptotic keratinocytes.

Discussion

In the present study, we demonstrated by pharmacological means that Dsg3 is a new target of MMP-9. Inhibition of MMP-9 results in a reduced cleavage of Dsg3 in keratinocytes undergoing exogenously induced apoptosis.

MMP-2 and MMP-9 are unique members of gelatinases, a subgroup of the MMP family. MMP-2 has been shown to proteolytically activate its cousin pro-MMP-9 (Fridman *et al*, 1995); this is in agreement with the reduced cleavage of Dsg3 observed when only MMP-2 was inhibited. However, in these experimental conditions, the shedding of Dsg3 is abolished only when both MMP-2 and MMP-9 became inhibited, thus showing a synergy among gelatinases in disrupting Dsg3-mediated adhesion in keratinocytes.

Specific proteolytic events against members of the desmoglein family are well established (Amagai *et al*, 2000; Dusek *et al*, 2006). Exfoliative toxin A targets Dsg1 but not closely related cadherins such as Dgs3 and induces blisters in bullous impetigo and staphylococcal scalded-skin syndrome. Of note, gelatinase expression has been shown to correlate with cell detachment from substrate after exposure to death-inducing stimuli (Pereira *et al*, 2005) and MMP-9 was found to be critical for dermal–epidermal separation in bullous pemphigoid, an autoimmune skin-blistering disease (Liu *et al*, 2005). Further studies are needed to address whether a specific cleavage of Dsg3 occurs in diseases where Dsg3-mediated adhesion is affected. It could be the case of pemphigus vulgaris, an autoimmune disease targeting Dsg3, in which proteases play a role in keratinocyte dishesion (Seishima *et al*, 1997) and apoptosis has been suggested as the pathogenic key responsible for acantholysis (Pelacho *et al*, 2004; Wang *et al*, 2004a,b). A specific proteolytic cutting of Dsg3 is also suggested by the discovery of its circulating immunodominant 30-kDa fragment (sDsg3), that is thought to be released from desmosome-assembling tissues (Lanza *et al*, 2006).

In conclusion, our results demonstrated the specific extracellular cleavage of Dsg3 by gelatinases with subsequent shedding from the cell surface in apoptotic keratinocytes. The major activity is due to MMP-9, while MMP-2 could also play a role by activating MMP-9 and partially by cutting Dsg3. Furthermore, our findings provide a structural basis for the interpretation of functional data in diseases where Dsg3 function is impaired.

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