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Disturbances of the Expression of Metalloproteinases and Their Tissue Inhibitors Cause Destruction of the Basement Membrane in Pemphigoid*

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Bullous pemphigoid (BP) is an autoimmune subepidermal blistering disease which pathogenesis is associated with destruction of the basement membrane components and the anchoring fibers. The binding of autoantibodies to antigens localized in the basement membrane of the epidermis activates a series of immunological and enzymatic phenomena that lead to blister formation. There are some data that MMPs are involved in the development of skin lesions in BP, however their exact role in this process is not fully understood. We aimed to investigate whether MMPs and their inhibitors (TIMPs), assessed by their tissue expression, are involved in the pathogenesis of BP. The localization and expression of collagenase (MMP1), gelatinase (MMP2), 92 kD gelatinase (MMP9) and stromelysin 2 (MMP10) and TIMP1, 2, 3 were examined by immunohistochemistry in skin biopsies as well as in normal human skin specimens. The study included 21 patients with BP at an active stage of the disease. The MMPs and TIMPs serum levels were measured by ELISA method. Expression of MMP1, MMP2, MMP9 and MMP10 was observed either in the whole epidermis or in the basal keratinocytes. Most of the enzymes examined, apart from TIMP3, were detected in dermal part of the blister. Expression of the majority of the enzymes examined was observed in blister fluid however, the most intense signal was noted for MMP10. In cellular infiltrate we found expression of all the MMPs and TIMPs, the most distinct for MMP1, MMP2, MMP10 and for TIMP2. In all biopsies obtained from healthy volunteers only single basal keratinocytes gave positive, weak signal for the examined proteins. The MMPs and TIMPs serum levels in the control group were normal while in some cases of BP patients they were increased. Based on the results we conclude that imbalance between these enzymes really occurs in BP and it is likely to take important part in the pathogenesis of the disease.

Introduction

Bullous pemphigoid (BP) is one of the autoimmune subepidermal blistering diseases, characterized by inflammatory infiltrate in the dermis, bound *in vivo* IgG and C3 deposits along the basement membrane zone and circulating IgG autoantibodies. Pathogenesis of BP is associated with destruction of the basement membrane components and the anchoring fibers. The binding of autoantibodies to antigens localized in the basement membrane of the epidermis activates a series of immunological and enzymatic phenomena that in consequence lead to blister formation [6].

Autoantigens in BP are glycoproteins: 230 kD (BPAG1) and 180 kD (BPAG2). BPAG1 belongs to family of plakins, intracellular proteins connecting the intermediate filament of the cytoskeleton with desmosomes and hemidesmosomes. The main autoantigen in BP is BPAG2 (collagen XVII) [18]. It is a key protein constituting the anchoring fibers responsible for adhesion of the epidermis to the basement membrane. Structural studies revealed that extracellular fragment of collagen XVII, with COOH-terminal collagenous domain connects the basement membrane with the epidermal hemidesmosomes. NC16a fragment of BPAG2, located within its

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extracellular fragment, is thought to be the most immunogenic part of the antigen [16].

Literature data revealed the role of certain metalloproteinases (MMPs) in the pathogenesis of BP. Recent studies established biochemical properties of metalloproteinases and their tissue inhibitors (TIMPs) and their high affinity to components of the basement membrane zone, especially to collagens XVII and VII.

Some authors [5, 10, 21] suggest that both MMPs, such as collagenases, stromalysins, gelatinases and their tissue inhibitors, present in BP skin lesions play an important role in destruction of the basement membrane and blister formation. However, a precise localization of these enzymes in the epidermis in the course of BP and their exact role have not been fully elucidated. Thus, the aim of our study was to determine expression of selected metalloproteinases: MMP1, MMP3, MMP9, MMP10 and TIMP1, TIMP2, TIMP3 in skin lesion biopsies taken from patients with BP in order to assess their involvement in the pathogenesis of the disease. The localization and expression of collagenase (MMP1), gelatinase (MMP2), 92 kD gelatinase (MMP9), stromelysin 2 (MMP10) and TIMP1, TIMP2, TIMP3 were examined by immunohistochemistry in diseased skin biopsies as well as in normal human skin biopsy specimens. The MMPs and TIMPs serum levels were measured by ELISA method.

Material and Methods

Patients

Twenty one patients (15 women, 6 men) with BP, mean age of 68.5 years (58–84) entered the study. The patients were at an active stage of the disease, i.e. they presented with skin lesions (bullae, vesicles, papules). The biopsies were taken before administration of any (topical or systemic) medications. Pemphigoid was diagnosed based on clinical picture, and histological and immunological findings. Eleven out of 21 patients presented with skin bullae, vesicles and itching papules, whereas others had only erythematous papules. In the all patients direct immunofluorescence test revealed bound *in vivo* IgG/C3 linear deposits along the basement membrane zone. In salt split method deposits were observed in the epidermal part of the blister. By indirect immunofluorescence test circulating IgG antibodies were found in 17/21 patients, whereas ELISA test showed the presence of anti-Nc16a autoantibodies in 19/21 cases. Histological examination gave features consisting with the diagnosis of BP, it means: neutrophilic infiltrates, eosinophils, lymphocytes and in 11 cases – subepidermal blisters.

The control group consisted of 10 healthy volunteers (5 women, 5 men), mean age of 42 years (19–49).

Before entering the study all the patients and volunteers gave a written inform consent. The study was approved by local Ethics Committee of Medical University of Lodz.

Immunohistochemistry

Paraffin-embedded sections (3–4 μm thick) were used for routine HE staining and for immunohistochemistry in DAKO EnVision detection system using immunoperoxidase method. The following primary mouse monoclonal antibodies were used: anti-MMP1, anti-MMP2, anti-MMP9, anti-MMP10 and anti-TIMP1, anti-TIMP2, anti-TIMP3 (Novocastra). For immunohistochemistry, the paraffin-embedded sections were placed on adhesive plates and dried at 56°C for 24 hours, later deparaffinized in a series of xylene and alcohols with decreasing concentrations (96%, 80%, 70%, 60%). Activity of endogenous peroxidase was blocked with 3% hydrogen peroxide solution in methanol for 5 minutes.

In order to retrieve the antigens of tissue and allow them to react with antibodies, specific procedures were used for each antibody, according to manufacturer' instructions. After incubation with diluted antibodies for 60 minutes at room temperature, slides were washed twice with TRIS buffer. Then, DAKO EnVision double-step visualization system was applied to visualize the antigen-antibody reaction. In cases of positive immunohistochemical reaction cellular nuclei were stained with Meyer's hematoxylin for 2 minutes. After dehydratation and processing through series of acetones and xylene, as described above, the sections were fixed in Canadian balm.

The three-step semiquantitative scale was applied in the evaluation of the intensity of immunohistochemical reaction. The first step (weak intensity) – immunohistochemical reaction was limited to the single epithelial cells and/or was only focally present in the stroma. The second step (moderate intensity) – immunohistochemical reaction was observed in part of epithelial cells and/or was present in some areas of the stroma. The third step (strong intensity) – immunohistochemical reaction was observed in numerous epithelial cells and/or was present in large areas of the stroma.

Expression of MMPs and TIMPs was assessed by two independent pathologists with a use of Nikon Microfob FXA microscope (Nikon LTD, Japan).

In the serum samples concentrations of metalloproteinases (MMP2, MMP3, MMP9) and their tissue inhibitors were determined by immunoenzymatic method – ELISA (Quantikine, R&D Systems).

TABLE 1
Expression of MMPs and TIMPs in examined tissues

	Positive biopsies/all biopsies						
Enzyme	basal keratinocytes	whole epidermis	blister fluid	epidermal part of blister	dermal part of blister	infiltration	stromal cells
Patients (N=21) examined group							
MMP 1	11/21	10/21	2/11	2/11	9/11	14/21	4/21
MMP 2	18/21	3/21	4/11	0/11	9/11	14/21	9/21
MMP 9	11/21	10/21	9/11	3/11	6/11	10/21	16/21
MMP 10	2/21	19/21	11/11	3/11	6/11	14/21	10/21
TIMP 1	9/21	12/21	3/11	2/11	7/11	10/21	0/21
TIMP 2	1/21	20/21	9/11	2/11	8/11	14/21	6/21
TIMP 3	9/21	13/21	11/11	4/11	2/11	10/21	0/21
Controls (N=10)							
MMP 1	single keratinocytes	ND	ND	ND	ND	ND	4/10
MMP 2	single keratinocytes	ND	ND	ND	ND	ND	4/10
MMP 9	single keratinocytes	ND	ND	ND	ND	ND	4/10
MMP 10	single keratinocytes	ND	ND	ND	ND	ND	4/10
TIMP 1	single keratinocytes	ND	ND	ND	ND	ND	4/10
TIMP 2	single keratinocytes	ND	ND	ND	ND	ND	4/10
TIMP 3	single keratinocytes	ND	ND	ND	ND	ND	4/10

ND- non detectable

Results

Epidermis

Expression of MMP1, MMP2, MMP9 and MMP10 was observed either in the whole epidermis or in the basal keratinocytes. Signals for MMP1 and MMP9 were found in both structures in equal percentage, i.e. in 50% (Table 1). MMP2 expression was found mainly in the basal keratinocytes (18 out of 21 biopsies) (Fig. 1) whereas MMP10 – in the whole epidermis (19 out of 21 biopsies) (Fig. 2). Signal intensity for these enzymes was assessed as very high.

Blister

Most of the examined enzymes, apart from TIMP3 were detected in dermal part of the blister. TIMP3 was found in

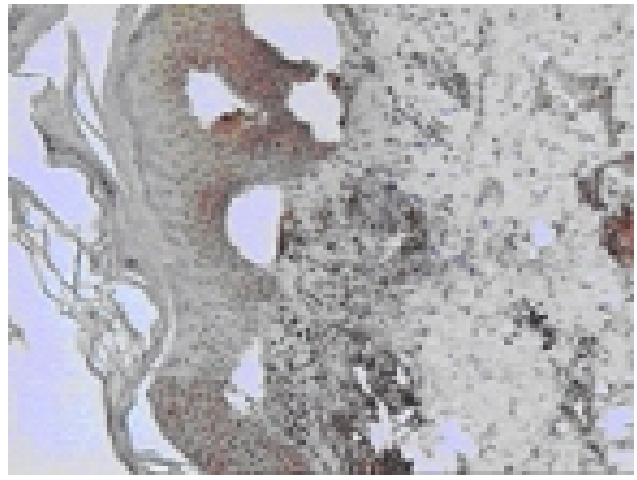

Fig. 1. Skin lesions. Immunohistochemistry. Moderate expression of MMP2 in the basal keratinocytes and infiltrates. Magn. 400×.

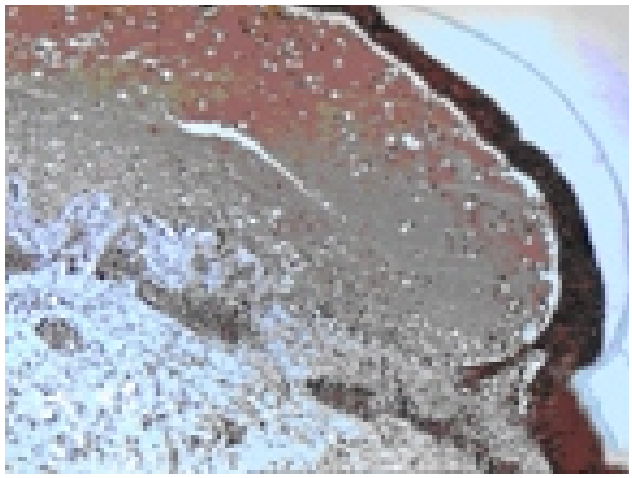

Fig. 2. Skin lesions. Immunohistochemistry. High expression of MMP10 in the whole epidermis and in blister fluid. Magn. 100×.

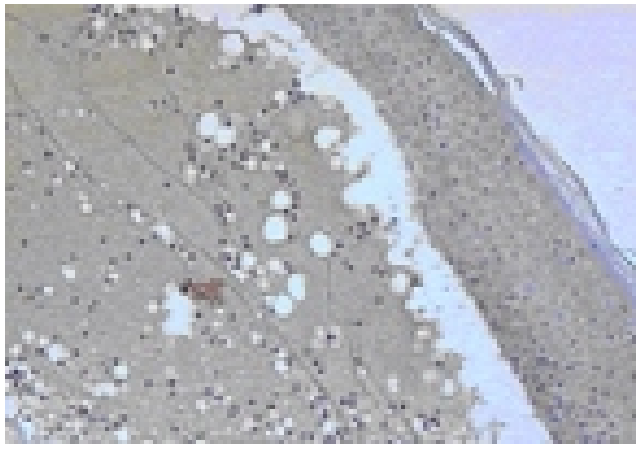

Fig. 3. Skin lesions. Immunohistochemistry. Weak expression of TIMP in the whole epidermis and blister fluid. Magn. 400×.

blister fluid in all the specimens (11 out of 11 biopsies with blisters) (Fig. 3). Expression of other metalloproteinases and their inhibitors was also observed in blister fluid however, the most intense signal was noted for MMP10. In the epidermal part of blister expression of the enzymes examined was observed only occasionally (Table 1).

Infiltrate

In cellular infiltrate (eosinophils and neutrophils) we revealed expression of all the MMPs and TIMPs, however the most distinct for MMP1, MMP2, MMP10 and for TIMP2. In stromal cells no TIMP1 and TIMP3 were found. The most intense signal was noted for MMP2 and MMP10.

Controls

Expression of MMP1, MMP2, MMP9, MMP10 and TIMP1, TIMP2, TIMP3 was examined in 10 skin samples

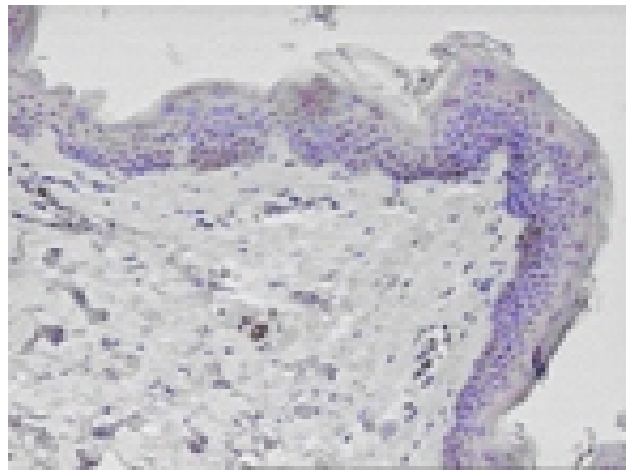

Fig. 4. Healthy skin. Immunohistochemistry. Expression of MMP1 in single basal keratinocytes – biopsy from healthy individual. Magn. 400×.

TABLE 2
Serum levels of metalloproteinases and tissue inhibitors in patients with pemphigoid

Enzyme	Increased levels of enzyme/No of patients
MMP 2	0/21
MMP 3	2/21
MMP 9	4/21
TIMP 2	0/21

obtained from healthy volunteers. In all the biopsies only few basal keratinocytes showed moderate expression of the proteins examined (Fig. 4). In 4 hair follicles biopsies a positive, weak staining for the above enzymes was present.

Serum levels of selected metalloproteinases: MMP2, MMP3, MMP9 and their tissue inhibitor 2 were increased only in some cases of BP patients (Table 2).

Discussion

Both soluble and membrane-anchored metalloproteinases participate in the degradation of extracellular matrix (ECM) and are likely to be involved in the development of skin lesions, characteristic for subepidermal bullous diseases, such as pemphigoid or dermatitis herpetiformis [3, 4, 5]. Metalloproteinases are secreted mainly by inflammatory cells. Paracrine/autocrine effect of various cytokines such as IL-6 or IL-18 on cells in inflammatory infiltrate may result in imbalance between metalloproteinases and their tissue inhibitors, causing at last disturbances in architecture of the extracellular matrix [1, 10, 12]. Decreased level of TIMPs leads also to remodeling of ECM [20].

Verraes et al. [21] in studies performed *in vitro* showed the presence of proteolysis of BPAG2 by neutrophilic elastase, secreted by inflammatory cells, mainly neutrophils. The results were not confirmed by *in vivo* experiments what probably results from the fact that main cells of the inflammatory infiltrates in BP are eosinophils.

Studies of fluid collected from spontaneous and artificial bullae revealed significantly elevated levels of collagenases and elastases. The authors suggested an important role of these proteins in preservation of skin integrity [3, 8, 10]. This hypothesis is strongly supported by the fact that the main sources of these enzymes are neutrophils, the main infiltrate cells in active BP [4, 15]. Similar observations on high level of MMPs in blister fluid were noted in dermatitis herpetiformis [12, 20].

Schmidt et al. [17] observed that binding of autoantibodies with BPAG2 activates mastocytes and neutrophiles. Upon activation these cells release specific enzymes: collagenases, stromelysins, gelatinases which digest a series of proteins constituting structures of the basement membrane. Released MMPs lead in consequence to formation of blister [5]. We confirmed the presence and high expression of MMP1, MMP2, MMP9 and MMP10 in the whole epidermis in skin biopsies taken from BP patients at an active stage of the disease. The highest signal intensity was observed for MMP2 and MMP10. We may suppose it was caused by dense infiltrates composed of activated neutrophiles. The results prove the role of the study enzymes in formation of skin lesions in BP. Interestingly, TIMPs were also expressed in all the specimens, predominantly TIMP2, however their expression intensity was much lower when compared to MMPs. TIMPs presence in blister fluid may suggest their role in the last stage of the basement membrane degradation, whereas MMPs are probably key proteins in initiating this phenomenon.

MMP9 and MMP10 probably contribute to the formation of blisters degrading the basement membrane components [11]. We are in favor of this suggestion as we observed high expression of these enzymes, especially MMP10 in the whole epidermis and in the basal keratinocytes.

One of hypotheses on blister lesion development in BP involves the stimulation of collagenase inhibitor synthesis by dermal fibroblasts. Interleukin-1 and other cytokines released by epidermal and migrating cells under inflammatory process stimulate fibroblasts to produce both collagenase [19] and its inhibitor [13]. Imbalance between these two enzyme families subsequently may result in blister formation [7]. TIMPs block the activity of a number of matrix metalloproteinases [2]. That is why it is possible that their low expression might play a role in the development of subepidermal blistering diseases.

Our findings provide evidence that MMP1, MMP2, MMP9, and MMP10 play important roles at phases of destruction of basement membrane, but serum MMPs levels could not have clinical value in identifying patients of high risk for bullous skin diseases. We conclude that the best prognostic value for these dermatoses development is immunohistochemistry detecting expression intensity of MMPs and TIMPs. The results of other authors suggest for the first time that pretreatment serum MMPs level could serve as a prognostic factor in some carcinomas [9, 14].

Impaired expression of metalloproteinases or low expression of their tissue inhibitors, stimulated by immune complexes present in structures of the basement membrane zone may be responsible for destruction of anchoring fibers and blister formation. Further studies focused on the precise role of MMPs and TIMPs in the pathogenesis of pemphigoid are necessary, especially important it would be to discover the factors activating these enzymes in diseased skin and to establish the possible therapeutic use of their inhibitors.

References

1. *Aeschliman D, Kaupp O, Paulsson M:* Osteonectin is a major glutaminyl substrate for transglutaminase-catalyzed cross-linking in cartilage matrix. J Cell Biol 1995,129, 881–892.
2. *Cerretti DP, Poindexter K, Castner BJ et al:* Characterisation of the cDNA and gene for mouse TNF alpha converting enzyme (TACE/ADAM) and its location to mouse chromosome 12 and human chromosome 2p25. Cytokine 1999, 11, 541–551.
3. *Dietrich W, Ehnis T, Bauer M et al:* Identification of tissue transglutaminase as the autoantigen of celiac disease. Nature Med 1997, 3, 797–801.
4. *Dietrich W, Laag E, Bruckner-Tuderman L et al:* Antibodies to tissue transglutaminase as serologic markers in patients with dermatitis herpetiformis. J Invest Dermatol 1999, 113, 133–136.
5. *Grando SA, Glukhenky BT, Orannik GN et al:* Mediators of inflammation in blister fluids from patients with pemphigus vulgaris and bullous pemphigoid. Arch Dermatol 1989, 125, 925–930.
6. *Jordon RE:* Basement zone antibodies in bullous pemphigoid. JAMA 1967, 200, 751–756.
7. *Kuno K:* Molecular cloning of gene encoding a new type of metalloproteinases – disintegrin family protein with trombospondin motifs as an inflammation associated gene. J Biol Chem 1998, 272, 556–562.
8. *Martinez J, Chalupowicz R, Roush A:* Transglutaminase-mediated processing of fibronectin by endothelial cell monolayers. Biochemistry 1994, 33, 2538–2545.
9. *Nikkola J, Vihinen P, Vuoristo MS, Kellokumpu-Lehtinen P, Kahari VM, Pyrhonen S:* High serum levels of matrix metalloproteinase-9 and matrix metalloproteinase-1 are associated with rapid progression in patients with metastatic melanoma. Clin Cancer Res 2005, 11, 5158–5166.
10. *Oikarinen A, Kyimaniemi M, Autjo-Harmainen H et al:* Demonstration of 72-kDa and 92-kDa forms of type IV collagenase in human skin: variable expression in various blistering diseases, inducting during re-epithelialization and decrease by topical glucocorticosteroids. J Invest Dermatol 1993, 101, 205–210.

11. *Oikarinen AI, Reunala T, Zone JJ et al:* Proteolytic enzymes in blister fluids from patients with dermatitis herpetiformis. Br J Dermatol 1986, 114, 295–230.
12. *Pauschinger M, Chandrasekharan K, Li J et al:* Mechanisms of extracellular matrix remodeling in dilated cardiomiopathy. Herz 2002, 27, 677–682.
13. *Primakoff P, Myles DG:* The ADAM gene family: surface proteins with adhesion and protease activity. Trends Genet 2000, 16, 83–87.
14. *Ruokolainen H, Paakko P, Turpeenniemi-Hujanen T:* Serum matrix metalloproteinase-9 in head and neck squamous cell carcinoma is a prognostic marker. Int J Cancer 2005, 1, 116, 422–427.
15. *Sardy M, Karpati S, Merkl B et al:* Epidermal transglutaminase (Tgase 3) is the autoantigen of dermatitis herpetiformis. J Exp Med 2002, 195, 747–757.
16. *Schmidt E, Obe K, Brecker EB et al:* Serum levels of autoantibodies to BP 180 correlate with disease activity in patients with bullous pemphigoid. Arch Dermatol 2000, 136, 253–254.
17. *Schmidt E, Reimer S, Kruse N et al:* Autoantibodies to BP 180 associated with bullous pemphigoid relase interleukin-6 and interleukin-8 from cultured human keratinocytes. J Invest Dermatol 2000, 115, 842–848.
18. *Stanley JR:* Cell adhesion molecules as targets of autoantibodies in pemphigus and pemphigoid, bullous diseases due to defective epidermal cell adhesion. Adv Immunol 1992, 53, 291–325.
19. *Stone AI, Kroeger M, Xiang Q et al:* Structure- function analysis of the ADAm family of disintegrin – like and metalloproteinase-containing proteins. J Protein Chem 2000, 18, 447–465.
20. *Stricklin GP, Nanney LB:* Immunolocalization of collagenase and TIMPs in healing human burn wounds. J Invest Dermatol 1994, 103, 488–492.
21. *Verraes S, Homebeck W, Polette M et al:* Respective contribution of neutrophil elastase and matrix metalloproteinase 9 in the degradation of BP 180 (type XVII) in human bullous pemphigoid. J Invest Dermatol 2001, 117, 1091–1096.

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