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Permalink
https://escholarship.org/uc/item/9v42m5mh

Journal
The Journal of experimental medicine, 188(3)

ISSN
0022-1007

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Publication Date
1998-08-01

DOI
10.1084/jem.188.3.475

Peer reviewed
Gelatinase B–deficient Mice Are Resistant to Experimental Bullous Pemphigoid

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Summary

Bullous pemphigoid (BP) is an autoimmune subepidermal blistering disease characterized by deposition of autoantibodies at the basement membrane zone. In an experimental BP model in mice, the subepidermal blistering is mediated by antibodies directed against the hemidesmosomal protein BP180 (collagen XVII, BPAG2), and depends on complement activation and neutrophil infiltration. Gelatinase B is present in BP blister fluid and can cleave BP180. In this study we investigated the role of gelatinase B in the immunopathogenesis of experimental BP using mice containing targeted disruption of the gelatinase B (MMP-9, 92 kD gelatinase) gene. Gelatinase B–deficient mice were resistant to the blistering effect of intracutaneous anti-mBP180 antibodies, although these mice showed deposition of autoantibodies at the basement membrane zone and neutrophil recruitment to the skin comparable to that observed in the control mice. Interleukin 8 given intradermally concomitantly with pathogenic anti-mBP180 elicited a significant neutrophil recruitment into the skin in gelatinase B–deficient mice, but blistering did not occur. However, gelatinase B–deficient mice reconstituted with neutrophils from normal mice developed blistering in response to anti-mBP180 antibodies. These results implicate neutrophil-derived gelatinase B in the pathogenesis of experimental BP and might lead to novel therapeutic strategies for BP.

Key words: autoimmunity • basement membrane zone • hemidesmosome • inflammation • mouse model

Bullous pemphigoid (BP)1 is an autoimmune subepidermal blistering disorder characterized by deposition of autoantibodies at the basement membrane zone (BMZ), complement activation, and inflammatory cell infiltration (1). BP autoantibodies are directed against two major hemidesmosomal components, the 230-kD intracellular protein BP230 (2, 3) and the 180-kD transmembrane protein BP180 (BPAG2, HD4, or type XVII collagen) (4–8). Histologic and ultrastructural studies show that the blisters in BP occur within the lamina lucida of the basement membrane (9, 10). It has been hypothesized that proteinases and reactive free radicals released from infiltrating inflammatory cells contribute to the tissue damage in BP lesions (11, 12). BP blister fluids and lesional/perilesional sites contain serine proteinases including neutrophil elastase, plasmin, plasminogen activators, and matrix metalloproteinases including gelatinase A (72-kD gelatinase; matrix metalloproteinase [MMP]-2) and gelatinase B (92-kD gelatinase; MMP-9) (13–19). However, the role of each of these enzymes in the pathogenesis of BP is unclear.

Gelatinase B is normally expressed by neutrophils, macrophages, osteoclasts, and trophoblasts (20). It cleaves a number of extracellular matrix proteins, including entactin, aggrecan, pepsinized type IV collagen, and elastin (21–24). It also acts on various nonmatrix macromolecules, including α1-proteinase inhibitor (α1-PI), galactoside-binding proteins, IL-1β, and substance P (20). Gelatinase B is an abundant component of BP blister fluid and is localized to BP lesional sites (19). Furthermore, gelatinase B cleaves the extracellular, collagenous domain of recombinant BP180 antigen (19). These data suggest that gelatinase B plays an important role in BP.

1Abbreviations used in this paper: α1-PI, α1-proteinase inhibitor; APMA, p-aminophenylmercuric acetate; BMZ, basement membrane zone; BP, bullous pemphigoid; IF, immunofluorescence; mBP180, murine BP180 antigen; MMP, matrix metalloproteinase; MPO, myeloperoxidase.
In this study we assessed whether gelatinase B plays a role in complement activation and neutrophil infiltration (26, 27). Subepidermal blister formation, which is triggered by anti-murine BP180 IgG (anti-mBP180), is dependent on complement activation and neutrophil infiltration (28). Neonatal mice, 24–36-h-old, weighing 1.4–1.6 g, were used for passive transfer experiments. One pathogenic anti-BP180 IgG (referred to as R621) and one control IgG (referred to as R50) were used.

Preparation of Pathogenic Rabbit Anti-murine BP180 IgG. The preparation of recombinant murine BP180 and the immunization of rabbits were performed as previously described (25). In brief, a segment of the murine BP180 antigen encompassing amino acids 495–643 of the ectodomain of this protein (29) was expressed as a glutathione S-transferase (GST) fusion protein using the pGEX prokaryotic expression system (Pharmacia Biotech, Piscataway, NJ). The murine BP180 fusion protein, designated GST-mBP180AABC, was purified to homogeneity by affinity chromatography (30). New Zealand White rabbits were immunized with the purified murine BP180 fusion protein and the IgG fraction from the sera was purified as previously described (25). The titers of rabbit anti-murine BP180 antibodies in the rabbit sera and in the purified IgG fractions were assayed by indirect immunofluorescence (IF) using mouse skin cryosections as substrate as reported elsewhere (25). The pathogenicity of IgG preparations was tested by passive transfer experiments. One pathogenic anti-mBP180 IgG (referred to as R 621) and one control IgG (referred to as R 50) were used.

Induction of Experimental BP and Clinical Evaluation. Neonates were given one intradermal injection (50 μl each, 2.5 mg/g body weight) of a sterile solution of IgG in PBS. The injection techniques have been described elsewhere (25, 31, 32). The skin of neonatal mice from the test and control groups was examined 12 h after the IgG injections. The activity of cutaneous disease was scored as follows: (−), no detectable skin disease; (+), mild erythematous reaction with no evidence of “epidermal detachment” (epidermal detachment was elicited by gentle friction of the skin that, when positive, produced fine, persistent wrinkling of the epidermis); (+), intense erythema and epidermal detachment involving 10–50% of the epidermis in the injection site; and (++) intense erythema with frank epidermal detachment involving >50% of the epidermis in the injection site. In some experiments, neonatal gelatinase B−/− and B+/+ mice were coinjected with human recombinant IL-8 (100 ng) and pathogenic anti-mBP180 IgG (2.5 mg/g body weight). After 12 h the skin was examined and biopsied for histological examination and quantification of PMN by MPO assay as described below.

Immunohistochemistry. After clinical examination the animals were killed. Skin sections were taken for light microscopy (hematoxylin and eosin staining) and direct IF to detect rabbit IgG and mouse C3 deposition at the BMZ, and sera was obtained for indirect IF analysis to determine the circulating titers of anti-BP180 IgG. Direct and indirect IF analyses were performed as previously described (25, 31, 32).

Quantification of Skin Site PMN Accumulation. Tissue MPO activity in skin sites of the injected animals was assayed as previously described (33). A standard reference curve was first established by measuring known concentrations of purified MPO. The skin sites were excised and extracted by homogenization in an extraction buffer containing 0.1 M Tris-Cl, pH 7.6, 0.15 M NaCl, and 0.5% hexadecyl trimethyl ammonium bromide. MPO activity in supernatants was measured by the change in optical density (OD) at 460 nm resulting from decomposition of H2O2 in the presence of o-dianisidine. MPO content was expressed as relative MPO activity (OD460nm × mg/g tissue protein). Protein concentrations were determined by the Bio-Rad dye binding assay (Bio-Rad, Hercules, CA) using BSA as a standard.

Gelatin Zymography. Gelatinase profiles were determined by zymography as described previously (34). In brief, protein extracts of neutrophils and skin sections from injected animals were subjected to SDS-PAGE on gelatin-containing acrylamide gels (10% acrylamide and 1% gelatin) under nonreducing conditions. After electrophoresis, gels were washed twice with 2.5% Triton X-100 for 30 min to remove SDS. Gels were then rinsed briefly with water followed by incubation overnight at 37°C in reaction buffer containing 50 mM Tris, pH 7.4, 150 mM NaCl, and 5 mM CaCl2. The gels were stained with 0.125% Coomassie Brilliant blue. Areas of gelatinolytic activity appeared as clear zones against a dark blue background.

To determine the class of proteinases visualized on zymograms, samples were mixed with inhibitors or the solvent used for the inhibitor and incubated for 15 min at room temperature before electrophoresis. The final concentrations of inhibitors used were 1 mM PMSF (isopropanol as the solvent), 5 mM EDTA (water as the solvent), and 10 mM 1,10-phenanthroline (methanol as solvent). After electrophoresis, the gels were washed in 2.5% Triton X-100, incubated in reaction buffer, and stained with 0.125% Coomassie Brilliant blue as described above.

Neutrophil Isolation and Extraction. Neutrophils were isolated from heparinized blood by dextran sedimentation followed by separation on a density gradient as previously described (35). In brief, the blood from adult animals was drawn by cardiac puncture into syringes containing heparin (20 U/ml). After sedimentation in an equal volume of 3% Dextran T 500 (Pharmacia Biotech AB, Uppsala, Sweden) in PBS for 20 min at room temperature, the leukocyte-rich layer was washed in PBS and layered over Ficoll-Hypaque (Pharmacia Biotech AB). The neutrophils and mononuclear cells were separated by centrifugation for 40 min at 400 x g at 20°C. Red blood cells were then removed from the cell preparation by hypotonic lysis in 0.2% NaCl. N neutrophils were washed and resuspended in cold PBS/10 mM glucose, counted in a hemocytometer, and adjusted to a concentration of 107 cells/ml. N neutrophil purity of the final cell preparation was consistently >95% as determined by cell-cytospin and LeukoStat staining (Fisher Diagnostics, Orangeburg, NY). The viability of the neutrophils was >95% as determined by trypan blue exclusion. The purified neutrophils were homogenized and extracted in the same manner as described for skin samples.
In Vivo R constitution of N neutrophil G elastase B. Gelatinase B−/− mice were injected intradermally with pathogenic anti-mBP180 IgG (2.5 mg/l 10 μl PBS). 2 h later, these mice received 5 × 105 neutrophils intradermally (in 50 μl of PBS/10 mM glucose) at the same site. The neutrophils were purified from gelatinase B−/− or gelatinase B+/+ mice. 12 h after the IgG injections the animals were analyzed as described above.

Statistical Analysis. The data were expressed as mean ± SEM and were analyzed using Student’s t test. P < 0.05 was considered significant.

Results

Gelatinase B is present in experimental BP blisters. Gelatinase B is abundant in blister fluid from patients with BP (19). To determine if gelatinase B was present in the subepidermal blisters of experimental BP, control BALB/c mice were injected with pathogenic rabbit anti-mBP180 IgG and lesional skin samples were analyzed by gelatin zymography. A prominent gelatinolytic band migrating at 97 kD was present only in lesional skin of mice injected with pathogenic anti-mBP180 IgG, R 621 (Fig. 1, lane 3). This gelatinolytic band comigrated with murine gelatinase B (Fig. 1, lane 4). The zymogen of the enzyme (pro) was converted to smaller fragments (act) by organomercurial activation (p-aminophenylmercuric acetate [APMA]) (Fig. 1, lane 11). No gelatinolytic band at 97 kD was present in samples from mice injected with PBS (Fig. 1, lane 1) or control IgG R 50 (Fig. 1, lane 2). In all skin samples gelatinolytic activities were also detected at 69, 64, and 50 kD, presumptively due to gelatinase A (MMP-2; 72-kD gelatinase), which is expressed by fibroblasts. All of the gelatinolytic activities could be blocked by 5 mM EDTA (Fig. 1, lanes 5–7) and 1,10-phenanthroline (data not shown), which inhibit matrix metalloproteinases, but not by the serine proteinase inhibitor PM SF (Fig. 1, lanes 8–10). These findings confirmed that the 97-kD gelatinase (gelatinase B), was associated with subepidermal blister formation in experimental BP, most likely from infiltrating neutrophils.

Gelatinase B-deficient mice are resistant to the pathogenic activity of anti-mBP180 A antibodies. To directly assess whether gelatinase B is involved in subepidermal blister formation in experimental BP, gelatinase B−/− and gelatinase B+/+ mice were injected intradermally with pathogenic anti-BP180 IgG (2.5 mg/l body weight). BALB/c mice were also used as a positive control. As expected, gelatinase B+/+ mice (n = 9) and the BALB/c controls (Table 1) developed extensive blisters 12 h after injection with anti-mBP180 IgG (Fig. 2 A). The skin of these animals was markedly erythematous and developed persistent epidermal wrinkling due to the detachment of the epidermis from the underlying dermis.

Direct IF on cryosections of lesional/perilesional skin of the mice showed in situ deposition of rabbit anti-mBP180 IgG (Fig. 2 B) and mouse C3 (Fig. 2 C) at the BMZ, dermal-epidermal separation, and neutrophilic infiltration (Fig. 2 D). In contrast, gelatinase B−/− mice (n = 9) exhibited no blisters 12 h after injection with anti-mBP180 IgG (Fig. 2 E), although they had a comparable level of circulating rabbit IgG (data not shown) to the wild-type mice, and direct IF showed in situ deposition of rabbit IgG (Fig. 2 F) and mouse C3 (Fig. 2 G) at the BMZ. The skin sections from gelatinase B−/− mice exhibited neutrophilic in-

Table 1. Summary of the Role of Gelatinase B in Experimental BP

| Host mice* | IgG injected | Treatment | Number of mice | Disease activity |
|------------|--------------|-----------|----------------|----------------|
| BALB/c     | R 50         | –         | 9              | –              |
|            | R 621        | –         | 12             | 3+             |
| Gelatinase B+/+ | R 50   | –         | 8              | –              |
|            | R 621        | –         | 14             | 3+             |
| Gelatinase B−/− | R 621 | –         | 16             | –              |
|            | R 621 + mPM N (−/−) | 5         | –              |
|            | R 621 + mPM N (+/+) | 5         | 2+             |
|            | R 621 + IL-8 | –         | 5              | –              |

*Primary BALB/c, gelatinase B-sufficient (gelatinase B+/+), and gelatinase B-deficient (gelatinase B−/−) mice were injected intradermally with either control IgG (R 50) or pathogenic anti-mBP180 antibody (R 621). Purified mouse neutrophils (5 × 10^5 cells/50 μl) from either gelatinase B−/− [mPM N (−/−)] or gelatinase B+/+ [mPM N (+/+) ] mice were given intradermally 2 h after IgG injection. Disease activity is scored on a scale of − to +, − means no detectable skin lesion; 2+ means intense erythema and epidermal detachment involving >50% of the epidermis at the injection site; 3+ means intense erythema with frank epidermal detachment involving >50% of the epidermis at the injection site. See Materials and Methods for details.
As expected, skin extracts from gelatinase B$^{-/-}$ filtration, but no dermal–epidermal separation (Fig. 2 H). As expected, skin extracts from gelatinase B$^{-/-}$ mice, which did not form blisters after pathogenic anti-mBP180 IgG, did not show gelatinase B activity (data not shown).

MPO activity, a method of quantifying neutrophil infiltration, was similar in the skin at the IgG-injected sites of gelatinase B$^{-/-}$, gelatinase B$^{+/+}$, and BALB/c control mice at 4 h after injection (Fig. 3, bars 1, 3, and 5). However, significant differences ($P < 0.001$) in the extractable MPO activity were observed at 12 h after injection. Gelatinase B$^{-/-}$ skin extracts showed approximately half as much MPO activity as controls (Fig. 3, bars 2, 4, and 6). These data indicate that neutrophil gelatinase B deficiency did not prevent neutrophil recruitment during the initial 4-h period after intradermal pathogenic anti-mBP180 IgG injection. This is important because the onset of blister formation in gelatinase B$^{+/+}$ mice is seen in this 4-h period. The higher MPO values at 12 h in control mice are consistent with the development of tissue injury in the skin of these animals leading to additional neutrophil recruitment between 4 and 12 h.

To determine whether gelatinase B participates in the immunopathology of BP or in neutrophil recruitment from the circulation into inflammatory sites, gelatinase B$^{-/-}$ ($n = 5$) and gelatinase B$^{+/+}$ ($n = 5$) mice were coinjected intradermally with a neutrophil chemoattractant, IL-8 (100 ng/mouse), and pathogenic anti-mBP180 IgG (2.5 mg/g body weight). These animals were examined 12 h after injection (Table 1). Although the IL-8 resulted in higher levels of MPO activity in the skin of the gelatinase B$^{-/-}$ (1.49 ± 0.21), comparable to positive control mice (1.19 ± 0.11), the gelatinase B$^{-/-}$ mice still showed no clinical or histological signs of blistering (data not shown). These data indicate that gelatinase B plays a direct role in the events leading to blister formation in experimental BP, after neutrophils are recruited into the skin.

Pathogenic Anti-mBP180 Antibodies Induce BP Blisters in Gelatinase B$^{-/-}$ Mice Reconstituted with Normal Neutrophils. If gelatinase B released from neutrophils is directly involved in the tissue injury in experimental BP, then gelatinase B$^{-/-}$ mice reconstituted with normal neutrophils should develop subepidermal blisters when challenged with the pathogenic anti-mBP180 IgG. Therefore, gelatinase B$^{-/-}$ mice ($n = 5$) were injected intradermally with pathogenic anti-mBP180 IgG and 2 h later received an intradermal injection of 5 × 10$^6$ neutrophils isolated from either gelatinase B$^{-/-}$ or gelatinase B$^{+/+}$ mice. Mice reconstituted with neutrophils from gelatinase B$^{-/-}$ mice showed no skin lesions (Fig. 4, A and B; Table 1). In contrast, mice reconstituted with neutrophils from gelatinase B$^{+/+}$ mice
developed typical BP skin lesions (Fig. 4 C) with dermal-epidermal separation (Fig. 4 D). The lack of gelatinase B had no effect on neutrophil survival in the skin. The retention of exogenous neutrophils in the skin was similar in both gelatinase B+/+ and gelatinase B−/− mice, as determined by MPO activity (data not shown).

Gelatin zymography revealed that gelatinase B−/− mice reconstituted with gelatinase B-deficient neutrophils showed no gelatinase B activity in the protein extracts of skin of mice injected with normal control IgG (Fig. 5, lane 1) or pathogenic IgG (Fig. 5, lane 2). These mice did not develop disease. In contrast, gelatinase B+/+ mice reconstituted with gelatinase B-sufficient neutrophils exhibited high levels of gelatinase B activity in protein extracts of skin of mice injected with control IgG (Fig. 5, lane 3) or pathogenic IgG (Fig. 5, lane 4). However, only pathogenic anti-mBP180 IgG but not control IgG induced clinical disease in gelatinase B−/− mice reconstituted with normal neutrophils. This is because pathogenic antibodies but not control IgG bind to the basement membrane zone in situ, trigger neutrophil degranulation and elicit experimental BP when normal neutrophils are present. Taken together, these results further demonstrate the requirement for release of neutrophil gelatinase B into the BMZ to trigger the pathogenesis of experimental BP.

**Discussion**

Experimental BP in mice involves passive immunization with pathogenic anti-mBP180 IgG to trigger subepidermal blistering (25). We have previously found that blister formation depends on complement activation (26) and neutrophil recruitment into the dermis (27). The neutrophil infiltration is temporally and spatially related to subepidermal blister formation, whereas blockage of neutrophil recruitment into the skin site completely inhibits the pathogenic activity of anti-mBP180 antibodies (27). The central role of infiltrating neutrophils in the tissue injury in experimental BP led us to investigate which neutrophil components are involved in the development of the disease. In this study we demonstrate that gelatinase B released from neutrophils plays an essential role in subepidermal blister formation in experimental BP based on the following findings: (a) a significant level of gelatinase B is present in le-

![Gelatinase B(-/-)](image1)

**Figure 4.** Experimental BP in gelatinase B−/− mice reconstituted with normal neutrophils. Pathogenic rabbit anti-mBP180 IgG R621 (intradermal injection, 2.5 mg/g body weight) did not induce blisters in neonatal gelatinase B−/− mice reconstituted with neutrophils from gelatinase B−/− mice [A, mPMN(−/−)]. The skin of these animals showed no dermal-epidermal separation by H&E (B). In contrast, the pathogenic R621 IgG triggered separation in neonatal gelatinase B−/− mice reconstituted with neutrophils from gelatinase B+/+ mice (C, mPMN(+/+)). H&E-stained section from these mice showing a subepidermal vesicle (D). d, dermis; e, epidermis; v, vesicle. Original magnification: ×100.
Gelatinase B activity is seen when gelatinase B reconstituted with gelatinase B purified from gelatinase B experimental BP (Table 1). In contrast, gelatinase B is observed from noninjected animals or noninvolved skin in lesional skin is neutrophils. Gelatinase B is not seen in skin data suggest that the major source of gelatinase B in mouse injection of pathogenic anti-mBP180 IgG, which coincides with the beginning of subepidermal blistering. Gelatinase B already begun in gelatinase B mice 4 h after the injection of pathogenic IgG, when blister formation has already begun in gelatinase B mice. Increasing neutrophil recruitment in gelatinase B mice to the level seen in gelatinase B mice at 12 h by the intradermal coinjection of IL-8 and pathogenic antibodies did not induce BP blistering. In addition, gelatinase B mice also have normal neutrophil recruitment into the lungs and the peritoneal cavity (Senior, R.M., unpublished observation). Moreover, the pathogenic anti-BP180 IgG induced blistering in gelatinase B mice, but not with neutrophils from gelatinase B mice. Taken together, these data demonstrate that gelatinase B deficiency does not impair neutrophil migration into the skin and implicate neutrophil-derived gelatinase B directly in the subepidermal blistering of experimental BP.

Gelatinase B has a broad substrate specificity for extracellular matrix proteins, including entactin, aggrecan, pepsinized type IV collagen, elastin (20–24), the extracellular domain of recombinant BP180 (19), and nonmatrix proteins such as α₁-PI (20). Thus, gelatinase B could contribute to tissue damage in BP directly by cleaving structural proteins in the dermal–epidermal junction, or indirectly by inactivating α₁-PI, the principal neutrophil elastase inhibitor, or other inhibitors of other neutrophil-derived proteolytic enzymes that may also contribute to the long-term pathogenesis (38, 39). Determining which gelatinase B substrates are critical in the dermal–epidermal detachment will allow further dissection of the immunopathological mechanisms of subepidermal blister formation in BP and aid in the development of more effective therapeutic strategies for this autoimmune skin disorder.

This work was supported in part by U.S. Public Health Service National Institutes of Health grants R29 AI-40768 (Z. Liu), R01 AR-32599 and R37 AR-32081 (L.A. Diaz), R01 HL-47328 (R.M. Senior), and P60 AR-20684 and P01 CA-72006 (Z. Werbl); by a Veterans Affairs Merit Review Grant (L.A. Diaz); and by the Alan A. and Edith L. Wolfe Charitable Trust (R.M. Senior). Z. Liu was the recipient of a Dermatology Fellowships in Experimental Bullous Pemphigoid

**Figure 5.** Gelatin zymography of lesional skin samples of gelatinase B−/− mice reconstituted with normal neutrophils. Neonatal gelatinase B−/− mice received intradermally 2.5 mg/g body weight of control IgG R50 (lanes 1 and 3), or pathogenic anti-mBP180 IgG R621 (lanes 2 and 4). 2 h later, these mice were injected at the same site with 5 × 10⁵ neutrophils purified from gelatinase B−/− mice (mPMN N/−/−; lanes 1 and 2), or gelatinase B−/− mice (mPMN N/+/+; lanes 3 and 4). 12 h after IgG injection, these animals were examined for blistering, and skin biopsies at the injection site were analyzed by gelatin zymography (12 μg/lane). Purified control murine neutrophil extract (1 μg/lane 5) was used as a standard for gelatinase B. Gelatinase B−/− mice reconstituted with mPMN N/−/− showed no disease and no gelatinase B in the skin samples when injected with control R50 IgG (lane 1) or R621 IgG (lane 2). In contrast, gelatinase B−/− mice reconstituted with mPMN N/+/+ developed disease when injected with R621 IgG (lane 4) but not control IgG R50 (lane 1). Gelatinase B activity is seen when gelatinase B−/− mice are reconstituted with normal neutrophils (lanes 3 and 4).

| Strain         | mPMN(-/-) | mPMN(+/*) | Disease |
|---------------|-----------|-----------|---------|
| -/-           | -         | -         | -       |
| +/-           | +         | +         | +       |
| +/-           | -         | -         | +       |
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