EPITHELIAL AND MESENCHYMAL CELL BIOLOGY

SerpinB2 Deficiency Results in a Stratum Corneum Defect and Increased Sensitivity to Topically Applied Inflammatory Agents

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SerpinB2 (plasminogen activator inhibitor type 2) is constitutively expressed at high levels by differentiating keratinocytes in mice and humans; however, the physiological function of keratinocyte SerpinB2 remains unclear. Herein, we show that *SerpinB2*^{-/-} mice are more susceptible to contact dermatitis after topical application of dinitrofluorobenzene, and show enhanced inflammatory lesions after topical applications of phorbol ester. Untreated *SerpinB2*^{-/-} mice showed no overt changes in epithelial structure, and we were unable to find evidence for a role for keratinocyte SerpinB2 in regulating immunity, apoptosis, IL-1 β production, proteasomal activity, or wound healing. Instead, the phenotype was associated with impaired skin barrier function and a defective stratum corneum, with *SerpinB2*^{-/-} mice showing increased transepidermal water loss, increased overt loss of stratum corneum in inflammatory lesions, and impaired stratum corneum thickening after phorbol ester treatment. Immunoblotting suggested that SerpinB2 (cross-linked into the cornified envelope) is present in the stratum corneum and retains the ability to form covalent inhibitory complexes with urokinase. Data suggest that the function of keratinocyte SerpinB2 is protection of the stratum corneum from proteolysis via inhibition of urokinase, thereby maintaining the integrity and barrier function of the stratum corneum, particularly during times of skin inflammation. Implications for studies involving genetically modified mice treated with topical agents and human dermatological conditions, such as contact dermatitis, are discussed. (*Am J Pathol* 2016, 186: 1511–1523; <http://dx.doi.org/10.1016/j.ajpath.2016.02.017>)

SerpinB2 (alias plasminogen activator inhibitor type 2) is a member of the clade B or ovalbumin-like serine protease inhibitor (ov-serpin) subgroup of the serpin superfamily. SerpinB2 is expressed by a range of cells, including differentiating keratinocytes, cancer cells, monocytes/macrophages, fibroblasts, endothelial cells, and dendritic cells, and is often up-regulated during inflammation.^{1–4} Despite >1000 articles in PubMed on SerpinB2/plasminogen activator inhibitor type 2, its physiological function remains unresolved, with this serpin labeled the undecided or enigmatic serpin.^{5–7}

Extracellular SerpinB2 is widely described as an inhibitor of the extracellular protease urokinase plasminogen activator

(uPA) and (to a lesser extent) tissue plasminogen activator (tPA), with SerpinB2 (at least *in vitro*) acting as a suicide substrate to form a covalent SerpinB2-uPA or SerpinB2-tPA complex.⁴ tPA and uPA convert plasminogen to plasmin, a broad-spectrum protease involved in many processes, including inflammation and fibrinolysis,⁸ extracellular matrix degradation and cell migration/invasion,^{9,10} wound healing,¹¹ cleavage of specific cell surface proteins,¹² and, if deregulated, tissue damage and disease.^{13–16} *SerpinB2*^{-/-} mice

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show no generalized/overt defects in plasminogen activation, and mice deficient in both SerpinB2 and plasminogen activator inhibitor type 1 show no additional plasminogen activation defects over those seen in plasminogen activator inhibitor type 1 deficient (*PAI-1*^{-/-}) mice,¹⁷ with tPA/PA inhibition therefore conceivably restricted to specific settings.¹

SerpinB2, like other ov-serpins, lacks a classic secretory signal peptide and is usually inefficiently secreted.¹⁸ However, in cancer cells and macrophages, efficient SerpinB2 secretion via microparticle formation was recently reported, providing at least one mechanism and setting whereby intracellular SerpinB2 can efficiently reach and inhibit extracellular uPA.¹⁰ Such microparticle-based secretion may explain the inhibitory effect of SerpinB2 expression on uPA-dependent migration and invasion.^{10,19} Macrophage SerpinB2 has also been associated with modulation of type 1/2 helper T-cell responses in *SerpinB2*^{-/-} mice,^{10,20–23} a phenotype potentially also associated with microparticle secretion.²⁴

SerpinB2 has also been reported to have several ostensibly uPA-independent intracellular binding partners and activities,¹ including negative regulation of NLRP3 inflammasome activation and IL-1 β release,^{25,26} inhibition of calpain,²⁷ inhibition of apoptosis,^{27,28} stabilization of transglutaminase 2,²⁹ and inhibition of the proteasome.³⁰

Herein, we sought to gain insights into the physiological role of keratinocyte SerpinB2. Differentiating keratinocytes constitutively express high levels of SerpinB2, up to approximately 7000 ng/mg of total protein, whereas SerpinB2 is largely absent in simple epithelia.^{3,31} Changes in SerpinB2 distribution and/or expression have been reported for several skin conditions, including psoriasis,³² bullous pemphigoid,³³ lupus,³⁴ congenital ichthyosis, Netherton syndrome,³⁵ chronic venous ulcers,³⁶ scleroderma,³⁷ and hyperkeratotic corn tissue.³⁸ Several roles for keratinocyte SerpinB2 have been proposed, including regulation of growth and/or differentiation,^{39–42} inhibition of apoptosis,^{27,28,43} and inhibition of migration.⁴⁴ SerpinB2 has also been proposed to regulate wound healing,^{4,36,45} although cutaneous wound healing (which involves keratinocyte migration) was unaffected in *SerpinB2*^{-/-} mice.¹⁷ SerpinB2 is largely retained intracellularly in living keratinocytes.^{46,47} During terminal differentiation of human keratinocytes, cytoplasmic SerpinB2 is covalently cross-linked by transglutaminase into the cornified envelope (CE) via glutamic acid residues (Gln83, Gln84, and Gln86¹) present in the large CD interhelical loop region of SerpinB2.^{47–49} Ultimately, the CE replaces the keratinocyte's plasma membrane and the resultant terminally differentiated corneocytes form the stratum corneum (SC), the uppermost lipid-rich skin layer essential for the physical and water barrier functions of the skin.^{49,50} Herein, we provide evidence that the physiological role of keratinocyte SerpinB2 is protection of the integrity of the SC, particularly in inflammatory settings.

Materials and Methods

Animal Ethics Statement

All mouse work was conducted in accordance with good animal practice, as defined by the National Health and Medical Research Council of Australia. The mouse work was approved by the QIMR Berghofer Medical Research Institute animal ethics committee.

DNFB Sensitization and Challenge

Female *SerpinB2*^{-/-} and *SerpinB2*^{+/+} mice (6 to 8 weeks old) were bred in-house at QIMR Berghofer Medical Research Institute.²¹ Dinitrofluorobenzene (DNFB) sensitization and challenge were performed essentially as described.⁵¹ Briefly, DNFB (Sigma, St. Louis, MO) [0.5% (w/v)] in 100 μ L of an acetone–olive oil (4:1) mixture was applied to backs. Lesion scores were obtained by blind scoring of lesion images by three independent assessors, supplied with a reference series of lesion scores.

Antimurine SerpinB2 Antibody

An antimurine SerpinB2 antibody was generated in rabbits by Peptide Specialty Labs GmbH (Heidelberg, Germany), using the peptide ⁶²EIGSYGITRNPENFSGC⁷⁹ (corresponding to the N-terminal region of the CD loop of mouse SerpinB2) coupled to ovalbumin, with the antiserum affinity purified using the same peptide. [In our hands, current commercially available anti-SerpinB2 antibodies do not specifically recognize murine SerpinB2 (data not shown).]

Histology

Skin samples were fixed in 10% neutral buffered formalin and embedded in paraffin wax, and sections (6 mm thick) were cut and stained with hematoxylin and eosin. Sections were digitally scanned using the Aperio AT-Turbo (Leica Microsystems, North Ryde, Australia). Image analyses were undertaken using Aperio ImageScope software version 10 (Aperio Technologies, Vista, CA) and the Positive Pixel Count algorithm version 9 (Aperio Technologies).

Immunohistochemistry

Paraffin sections (3 to 4 μ m thick) were affixed to Menzel Superfrost Plus adhesive slides (Menzel GmbH, Braunschweig, Germany) and air dried overnight at 37°C. Sections were dewaxed and rehydrated through descending graded alcohols to running tap water using standard protocols. After washing in phosphate-buffered saline, the sections were treated with 0.001 mol/L EDTA/0.01 mol/L Tris buffer, pH 8.8, for 15 minutes at 105°C, followed by washing. Endogenous peroxidase activity was blocked using 5% H₂O₂ in phosphate-buffered saline for 10 minutes, followed by washing. Normal goat serum (10%) was applied for 30 minutes, followed by the rabbit polyclonal

anti-SerpinB2 antibody diluted 1:200 in phosphate-buffered saline for 1 hour at room temperature. After washing, Biocare Medical MACH2 Rabbit HRP was added for 30 minutes. After washing, color was developed using 3,3'-diaminobenzidine for 5 minutes. Sections were washed in tap water, lightly counterstained in Mayer's hematoxylin, dehydrated through ascending graded alcohols, cleared in xylene, and then mounted.

Apoptotic cells were stained using the ApoTag Plus Peroxidase *In Situ* Apoptosis Detection Kit (Millipore, Billerica, MA), according to the manufacturer's instructions. Slides were scanned and analyzed as above. Neutrophils were stained using anti-Ly6G, as described previously.⁵²

Immunoblotting of Skin and SC Preparations

Full-thickness skin samples were cut from within the treatment sites and cut into small pieces and homogenized at 4°C using ceramic beads (2.8 mm; MOBIO Labs, Inc., Carlsbad, CA) and a tissue homogenizer (Precellys 24, 6500 rpm, twice for 20 seconds) in 0.1% Igepal, 20 mmol/L HEPES, pH 7.5, supplemented with cOmplete Protease Inhibitor Cocktail (Roche, Dee Why, Australia). The samples were then spun at top speed on a bench microfuge, and supernatants run on standard denaturing SDS-PAGE. Immunoblotting was performed using the anti-SerpinB2 and anti- β -actin (rabbit, 13E5; Cell Signaling, Danvers, MA) antibodies. Resident peritoneal macrophages, which express high levels of SerpinB2, were prepared as described.²¹ The secondary antibody was goat anti-rabbit horseradish peroxidase (Chemicon, Temecula, CA).

SC samples were collected by gently scraping the dampened skin surface of the mice at the treatment or control sites with a flat scalpel blade. The SC samples collected at the blade edge were scraped into 0.1% SDS, 2 mmol/L EDTA, 20 mmol/L HEPES, pH 7.5, on ice. The samples were homogenized (Precellys 24) and sonicated (20 seconds $\times$ 6) and protein concentrations determined by standard Bradford protein assay (Bio-Rad, Hercules, CA). Samples (15 μ g) were run on a standard SDS-PAGE and analyzed by immunoblotting, using anti-SerpinB2 and anti-uPA (rabbit anti-mouse uPA; Abcam, Cambridge, UK) antibodies, with the secondary antibody as above.

TEWL, PMA Treatment, and Inflammation Scoring

Transepidermal water loss (TEWL) was measured using the hand-held VapoMeter (Delfin Technologies Ltd, Kuopio, Finland); four readings were taken for each mouse at each time point. For phorbol 12-myristate 13-acetate (PMA) treatment, mice were shaved on the backs and 5 days later 10 μ g PMA was applied in 20 μ L of acetone daily for 3 days. Images of the lesions were taken and analyzed using Color Deconvolution Algorithm software version 9 (Aperio Technologies) to separate red (erythema) from brown/black (no erythema).

Statistical Analysis

Analyses were performed using IBM SPSS Statistics version 19 (Armonk, North Castle, NY). The *t*-test was used

if the difference in the variances was <4 , skewness was >-2 , and kurtosis was <2 . Where the data were nonparametric and difference in variances was <4 , the Mann Whitney *U* test was used, if >4 the Kolmogorov-Smirnov test was used. Where indicated, a repeat measures analysis of variance was used after log transformation of the data because of the differences in variance.

Results

DNFB Irritant Contact Dermatitis Is More Severe in *SerpinB2*^{-/-} Mice

To investigate the physiological function of keratinocyte SerpinB2, DNFB was topically applied to the skin of *SerpinB2*^{-/-} and *SerpinB2*^{+/+} mice. DNFB is a hapten (widely used in models of irritant contact dermatitis and allergic contact hypersensitivity) that reacts with proteins in the skin, triggering innate proinflammatory responses, as well as adaptive T- and B-cell responses.^{53,54} *SerpinB2*^{-/-} mice developed significantly more severe skin reactions than *SerpinB2*^{+/+} mice after a single topical treatment with DNFB (Figure 1). Skin reactions were scored (0 to 6) blind by three independent assessors, with minimal variation evident between assessors (Figure 1A). Skin reactions were significantly more severe in *SerpinB2*^{-/-} (compared with *SerpinB2*^{+/+}) mice on multiple days after DNFB application in three independent experiments (Figure 1, B and C). Lesions in *SerpinB2*^{+/+} mice were largely restricted to mild patchy serous exudates, whereas lesions in *SerpinB2*^{-/-} mice were generally characterized by robust and extensive eschar formation (Figure 1D).

Histology and Immunohistochemistry of DNFB-Induced Lesions

Hematoxylin and eosin staining showed that, in contrast with *SerpinB2*^{+/+} mice, *SerpinB2*^{-/-} mice had more epidermis showing loss of SC after DNFB treatment (Supplemental Figure S1, A and B), consistent with the increased eschar formation seen in these mice (Figure 1C). These lesions in the SC were always associated with pronounced neutrophil infiltrates (Supplemental Figure S1A), with neutrophil recruitment to the skin after DNFB application well described.⁵⁵ However, neutrophil recruitment to the treatment sites after DNFB application was not significantly different between *SerpinB2*^{-/-} and *SerpinB2*^{+/+} mice, as demonstrated by immunohistochemistry using quantitative image analysis (Supplemental Figure S1C) of anti-Ly6G antibody staining (Supplemental Figure S1D).⁵² In neither *SerpinB2*^{-/-} nor *SerpinB2*^{+/+} mice was there significant recruitment of macrophages (as determined by immunohistochemistry and F4/80 staining⁵²) to the DNFB treatment sites or to the epidermis apparent at 24, 48, or 72 hours after DNFB treatment (Supplemental Figure S2).

Figure 1 Skin reactions in *SerpinB2*^{-/-} and *SerpinB2*^{+/+} mice after topical dinitrofluorobenzene (DNFB) treatment. **A:** Individual skin reaction severity scores for each of 10 *SerpinB2*^{-/-} and 10 *SerpinB2*^{+/+} mice on day 3 after topical 0.5% DNFB application. Values were established for each mouse from scores provided by three independent assessors. **B:** Mean skin reaction severity scores (mean of 10 mice per group) from *SerpinB2*^{-/-} and *SerpinB2*^{+/+} mice over time after DNFB treatment. (Scoring as for **A**; day 3 data derived from **A**). **C:** Two independent repeat experiments (set up as for **B**). **D:** Images of the treatment areas on the backs of the shaved mice showing five *SerpinB2*^{+/+} mice (top panels) and five *SerpinB2*^{-/-} mice (bottom panels). The skin reaction severity score for each mouse (three independent assessors) is shown bottom right of each image (each of these mice is represented in **A**). Data are given as means $\pm$ SEM (**A** and **D**). $n = 5$ mice per group [**C**, Expt. 2]; $n = 8$ mice per group (**C**, Expt. 3). * $P < 0.05$, ** $P \leq 0.01$, and *** $P \leq 0.001$. Expt., experiment.

SerpinB2 Expression in Mouse Skin

SerpinB2 is constitutively expressed by epidermal keratinocytes in both mice and humans, with levels in human keratinocytes up to approximately 7000 ng/mg.^{1,3} We generated a new polyclonal, affinity-purified, anti-mouse SerpinB2 antibody raised against the unique CD interhelical loop region of SerpinB2 (as described in *Materials and Methods*). Using this antibody, we were able to confirm

the abundant intracellular expression of SerpinB2 in murine epidermal keratinocytes of *SerpinB2*^{+/+} mice before DNFB treatment (*Figure 2A*), with no other cell types in skin showing significant SerpinB2 staining (data not shown). The absence of significant staining in *SerpinB2*^{-/-} mice illustrated the specificity of the antibody (*Figure 2, B, D, F, and H*). At 6 hours after DNFB application, SerpinB2 expression in *SerpinB2*^{+/+} mouse epidermis was substantially reduced, with some keratinocytes showing no SerpinB2 staining (*Figure 2C*) and some condensed SerpinB2 staining associated with degenerating cells (*Figure 2C*). At 24 hours (*Figure 2E*) and 48 hours (data not

Figure 2 Immunohistochemistry and immunoblotting of skin from *SerpinB2*^{-/-} and *SerpinB2*^{+/+} mice after dinitrofluorobenzene (DNFB) application. Immunohistochemistry using anti-SerpinB2 antibody (and hematoxylin counter stain) of skin from *SerpinB2*^{+/+} (**A, C, E, and G**) and *SerpinB2*^{-/-} (**B, D, F, and H**) mice at the indicated times after application of DNFB. Dotted oval in **C** shows keratinocytes with loss of SerpinB2 staining. **Arrowheads** in **C** and **D** indicate keratinocytes with degenerating nuclei. **I:** Immunoblotting of skin samples from *SerpinB2*^{+/+} and *SerpinB2*^{-/-} mice at the indicated times after application of DNFB using anti-SerpinB2 and anti-actin antibodies. Resident peritoneal macrophages (RPMs) were included as a positive control; these cells express high levels of SerpinB2.

shown) after DNFB treatment, SerpinB2 staining of epidermis was lost. At 72 hours after DNFB, SerpinB2 staining reappeared in patches of regenerating epidermis (Figure 2G), consistent with previous findings.⁵⁶ Western blotting of extracts from whole (full-thickness) skin samples showed the expected approximately 47-kDa band in skin of *SerpinB2*^{+/+} mice, but not *SerpinB2*^{-/-} mice, with no other bands detected (Figure 2I). Primary resident peritoneal macrophages from *SerpinB2*^{+/+} mice express high levels of SerpinB2,²¹ and resident peritoneal macrophage lysates from *SerpinB2*^{+/+} mice, but not *SerpinB2*^{-/-} mice, also showed the expected approximately 47-kDa band, with no other bands detected (Figure 2I). The latter two immunoblotting experiments attest to the specificity of the anti-SerpinB2 antibody. At 6 hours after DNFB treatment, a SerpinB2 cleavage product of approximately 40 kDa appeared in skin lysates (Figure 2I), with loss of detectable expression evident at 24 hours (Figure 2I) and 48 hours (data not shown), consistent with the immunohistochemistry data (Figure 2E). N-terminal cleavage of SerpinB2 has been reported previously and has been associated with apoptosis,^{2,46,57} with keratinocyte apoptosis evident after DNFB treatment (see below). The polyclonal anti-SerpinB2 antibody was raised to a peptide (amino acids 62 to 79) from the CD loop region, suggesting an N-terminal cleavage event is located just N-terminal to, or just within, the CD loop region.¹

These data thus confirm the expression of SerpinB2 protein in wild-type mouse keratinocytes, with SerpinB2 cleavage and loss after DNFB treatment consistent with keratinocyte apoptosis.

Lesion Severity in *SerpinB2*^{-/-} Mice Is Not Associated with Changes in T- and B-Cell Responses

Mice were sensitized as above and then challenged with DNFB on the ear 6 days later, as per the standard contact hypersensitivity model.^{51,54} Skin lesions in the ears of *SerpinB2*^{-/-} mice were again more severe (Supplemental Figure S3A), essentially recapitulating data shown in Figure 1. However, the ear swelling (using three different challenge doses of DNFB) was not different between *SerpinB2*^{-/-} and *SerpinB2*^{+/+} mice (Supplemental Figure S3, B and C). Because T cells are a primary driver of inflammation after challenge,⁵⁴ these data do not support the notion that DNFB-induced T-cell activity was significantly altered in *SerpinB2*^{-/-} mice.

Antibodies also play a role in hapten-based contact hypersensitivity.⁵⁸ However, no significant differences in anti-DNFB IgG1 or IgG2c responses^{21,59} were apparent between *SerpinB2*^{-/-} and *SerpinB2*^{+/+} mice in two independent experiments (Supplemental Figure S4). Thus, in contrast with macrophage SerpinB2,^{1,20–23} these data suggest that keratinocyte SerpinB2 (at least in this setting) is not involved in modulating adaptive immune response. Adaptive immune responses are thus also unlikely to be responsible for the observations described in Figure 1.

Lesion Severity in *SerpinB2*^{-/-} Mice Is Not Associated with Altered IL-1 Responses

Inflammasome activation and IL-1 β release represents a prominent early keratinocyte response after DNFB application.^{53,60} SerpinB2 has been reported to inhibit IL-1 β release^{25,26}; however, treatment with anakinra (an IL-1 receptor antagonist) did not significantly decrease the severity of the DNFB-induced skin lesions in *SerpinB2*^{-/-} or *SerpinB2*^{+/+} mice (Supplemental Figure S5A). Furthermore, no increase in IL-1 β processing was seen in skin of *SerpinB2*^{-/-} mice after DNFB application (Supplemental Figure S5B), nor were the total levels of IL-1 α and IL-1 β significantly different in skin from *SerpinB2*^{-/-} and *SerpinB2*^{+/+} mice (Supplemental Figure S5C). These results do not support a role for keratinocyte SerpinB2 in regulating IL-1, and suggest IL-1 differences do not account for the exacerbated skin pathology shown in Figure 1.

The rate of lesion resolution after DNFB treatment was no different in *SerpinB2*^{-/-} and *SerpinB2*^{+/+} mice (irrespective of anakinra treatment) (Supplemental Figure S5D), consistent with the lack of a wound healing phenotype in *SerpinB2*^{-/-} mice reported previously.¹⁷

Lesion Severity in *SerpinB2*^{-/-} Mice Is Not Associated with Altered Keratinocyte Apoptosis

A role for SerpinB2 in protection against apoptosis has been widely reported,^{27,61} although the literature is not consistent.^{1,10,43,62,63} Apotag staining revealed substantial levels of apoptosis in keratinocytes 6 hours after DNFB application (Supplemental Figure S6A). DNFB-induced apoptosis of keratinocytes has, to our knowledge, not previously been reported. However, Apotag staining levels were not significantly different between *SerpinB2*^{-/-} and *SerpinB2*^{+/+} mice (Supplemental Figure S6B). Thus (in this setting), keratinocyte SerpinB2 does not appear to regulate apoptosis, suggesting differences in apoptosis are not responsible for the observations in Figure 1.

No Overt Differences in Epidermal Structure in *SerpinB2*^{-/-} Mice

Several articles have suggested that SerpinB2 has a role in regulating keratinocyte growth and/or differentiation.^{39–42} However, hematoxylin and eosin staining of foot pads (which have a much thicker epidermis than normal mouse skin) showed no overt differences in the structure of the epidermis or the SC from untreated *SerpinB2*^{-/-} and *SerpinB2*^{+/+} mice (Figure 3B), consistent with a previous study.¹⁷

SerpinB2 staining of foot pads illustrated that SerpinB2 protein expression was lower in the basal cells than the suprabasal stratifying cells (Figure 3C), a pattern similar to that seen in human skin.^{32,34,35} Staining of the SC with the anti-SerpinB2 antibody was not generally observed (Figure 3C), except on rare occasions (Supplemental Figure S6C).

Figure 3 Transepidermal water loss (TEWL), histology, and immunohistochemistry of untreated skin from *SerpinB2*^{-/-} and *SerpinB2*^{+/+} mice. **A:** TEWL was measured from the belly skin (unshaved) of *SerpinB2*^{+/+} and *SerpinB2*^{-/-} mice. Statistics by *t*-tests. **B:** Hematoxylin and eosin staining of footpads from *SerpinB2*^{-/-} and *SerpinB2*^{+/+} mice. **C:** Immunohistochemistry of footpads from *SerpinB2*^{-/-} and *SerpinB2*^{+/+} mice with anti-SerpinB2 antibody. *n* = 20 (**A**, aged 10 to 15 weeks); *n* = 8 (**A**, aged 17 to 19 weeks); *n* = 4 to 7 (**A**, aged 35 weeks). ***P* ≤ 0.01, ****P* < 0.001. SC, stratum corneum.

No Evidence for SerpinB2 Deficiency Changing Proteasome Activity

SerpinB2 has been reported to bind the proteasomal subunit member β1,^{2,64} with a report also suggesting SerpinB2 can inhibit proteasome activity.³⁰ In an extensive series of experiments, we were unable to show a consistent or significant effect of SerpinB2 expression on proteasomal enzyme activities (Supplemental Figure S7).

Microarray Analysis of Normal Skin from *SerpinB2*^{-/-} and *SerpinB2*^{+/+} Mice

To gain insights into the cell type(s) and processes in the skin potentially dysregulated by SerpinB2 deficiency, microarray analysis⁶⁵ of normal skin from *SerpinB2*^{-/-} and *SerpinB2*^{+/+} mice was undertaken. Only a handful of genes were significantly up-regulated or down-regulated in *SerpinB2*^{-/-} mice by more than twofold (Supplemental Table S1). This mild effect on gene expression is perhaps consistent with the normal skin structure and wound healing seen in *SerpinB2*^{-/-} mice. DAVID Functional Annotation Clustering analysis illustrated that genes down-regulated in *SerpinB2*^{-/-} skin were significantly associated with keratinocytes and keratinocyte differentiation, including keratinization and cornification (Supplemental Table S2). Keratinocytes express functional olfactory receptors,^{66–68} which can sense odorants^{66,69} and be up-regulated by allergens⁷⁰; and DAVID analysis of up-regulated genes identified genes associated with olfactory receptors as being up-regulated in *SerpinB2*^{-/-} skin (Supplemental Table S2). These results suggest that the differences in the skin of *SerpinB2*^{-/-} and *SerpinB2*^{+/+} mice largely resided within the keratinocyte population [rather than other cell type within the skin (eg, fibroblasts, endothelial cells, or macrophages, which can also express SerpinB2)] and that *SerpinB2*^{-/-} mice might have a CE/SC defect given that the processes described in Supplemental Table S2 are associated with terminal differentiation of keratinocytes and the formation of the CE/SC.

Increased TEWL in *SerpinB2*^{-/-} Mice

During the terminal differentiation of keratinocytes, SerpinB2 is cross-linked by transglutaminase into the CE^{47,48} via glutamic acid residues in the CD loop region,³⁵ with the CEs ultimately forming the SC. The importance of an intact skin barrier function for limiting the sensitivity to hapten-induced contact dermatitis is widely accepted,^{53,71} with the SC largely responsible for the skin barrier function.⁴⁹ Because the microarray analysis suggested a CE/SC defect, skin barrier function was investigated by measuring TEWL.⁷² A significant and consistent 15% to 30% increase in TEWL was apparent in *SerpinB2*^{-/-} mice, with TEWL decreasing with age (Figure 3A and Supplemental Figure S8A). SerpinB2 deficiency in keratinocytes thus results in an impaired skin barrier function, an observation that would explain increased sensitivity to topically applied haptens.

TEWL and Inflammation after Topical PMA Treatment of *SerpinB2*^{+/+} and *SerpinB2*^{-/-} Mice

Topical application of PMA is known to up-regulate both SerpinB2 expression and the normally low uPA expression in the epidermis.⁷³ *SerpinB2*^{+/+} and *SerpinB2*^{-/-} mice were thus treated topically with PMA (days 0, 1, and 2) and TEWL monitored. The initial 11% higher TEWL in *SerpinB2*^{-/-} (compared with *SerpinB2*^{+/+}) mice increased to a mean 27% (range, 8% to 48%) difference between days 2 and 9 after treatment initiation (Figure 4A). PMA treatment thus exacerbated the differences in TEWL between *SerpinB2*^{+/+} and *SerpinB2*^{-/-} mice. The rate of TEWL recovery after PMA treatment cessation was similar in *SerpinB2*^{+/+} and *SerpinB2*^{-/-} mice (Figure 4A and Supplemental Figure S5E), again consistent with the lack of a wound healing phenotype in *SerpinB2*^{-/-} mice.

In the same experiment, digital images of the PMA treatment sites were taken (Supplemental Figure S8B) and erythema (red color) quantitated by Color Deconvolution Algorithm software version 9. The red erythema score was

Figure 4 Skin treatment with phorbol 12-myristate 13-acetate (PMA). **A:** *SerpinB2*^{-/-} and *SerpinB2*^{+/+} mice were treated topically with PMA three times (at the times indicated by arrows) and transepidermal water loss (TEWL) measured at the treatment sites. Statistics: differences on day 0 between *SerpinB2*^{-/-} and *SerpinB2*^{+/+} mice determined by *t*-test; differences on days 2 to 7 between *SerpinB2*^{-/-} and *SerpinB2*^{+/+} mice determined by repeated measures analysis of variance. **B:** PMA treatment as for **A**, with erythema (red pixels) quantitated by color deconvolution software from images of skin lesions (Supplemental Figure S8B). Statistics by repeated measures analysis of variance. **C:** Hematoxylin and eosin (H&E) staining of treatment sites on day 2.5 (6 hours after last PMA treatment). Arrows indicate intact stratum corneum (SC). **D:** Length and number of lesions without SC with each lesion plotted as a single dot: black for *SerpinB2*^{+/+} and red for *SerpinB2*^{-/-} (two H&E sections per mouse). **E:** Statistical treatment of data from **D** comparing mean number of skin lesions where SC was absent. Statistics by Kolmogorov-Smirnov test. **F:** Statistical treatment of data from **D** comparing mean percentage of skin section lengths where SC was absent. Statistics by Kolmogorov-Smirnov test. *n* = 6 per group (**A** and **D**). **P* < 0.05, ***P* < 0.01.

significantly higher in *SerpinB2*^{-/-} compared with *SerpinB2*^{+/+} mice (Figure 4B), suggesting greater penetration of PMA across the SC in *SerpinB2*^{-/-} mice.

Increased Loss of SC after PMA Treatment of *SerpinB2*^{-/-} Mice

Mice were treated with PMA, as described above, and lesions were examined by hematoxylin and eosin. In *SerpinB2*^{-/-} mice, the SC was frequently absent, in particular in areas containing high numbers of neutrophils, which are efficiently recruited by PMA treatment.⁷⁴ The presence of neutrophils was again confirmed by anti-Ly6G staining (data not shown). In such neutrophil-rich areas in *SerpinB2*^{+/+} mice, the SC remained largely intact (Figure 4C). After PMA treatment, *SerpinB2*^{-/-} mice showed more, and longer, skin section lengths with overt loss of SC (Figure 4D). Both the number of lesions (Figure 4E) and percentage of skin section lengths without SC (Figure 4F) were significantly different between *SerpinB2*^{+/+} and *SerpinB2*^{-/-} mice.

Epidermal and SC Thickening after PMA Treatment

Topical PMA application is known to cause keratinocyte hyperproliferation, resulting in thickening of the epidermal layer,^{75,76} and promote terminal differentiation of keratinocytes, resulting in thickening of the SC.⁷⁷ The thickening of the epidermal cell layers was not significantly different between *SerpinB2*^{+/+} and *SerpinB2*^{-/-} mice after PMA treatment (Figure 5, A and C). However, the significant thickening of the SC seen in *SerpinB2*^{+/+} mice (after PMA) was not seen in *SerpinB2*^{-/-} mice (Figure 5, B and C). Thus, under inflammatory conditions known to induce uPA, an overt SC defect was apparent in *SerpinB2*^{-/-} mice.

Detection of SerpinB2 and uPA in SC Extracts by Immunoblotting

To determine whether murine SerpinB2 (like human SerpinB2) is present in the SC, SC samples were analyzed by immunoblotting. SC samples were collected by gently

Figure 5 The epidermal cell layer and stratum corneum (SC) after phorbol 12-myristate 13-acetate (PMA) treatment. **A:** The mean thickness of the epidermal cell layer in *SerpinB2*^{-/-} (-/-) and *SerpinB2*^{+/+} (+/+) mice as determined from hematoxylin and eosin sections from PMA treatment sites (PMA+) or control sites (PMA-) on day 2.5 after PMA treatment initiation. (Two sections per site and eight to nine measurements per section were used to generate a mean for each mouse.) **B:** Same as **A** except measuring the thickness of the SC. Statistics by *t*-test and Kolmogorov-Smirnov test. **C:** Examples of thickness measurements used in **A** (epidermis, long arrows) and **B** (SC, short arrows). **D:** Immunoblot of skin scrapings from PMA treatment sites (+PMA) and control sites (-PMA) from untreated mice. The stacking gel is indicated (STACK); the bottom of the stack is indicated by a dotted line. **E:** Immunohistochemistry of PMA treatment sites using the anti-SerpinB2 antibody. **F:** Immunohistochemistry of PMA treatment sites using an anti-urokinase plasminogen activator (uPA) antibody. *n* = 6 mice per group (**A**, PMA+); *n* = 4 mice per group (**A**, PMA-). **P* < 0.05, ***P* < 0.01. CE, cornified envelope; N, neutrophil-rich infiltrate.

scraping the surface of dampened skin with a flat scalpel blade. In multiple experiments, high-molecular-weight anti-SerpinB2 reactive bands were detected in the stacking gel, which were significantly more prominent in SC samples from *SerpinB2*^{+/+} mice after PMA treatment, and were undetectable in samples from *SerpinB2*^{-/-} mice (Figure 5D and Supplemental Figure S8C). The proteins in the SC exist as large covalently cross-linked complexes and would thus not be expected to migrate far into a polyacrylamide gel. When the same SC samples were analyzed with an anti-uPA antibody, high-molecular-weight bands in the stacking gel were also apparent in samples from *SerpinB2*^{+/+} mice after PMA treatment, but not in SC samples from *SerpinB2*^{-/-} mice (Figure 5D). The former also showed a long uPA-reactive smear in the separating gel (Figure 5D). A smear was also faintly visible when this sample was immunoblotted with the anti-SerpinB2 antibody (Figure 5D). These smears suggest degradation of the SC, perhaps by one

or more of the >30 proteases expressed by keratinocytes and/or infiltrating inflammatory cells.⁷⁸

The results (Figure 5D) thus support the view that SerpinB2, which is covalently cross-linked to proteins in the SC, retains the ability to generate covalent inhibitory complexes with uPA, thereby covalently attaching uPA (via SerpinB2) to the proteins in the SC. (The CD loop is known to be dispensable for uPA inhibition.⁷⁹) Thus, in *SerpinB2*^{-/-} mice, although uPA is present (Figure 5F), because of the absence of cross-linked SerpinB2, uPA does not become covalently attached to the SC.

Immunohistochemistry after PMA with Anti-SerpinB2 and uPA Antibodies

Anti-SerpinB2 antibody staining of skin sections from *SerpinB2*^{+/+} mice after PMA treatment illustrated strong SerpinB2 expression in the upper layers of the epidermis

(Figure 5E). Some staining of the CE was also evident (Figure 5E); the tightly packed lipid-rich lamellar bodies in the SC may limit antibody access. In contrast to previous reports (reviewed by Schroder et al¹), we were unable to see significant SerpinB2 protein expression in neutrophils (Figure 5E).

As expected,⁷³ uPA expression was clearly evident in keratinocytes after PMA treatment, and was similar in both mouse strains (Figure 5F). However, staining was particularly pronounced in the infiltrating neutrophils (Figure 5F), with uPA expression by neutrophils well established in other settings.⁸⁰ Normal rabbit serum controls showed no significant staining (data not shown). The loss of SC in *SerpinB2*^{-/-} mice was nearly always associated with the presence of neutrophils in the epidermal layer. Thus, both keratinocytes and infiltrating neutrophils provide a source of uPA after PMA treatment.

Discussion

Herein, we show for the first time that a physiological function of keratinocyte SerpinB2 is protection of the SC and its barrier function, an activity particularly evident during skin inflammation. The skin of *SerpinB2*^{-/-} mice showed increased TEWL and increased sensitivity to cutaneous applications of irritants, with an intact barrier function key to limiting water loss and penetration of chemicals into the skin.^{49,53,71,81} Furthermore, PMA-induced thickening of the SC did not occur in *SerpinB2*^{-/-} mice, and *SerpinB2*^{-/-} mice experienced loss of SC at sites with extensive neutrophil infiltrates after PMA or DNFB treatment. Our data support the view that SerpinB2 cross-linked into the CE and subsequently incorporated into the SC retains the ability to inhibit uPA, thereby limiting proteolytic degradation of the SC (an activity that most clearly manifests in inflammatory settings). uPA is up-regulated in keratinocytes and abundant in infiltrating neutrophils during skin inflammation. Plasmin(ogen) (the target of uPA) is found throughout the epidermis⁸² and the SC⁸³ in inflammatory lesions, with plasmin also shown to induce psoriasiform skin inflammation.¹³

Differentiating keratinocytes constitutively express high levels of intracellular SerpinB2 in both humans and mice, with SerpinB2 secretion by keratinocytes shown to be inefficient.^{3,47} *SerpinB2*^{-/-} mice showed no overt differences in the arrangement and structure of living keratinocyte layers in the epidermis, either in the resting state (in agreement with previous studies¹⁷) or after PMA treatment. Although a sizable literature has ascribed several activities to SerpinB2 and keratinocyte SerpinB2, we were unable to find evidence for a role for keratinocyte SerpinB2 in regulating IL-1 β production, apoptosis, proteasome activity, or wound healing. Although keratinocytes can secrete active uPA during migration and wound healing,^{11,84,85} physiologically, SerpinB2's role in regulating this process would appear to be minimal. We recently reported efficient secretion of SerpinB2 by

macrophages and transformed cells via microparticle formation, with SerpinB2 potentially externalized through association with phosphatidylserine (via annexins). Externalized SerpinB2 in these latter settings was able to inhibit tumor cell invasion/migration.¹⁰ However, to our knowledge, primary keratinocytes have not been reported to make microparticles, and phosphatidylserine externalization does not occur during keratinocyte differentiation⁸⁶ or cornification.⁸⁷ The physiological role of keratinocyte SerpinB2 would thus appear to be largely restricted to protection of the SC.

A role for SerpinB2 in regulating immunity has been well described,^{1,20–23} and is likely mediated by SerpinB2 expression in macrophages. Several observations argue that the *SerpinB2*^{-/-} mouse skin phenotype described herein is not associated with an immune-based activity: i) no significant differences were seen in anti-DNFB T-cell and antibody responses in *SerpinB2*^{-/-} and *SerpinB2*^{+/+} mice, ii) neutrophil recruitment after DNFB treatment was not significantly altered, iii) no significant recruitment of macrophages to the DNFB treatment sites or the epidermis was apparent, and iv) microarray analysis of skin suggested differences in *SerpinB2*^{-/-} mice were largely associated with keratinocytes.

The SC defect in *SerpinB2*^{-/-} mice suggests topically applied proinflammatory agents would penetrate more easily into the skin of *SerpinB2*^{-/-} mice (Figure 4B). This contention might prompt a reinterpretation of some experiments involving topical application of carcinogens (eg, DMBA [alias 7,12-Dimethylbenz(a)anthracene]) and tumor promoters (eg, PMA) in *SerpinB2*^{-/-} mice^{27,43} and mice overexpressing SerpinB2.²⁸ The reduced and increased expression of SerpinB2 might simply result in increased and reduced, respectively, penetration of topically applied agents, rather than revealing an antiapoptotic role for keratinocyte SerpinB2. Several microarray studies have also shown up-regulation of SerpinB2 mRNA by oncogene products, such as Ras^{88–90} (eg, 33-fold up-regulation by H-Ras),⁹¹ p53,⁹² Raf,^{93,94} and E1A.⁹⁵ *p63*^{-/-} mice also show an approximately 10-fold reduction in skin SerpinB2 mRNA expression.⁹⁶ We have also confirmed the role of Ras and transformation in up-regulating SerpinB2 expression using quantitative RT-PCR, confocal microscopy, and reporter assays^{97,98} (Supplemental Figure S9). Care may thus also be warranted when interpreting studies using mice with genetically modified keratinocytes and topically applied carcinogens and tumor promoters,^{43,99–103} because keratinocytes in such mice may express different levels of SerpinB2, resulting in a modified SC and different penetration of these agents. Increased SerpinB2 expression was linked to the relative resistance of C57BL/6J mice to PMA-mediated tumor promotion when compared with susceptible DBA/2J mice.¹⁰⁴ Similar logic might also be applied to studies on contact dermatitis in genetically modified mice, such as those lacking certain NF- κ B subunits,¹⁰⁵ given the probable regulation of SerpinB2 by NF- κ B.^{106,107} Simple TEWL pretesting might thus be warranted in genetically modified mice to reveal differences in barrier function.

SerpinB2 thus joins other Serpins reported to be important for the barrier function of the SC, including SerpinB3/B4,¹⁰⁸ SerpinB7,¹⁰⁹ and SerpinA12.¹¹⁰ Interestingly, SerpinB2 was the only keratinocyte Serpin down-regulated in a publicly available study of nonlesional skin of allergic contact dermatitis patients¹¹¹ (Supplemental Figure S10, A–C). A dysfunctional SC is also associated with atopic dermatitis,¹¹² and analysis of three published and publicly available microarray studies^{113–115} revealed SerpinB2 is down-regulated in the skin (often nonlesional skin) of such patients (Supplemental Figure S10, D–F). Low keratinocyte SerpinB2 expression in humans might thus (as in mice) increase sensitivity to topical inflammatory agents and therefore predispose individuals to allergic contact dermatitis and/or atopic dermatitis.

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Supplemental Data

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