DEGRADATION OF THE EPIDERMAL-DERMAL JUNCTION BY PROTEOLYTIC ENZYMES FROM HUMAN SKIN AND HUMAN POLYMORPHONUCLEAR LEUKOCYTES

By ROBERT A. BRIGGAMAN, NORMAN M. SCHECHTER,* JORMA FRAKI,‡ AND GERALD S. LAZARUS*

From the Department of Dermatology, University of North Carolina School of Medicine, Chapel Hill, North Carolina; the *Department of Dermatology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania; and the ‡Department of Dermatology, University of Kuopio, Kuopio, Finland

Skin is a rich source of proteolytic enzymes (1) and the release of these proteinases may produce serious structural damage. Incubation of skin with commercial preparations of pancreatic trypsin and elastase, and bacterial collagenase, produced separation of the epidermis from the dermis (2–6). Indeed, a solution of 0.25% bovine pancreatic trypsin is routinely used to separate viable epidermis from dermis for tissue culture purposes. Skin does not encounter active pancreatic proteinases in vivo, but there are several neutral cellular endoproteinases that can be found in the skin at reasonable concentrations which could act upon the tissue. One of these is a chymotrypsin-like proteinase isolated from human dermis where it appears to be a mast cell constituent (7–8). Two other neutral serine proteinases, cathepsin G and elastase, located in the cytoplasmic granules of human polymorphonuclear leukocytes, are also of pathophysiological interest (9–12), since polymorphonuclear leukocytes may migrate into the skin in response to inflammatory signals. Cathepsin G exhibits chymotrypsin-like substrate specificity, but it is distinct from the human skin chymotrypsin-like proteinase (8).

This study investigates the effect of human skin chymotrypsin-like proteinase, cathepsin G, and elastase on the integrity of whole skin. As will be shown, the primary action of all three proteinases is at the epidermal-dermal junction. Structural alterations in this region were examined by electron microscopy while the degradation of specific proteins was determined by immunohistochemistry using antibodies to several different basement membrane components. The concentrations of proteinase used for our studies were in the range of 10–700 nM; these enzyme concentrations were selected to approach those that might be attained under physiologic or pathologic conditions in skin. They are 100–1,000-fold lower than proteinase concentrations used in previous studies. The proteinases used for these studies are highly purified. Also presented is a procedure for the simultaneous purification of cathepsin G and elastase from the same human leukocyte preparation.

Materials and Methods

Materials. Protein standards, synthetic substrates, phenylmethylsulfonyl fluoride (PMSF), bovine pancreatic trypsin (type III), and Trasylol (lyophilized powder) were obtained from Sigma Chemical Co., St. Louis, MO. Cyanogen bromide (CNBr)-activated Sepharose 4B and Sephadex G-100 were purchased from Pharmacia Fine Chemicals, Piscataway, NJ. CM-52 cellulose was obtained from Whatman Laboratory Products, Inc., Clifton, NJ, Brij and diisopropylfluorophosphate (DFP)[1] from Aldrich Chemical Co., Milwaukee, WI, and [1,3 ^{14}C]DFP from New England Nuclear, Boston, MA (120 mCi/ mmol).

Enzymatic Assays. During purification, cathepsin G was primarily monitored by following the hydrolysis of 0.1 mM N-benzoyl-DL-phenylalanine-β-naphthyl ester at pH 7.6 (1, 3). Elastase was monitored by following the hydrolysis of 0.25 mM N-succinyl-L-alanyl-L-alanyl-alanine p-nitroanilide under the conditions of Nakajima et al. (14).

Estimation of Proteinase Concentration. The concentrations of the human skin chymotrypsin-like proteinase and cathepsin G were determined using their specific activities for N-benzoyl-L-tyrosine ethyl ester (8). Under the assay conditions used (a solution of 0.3 M Tris-HCl (pH 8.0), 1.5 M KCl, 15% ethanol, 0.5 mM substrate), the specific activities of the human skin chymotrypsin-like proteinase and cathepsin G were 7.7 and 1.3 U/nmol enzyme, respectively. The concentration of elastase was calculated from kinetic data for the hydrolysis of N-succinyl-L-alanyl-L-alanyl-alanine p-nitroanilide according to the relationship: $[E] = Vmax/kcat$, where $[E]$ is the enzyme concentration. $Vmax$ was experimentally determined from Lineweaver-Burk plots. The $kcat$ value, 2.1/s, and the assay conditions were those reported by Nakajima et al. (14). In one preparation the concentration of elastase was verified by radioactively labeling the purified proteinase with [1,3 ^{14}C]DFP and quantitating bound radioactivity. The value obtained was within 10% of that obtained using kinetic data. Trypsin concentration was determined using its extinction coefficient of 14.3 (280 nm 1% solution, 1 cm pathlength) reported in the Worthington Enzyme Manual (Worthington Biochemical Corporation, Freehold, NJ, 1972, p. 125).

Purification of Proteinases. (*a*) Human skin chymotrypsin-like proteinase: The purification method was that of Schechter et al. (8). Proteinase was precipitated from the high salt extract of human skin by the addition of 0.025% protamine chloride and was then chromatographed on a Sephacryl S-200 column and a CH-Sepharose-D-tryptophan methyl ester affinity column. The proteinase was between 30 and 50% pure at this stage and was not contaminated with other proteolytic enzymes. The concentration of this proteinase in human skin is 200 nM. This value is higher than previously reported.

(*b*) Human leukocyte elastase and cathepsin G: The purification scheme was developed from the previously reported methods of Schmidt and Havemann (9) and Baugh and Travis (10). 400 ml of heparinized blood was separated into leukocyte and erythrocyte fractions by differential sedimentation in a solution containing 3% (wt/vol) dextran T-500 and 0.15 M NaCl. Leukocytes were concentrated from the supernatant by centrifugation (2,000 rpm for 10 min in a Sorvall GS-3 rotor; Sorvall Instruments Div., DuPont Co., Wilmington, DE) and contaminating erythrocytes were removed by three cycles of hypotonic lysis. The resulting leukocyte pellets were usually stored at −20°C until needed. Proteinases were solubilized by the suspension of cell pellets in 8 ml of a cold, high-salt detergent solution (1.0 M NaCl, 10 mM sodium phosphate, 1 mM EDTA, and 0.1% Brij) followed by sonication (4 × 15 s with microprobe at setting 4 on a Branson Sonifier; Branson Sonic Power Co., Danbury, CT). The suspension was clarified by centrifugation (20,000 rpm for 0.5 h with an SS-34 rotor at 4°C) and the supernatant fractionated by chromatography on a Sephadex G-100 column (100 × 2.3 cm bed volume) equilibrated at 4°C with the high-salt detergent buffer. Elastase eluted at ~300 ml while cathepsin G eluted at ~340 ml. The elastase- and cathepsin G–containing fractions were both dialyzed against a solution of 5.0 mM MES [2-(N-morpholino)-ethanesulfonic acid] (pH 5.7), 0.15

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[1] *Abbreviations used in this paper:* DFP, diisopropylfluorophosphate; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; HBSS, Hanks' balanced salt solution; MES, 2-(N-morpholino)-ethanesulfonic acid; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate.

M NaCl in preparation for ion exchange chromatography on a 10 ml CM-52 cellulose column (see Fig. 1). Specifically the proteinases were eluted with a 300 ml linear gradient of NaCl (containing MES buffer) ranging from 0.15 to 1.5 M NaCl. Elastase and cathepsin G obtained from ion exchange columns were then purified to virtual homogeneity using a Trasylol-Sepharose 4B affinity column (2.5 ml swollen resin) prepared as described in the Pharmacia Fine Chemicals handbook. The separate pools of elastase and cathepsin G from CM-52 chromatography were adjusted to pH 8.0 by the addition of 1.0 M Tris-HCl (pH 8.0) and layered onto the affinity columns. The columns were then washed with 25 ml of a 0.05 M Tris-HCl (pH 8.0), 0.4 M NaCl solution and the proteinases were eluted with a solution of 0.05 M sodium acetate (pH 5.0), 0.4 M NaCl. The total recovery of both proteinases by this purification method was ~25%, and the amount of enzyme purified from 400 ml blood was 2.1 nmol cathepsin G and 7.5 nmol elastase. Purified elastase preparations did not hydrolyze N-benzoyl-L-tyrosine ethyl ester (0.7 mM substrate for 20 min), a substrate of cathepsin G, and cathepsin G preparations did not hydrolyze the elastase substrate.

(c) Trypsin: Purification of trypsin was accomplished using a Trasylol-Sepharose 4B column prepared as described above. Trypsin dissolved in phosphate-buffered saline (PBS) was bound to the resin. The column was then eluted with 0.2 M acetic acid (pH 3.2) followed by 0.2 M HCl. The HCl solution eluted trypsin that was not contaminated with chymotrypsin. Trypsin preparations were quickly dialyzed in Hanks' balanced salt solution (HBSS) as described subsequently.

Radioactive DFP Labeling. In separate experiments, elastase (0.4 ml of a 2.6 μM solution) and cathepsin G (0.4 ml of a 0.6 μM solution) were incubated with 0.05 ml of 2.0 M Tris-HCl (pH 8.3) and 10 μC of [1,3 ^{14}C]DFP (120 mCi/mmol; New England Nuclear) at 37°C for 1.5 h. The reaction was stopped by the addition of 0.5 ml unlabeled 0.1 M DFP in propylene glycol and unbound DFP was removed by dialysis.

Sodium Dodecyl Sulfate (SDS) Polyacrylamide Slab Gel Electrophoresis. SDS gel electrophoresis was performed as described by Anderson et al. (15) using a running gel composed of 17.5% acrylamide and 0.8% bisacrylamide. Proteinase samples were denatured by heating to 100°C for 10 min in a solution of 0.5% SDS and 2% 2-mercaptoethanol. Diluted samples were concentrated by lyophilization. Proteins used for calibration were phosphorylase b (100,000 mol wt), bovine serum albumin (67,000), catalase (60,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (21,500), and lysozyme (14,000). To visualize bands, gels were either stained with Coomassie Brillant Blue or dried in the presence of Autofluor (National Diagnostics Inc., Somerville, NJ) and fluorographed on Kodak XAR-5 x ray film at −70°C.

Preparation of Proteinases for Incubation with Skin. Proteinases were dialyzed against sterile HBSS (minus indicator) containing 15 mM Hepes (pH 7.5) and 0.35 g/l sodium bicarbonate. Enzyme concentrations for incubations were determined by standard assay procedures after dialysis. For control studies, comparable concentrations of proteinases were inhibited before dialysis with 2.0 mM PMSF or DFP. Inactivation of enzymes was >99% complete. Skin specimens were placed directly into 1.0 ml of enzyme solutions.

Human Skin Preparation and Enzyme Incubation. Skin was removed from the upper thigh or buttocks of normal healthy human volunteers after obtaining informed consent. The skin area was prepared with Betadine and alcohol, and anesthetized with xylocaine. Thin, split-thickness strips of skin were removed with a Castroviejo dermatotome set to cut at a depth of 0.4 mm. The skin strips were placed in cold buffer (HBSS) and immediately transported to the laboratory where the strips were further divided into pieces approximately 1.0–1.5 mm wide and 4–5 mm long. Histologic monitoring indicated that the skin thickness of the specimens varied from a dermal thickness equivalent to that of the epidermis to a dermal thickness twice that of the epidermis.

The time from skin removal to initiation of enzyme incubation did not exceed 30 min in any experiment. The skin specimens were placed in Linbro plate wells, three to four pieces per well, containing 1 ml active enzyme solution, inhibited enzyme solution, or buffer control and incubated in a humidified incubator at 36°C for varying times from 2 to 8 h before samples were removed. After incubation, the treated skin specimens were

washed with buffer and immediately prepared for transmission electron microscopy or embedded in OCT compound (Ames Laboratories, Inc., Milford, CT) and frozen in liquid nitrogen for immunocytochemical studies.

Specimens for electron microscopy were fixed in 2.5% glutaraldehyde in 0.2 M phosphate buffer, pH 7.4, for 4 h, washed, postfixed in 1% osmium tetroxide solution for 1 h, embedded in Epon resin 812, and then cut with diamond knives. After staining with uranyl acetate and Reynold's lead citrate, the specimens were visualized in either a JEM T7 or JEM 100B electron microscope (JEOL Ltd., Tokyo).

Immunohistochemical Studies. Antibodies with reactivity to components of human skin basement membrane zone were used for immunohistochemical studies. Bullous pemphigoid antibody was obtained from two patients with classical bullous pemphigoid; titers against human skin as substrate were 1:160 and 1:320. The specificity of the bullous pemphigoid antibody was confirmed by immunoelectron microscopy which localized the antibody to the lamina lucida. Rabbit antilaminin (Engelbreth-Holm Swarm [EHS] tumor derived) antibody was obtained from Bethesda Research Laboratories, Gaithersburg, MD. The specificity of this antibody was documented by enzyme-linked immunosorbent assays (ELISA) and immunoprecipitation with laminin (specific antibody concentration of ~0.25 μg/ml). Rabbit anti–type IV collagen (EHS tumor derived) antibody was a gift from Dr. David Woodley (Department of Dermatology, University of North Carolina School of Medicine, Chapel Hill, NC). The specificity of this antibody was documented by its reactivity with type IV collagen in an ELISA (data not shown). The antibody was unreactive to laminin, heparan sulphate proteoglycan, and fibronectin. Another rabbit anti–type IV procollagen antibody (bovine lens capsule derived) was a gift of Dr. Nicholas Kefalides (Department of Connective Tissue Research, University of Pennsylvania, Philadelphia, PA). Mouse monoclonal antibodies (AF1 and AF2) to anchoring fibrils of human skin (16, 17) were used in a working dilution of 1:10 and 1:20 (titers of the antibodies were 1:40). Mouse monoclonal antibody (KF1) with demonstrated reactivity to human skin lamina densa (18) was a gift of Dr. Stephen Katz (National Institutes of Health, Bethesda, MD) and was used in a dilution of 1:20 (titer of this antibody was 1:80). Acquired epidermolysis bullosa antibody obtained from two patients with acquired epidermolysis bullosa was shown by immunoelectron microscopy to react with antigen in the sub-lamina densa zone and to a limited extent in the lamina densa. Working dilutions of these antibodies were 1:20, 1:40 (titer of both was 1:160).

Working dilutions of the above antibodies were determined by chessboard titration with the appropriate fluoresceinated antibody conjugate and a series of working dilutions selected in the mid-zone of reactivity. Fluorescein isothiocyanate (FITC)-conjugated antibody with specificities for rabbit, human, and mouse immunoglobulin were used in dilutions determined by chessboard titration above.

Indirect immunofluorescence was performed according to Beutner and Nisengard (19). 4–6-μm-thick sections of skin were cut and air dried on glass slides. Sections were then incubated for 30 min in a humid chamber at 25°C with appropriate dilutions of each of the specific antisera described above. Sections were then washed twice for 10–15 min each in PBS; moisture was removed and the slides were incubated for 30 min in appropriate FITC-conjugated antisera, twice rinsed again in PBS, and coverslipped. Specimens were examined in a Leitz Orthoplan microscope equipped with epiillumination and the following filter systems for fluorescence microscopy (exciter filter EP 450–490, barrier filter LP 515, and beam splitter PKP 510; E. Leitz, Inc., Rockleigh, NJ). Results were recorded photographically using Kodak Ektachrome 400 film.

Results

Purification of Proteinases. Human skin chymotrypsin-like proteinase was purified as described by Schechter et al. (8). Human leukocyte cathepsin G and elastase were purified by a method developed from two previously reported procedures (see Materials and Methods); this is an improvement over previous methods because it results in the purification of each proteinase from the same

blood preparation and avoids steps that may result in the precipitation of cathepsin G (9, 10). The method involves (a) solubilization of both proteinases in a high-salt detergent solution, (b) gel filtration chromatography in the same solution to separate cathepsin G from elastase, (c) ion exchange chromatography to complete the separation (Fig. 1), and (d) affinity chromatography on a Trasylol-Sepharose 4B resin. Purified cathepsin G (G) and elastase (E) preparation analyzed by SDS polyacrylamide gel electrophoresis are presented in Fig. 2. Fig. 2a is a photograph of a gel with the resolved polypeptide chains stained Coomasie Brillant Blue and Fig. 2b is a fluorogram of the proteinases inactivated with radioactive DFP before analysis. The banding patterns obtained show some evidence of microheterogeneity. Both leukocyte proteinases are recognized as a family of isoenzymes differing in carbohydrate content (9, 10). The low molecular weight (<10,000) polypeptides in the fluorogram of cathepsin G may be the result of slight autodegradation during the incubation with radioactive label.

Ultrastructural Studies. Since these proteinases primarily affect the epidermal-dermal junction, it is appropriate to orient the reader to this well-characterized structure (20, 21). Ultrastructurally, the junction between basal keratinocytes and the dermis, proceeding from the epidermis to the dermis, consists of the basal cell plasma membrane studded along its length with hemidesmosomes; an electron lucent zone beneath the cell membrane, termed the lamina lucida (lamina rara), which is crossed by fine "anchoring" filaments; an electron dense layer, the lamina densa (basal lamina) and a fibrous zone (sub-lamina densa fibrous zone or reticular (lamina) beneath the lamina densa composed of three fibrous elements: anchoring fibrils, dermal microfibril bundles, and collagen fibers. The adepidermal ends of the anchoring fibrils and dermal microfibril bundles insert directly into the lamina densa. Anchoring fibrils have a character-
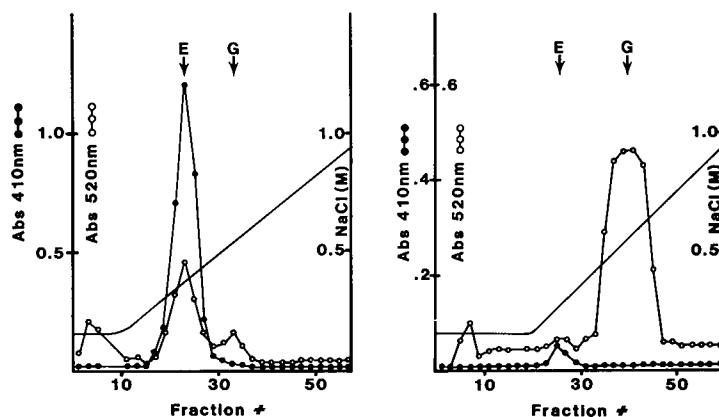

FIGURE 1.   CM-52 cellulose chromatography of cathepsin G and elastase after their initial separation by gel filtration chromatography. *Left* graph depicts the fractionation pattern of the elastase preparation and the *right* graph depicts the fractionation of the cathepsin G preparation. Absorbance at 410 nm (closed circles) marks the hydrolysis of N-succinyl-L-alanyl-L-alanyl-alanine p-nitroanilide, a substrate preferred by elastase, and absorbance at 520 nm (open circles) marks the hydrolysis of N-benzoyl-DL-phenylalanine-β-napthyl ester, a substrate preferred by cathepsin G. Solid line indicates the NaCl gradient. E, NaCl concentration (0.25 M) where the elastase peak elutes; G, NaCl concentration (0.55 M) where the cathepsin G peak usually elutes.
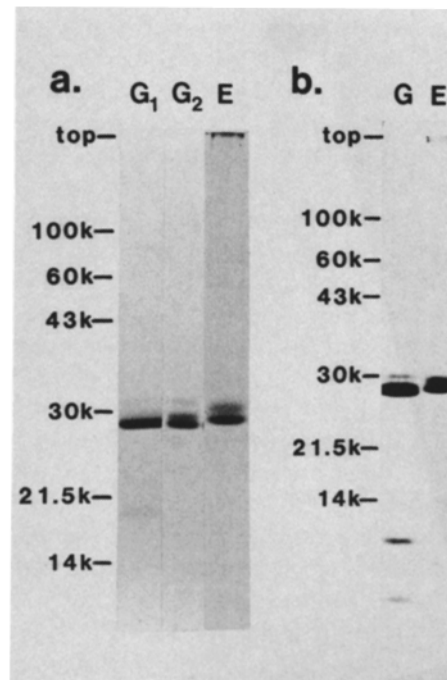
FIGURE 2. SDS polyacrylamide gel electrophoresis of purified cathepsin G (G) and elastase (E) preparations. (a) Polypeptide chains stained with Coomassie Brilliant Blue and (b) fluorogram of proteinases inactivated with radioactive DFP before analysis. Lane G-1 is an analysis of cathepsin G purified by the method of Schmidt and Havemann (9) and tracks G-2 and G are analyses of cathepsin G purified by the method presented here. Elastase preparations analyzed were purified by the method described here. 2.5 μg of cathepsin G and 10.0 μg elastase were analyzed in a and 3.5 μg of radioactive cathepsin G and 2.0 μg of radioactive elastase were analyzed in b. X ray film was allowed to develop for 48 h and the migration of standards is indicated by their molecular weights (see Materials and Methods).

istic central cross-banded portion and a fan-like arrangement of filament toward the ends. Dermal microfibril bundles are composed of parallel bundles of microfibrils, morphologically identical with elastic microfibrils of true elastic tissue. Indeed, these dermal microfibril bundles course perpendicularly deep into the dermis where actual connections to elastic fibers have been found (22). Collagen fibers are present in the sub-lamina densa region, but do not connect directly with either the lamina densa or with the other sub-lamina densa fibrous structures.

All degradation studies were monitored as a function of time and enzyme concentration. Incubations of skin with proteinases were for periods of 2–8 h, and they were performed at two different proteinase concentrations, designated high and low. For the human skin chymotrypsin-like proteinase and cathepsin G, the high concentrations ranged between 70 and 100 nM and the low concentrations ranged between 10 and 25 nM. For elastase, the high concentration ranged between 600 and 700 nM and the low concentration ranged between 100 and 200 nM. The morphological changes produced by the human skin chymotrypsin-like proteinase and elastase were studied in five separate experi-

ments, whereas those produced by cathepsin G were studied in three separate experiments. The major degradative changes produced by each proteinase were highly reproducible. All studies were performed in parallel with controls consisting of incubations containing proteinases inhibited by PMSF and incubations with buffer along.

Human Skin Chymotrypsin-like Proteinase. The degradation of the epidermal-dermal junction by the human skin chymotrypsin-like proteinase is shown in Figs. 3 and 4. The earliest changes were evident after 4–6 h of incubation with proteinase at high concentration. The initial changes included clearing in the lamina lucida and loss of the anchoring filaments that normally traverse this area (Fig. 3). Irregular widening along the length of the lamina lucida was also evident as well as focal areas of frank epidermal-dermal separation (Fig. 4). By 6–8 h of incubation, extensive epidermal-dermal separation was observed. Degradation appeared largely confined to the lamina lucida; the lamina densa and the sub-lamina densa fibrillar network remained intact. At lower proteinase concentrations, the same pattern of damage is observed, but the alterations are delayed by 2–4 h. The dependence of morphological alterations on enzyme concentration and time suggests that changes are a direct result of degradation by the proteinase. Limited morphological changes were seen in the epidermis, especially in skin treated with high enzyme concentrations for 6–8 h. These alterations consisted
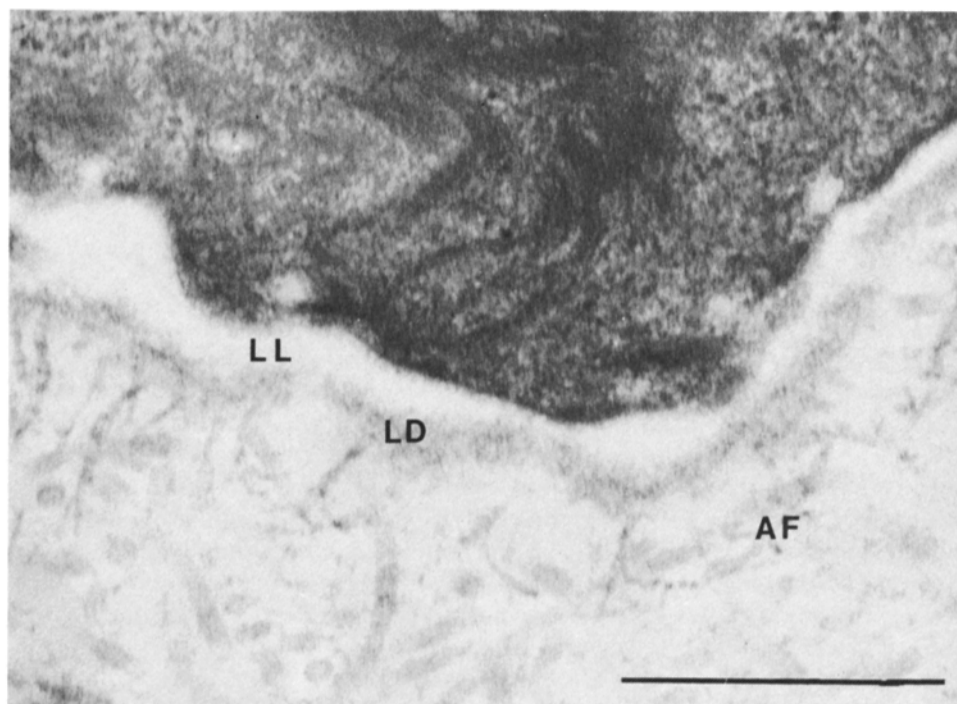

FIGURE 3. Normal human skin incubated with active human skin chymotrypsin-like proteinase (concentration, 100 nm/ml; 36°C) for 4 h. Loss of structure and widening of the lamina lucida (*LL*) are present. Lamina densa (*LD*) and anchoring fibrils (*AF*) are intact. × 87,000. Bar = 0.5 μm.
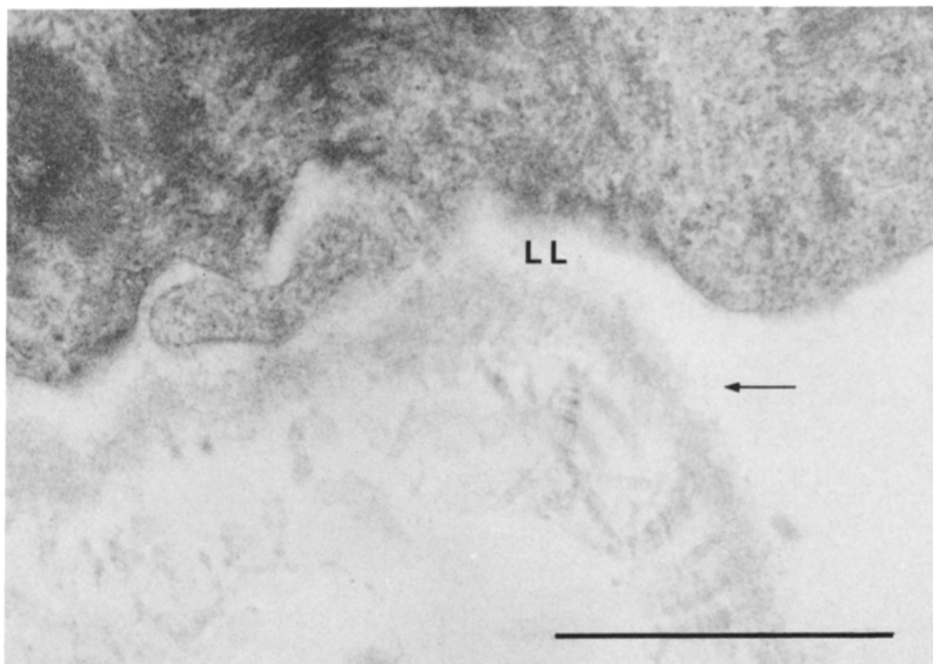
FIGURE 4.   Normal human skin incubated with active human skin chymotrypsin-like proteinase (concentration, 100 nM/ml; 36°C) for 6 h. Focal epidermal-dermal separation (arrow) is evident at the level of the lamina lucida (*LL*). × 90,000. Bar = 0.5 µm.

primarily of focal disruptions along the basal cell plasma membrane and occasional disruptions in the membranes of the lower spinous cells. Some lateral separation of cells in the lower portion of the epidermis was also observed. Dermal alterations were not detected in these studies.

Human Leukocyte Cathepsin G.   The earliest morphological changes observed with cathepsin G were those seen at 8 h incubation with high concentrations of proteinase. The pattern of alterations was qualitatively similar to the initial alterations observed with the human skin chymotrypsin-like proteinase; i.e., focal areas of epidermal-dermal separation.

Human Leukocyte Elastase.   The earliest changes noted for elastase were after 2 h of incubation at high proteinase concentrations. The most striking change evident was a decrease in the density of the lamina densa. As the incubations continued (Fig. 5), the lamina densa began to disappear and regions of epidermal-dermal separation were seen. By 6 h the lamina densa was completely absent in all preparations (Fig. 6). Similar results were observed in incubations containing lower elastase concentrations, except that the time required for degradation was longer. No epidermal alterations were noted.

Although lamina densa was extensively destroyed, anchoring fibrils were apparently not affected directly. Morphologically intact anchoring fibrils were observed in the upper dermis subjacent to intact basal cell plasma cell membranes (Figs. 5, 6). Many of these anchoring fibrils retained their characteristic central banding pattern. Anchoring fibrils were seen singly, in small clusters and in
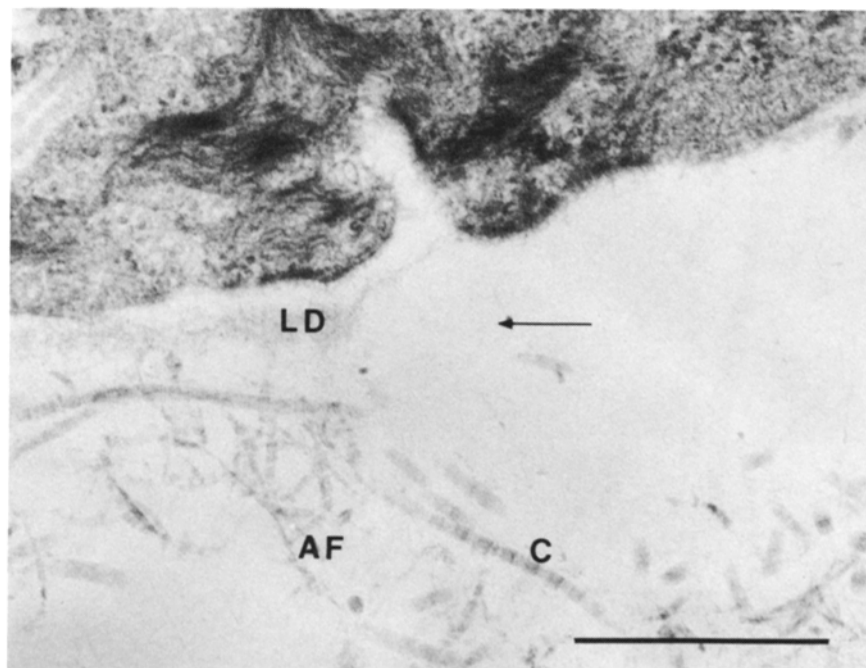
FIGURE 5.   Normal human skin treated with active leukocyte elastase for 4 h (concentration, 600 nM/ml, 36°C). Focal absence of lamina densa and epidermal-dermal separation (arrow) is seen in a portion of the junction zone. Residual lamina densa (*LD*) is present in the adjacent junction. Anchoring fibrils (*AF*) and collagen fibers (*C*) appear structurally intact. × 67,000. Bar = 0.5 μm.

dense aggregates. Their orientation was haphazard, probably resulting from loss of attachment to the lamina densa. Overall, the number of anchoring fibrils appeared to be similar to the skin in the inhibited enzyme and buffer-treated controls.

Microfibril bundles that also attach to the lamina densa were similarly altered (data not shown). Individual microfibrils appeared to remain intact, but they were no longer grouped as bundles. Rather, they appeared as individual fibrils or small groups of scattered fibrils. Dermal collagen fibers were morphologically unaltered by elastase as evidenced by the similarity of their appearance to controls. Interestingly, the lamina densa of dermal blood vessels was also destroyed by elastase. Damage to dermal blood vessels was not observed for the chymotrypsin-like proteinases.

Trypsin.   Because the damage to the epidermal-dermal junction by the chymotrypsin-like proteinases and elastase was markedly different, we believed it important to determine the type of damage to skin that might be produced by proteinases having trypsin-like specificity. To accomplish this, the degradation of skin by commercial pancreatic bovine trypsin purified by affinity chromatography (see Materials and Methods) was monitored. The studies were performed at 100 and 25 nM proteinase. By 4 h of incubation with 100 nM trypsin, extensive epidermal-dermal separation had occurred. Epidermal-dermal separa-
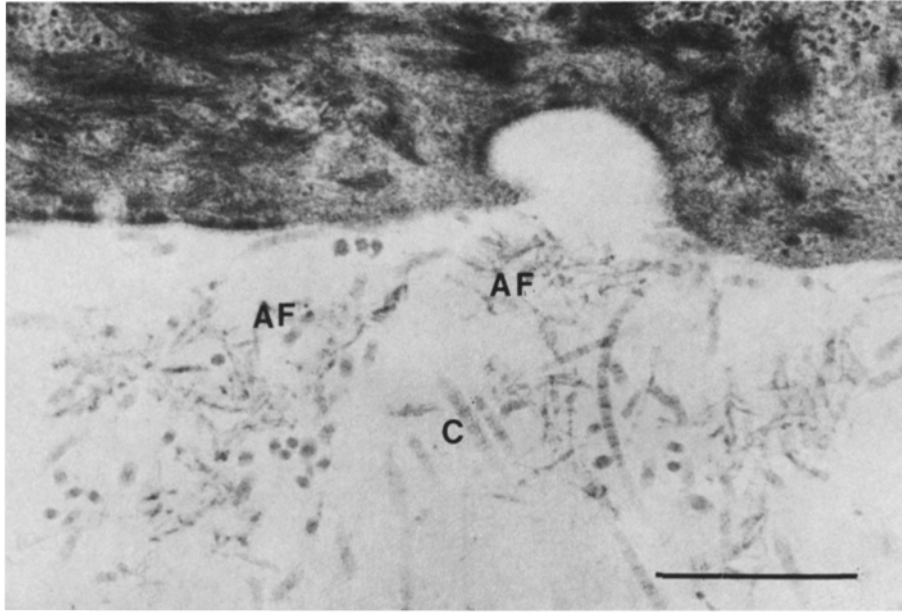
FIGURE 6.   Normal human skin treated with active leukocyte elastase for 6 h (concentration, 600 nm/ml; 36°C). Complete absence of lamina densa is evident at the epidermal-dermal junction. Anchoring fibrils (AF) and collagen fibers (C) appear structurally intact. × 54,000. Bar = 0.5 μm.

tion was also apparent, but to a lesser extent, with the lower concentration of trypsin. The alterations observed are qualitatively similar to that seen with the chymotrypsin-like proteinases, although they were more extensive at the same time period.

Immunohistochemical Studies.   Antibodies to known junctional components were used to define the specific epidermal-dermal junction constituents affected by each proteinase and to further describe the level at which epidermal-dermal separation was occurring. The bullous pemphigoid and antilaminin antibodies define antigenic components in the lamina lucida (23-26). Anti-type IV collagen antibodies and the KF1 monoclonal antibody define antigens in the lamina densa (18, 27,). EBA, AF1, and AF2 antibodies localize antigens in the sub-lamina densa fibrous zone (16, 17, 28-30).

Skin incubated with the high concentrations of the human skin chymotrypsin-like proteinase did not show differences in immunofluorescent staining until epidermal-dermal separation had occurred (that is, in incubations of 6 and 8 h with high proteinase concentrations). In separated regions, bullous pemphigoid antibody localized to the epidermal side of the separation plane in a somewhat discontinuous pattern on the basal cell surface (Fig. 7, a and b), whereas laminin (Fig. 7, c and d) and all the other antigens localized to the dermal side. The intensity of all immunofluorescent staining (even at serial antibody dilutions) in separated skin remained comparable to controls and did not diminish with time, suggesting there is no significant degradation of these antigens by the human skin chymotrypsin-like proteinase. Since bullous pemphigoid antigen and laminin
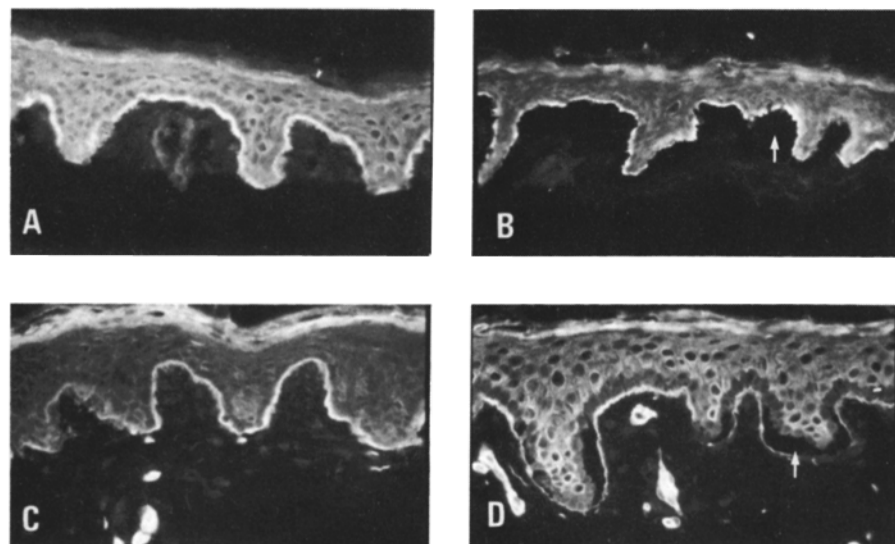
FIGURE 7.   Distribution of antibodies to specific basement membrane components in normal human skin treated for 6 h with active human skin chymotrypsin-like proteinase (B, D) and inhibited enzyme (A, C). A and B show localization of bullous pemphigoid antibody. In active enzyme-treated skin, bullous pemphigoid antibody localizes to the epidermal side of the epidermal-dermal separation (arrow). C and D show antilaminin antibody localization. In active enzyme-treated skin, antibody staining is seen on the dermal side of the epidermal-dermal separation (arrow).

TABLE I

Human Leukocyte Elastase (4 h Incubation, High Concentration)

Antibody to:	Inhibited	Active
Bullous pemphigoid	+ Linear BMZ	+ On epidermal side of separation
Laminin	+ Linear BMZ and vascular BM	Absent
Type IV collagen	+ Linear BMZ and vascular BM	Absent
KF1	+ Linear BMZ	Absent
EBA antigen	+ Linear BMZ	Absent
AF1, AF2	+ Linear BMZ	+ Scattered on dermal side of separation

BMZ, basement membrane zone; EBA, epidermolysis bullosa acquisita. KF-1 is a monoclonal antibody that recognizes an antigen in the lamina densa (18). AF1 and AF2 are monoclonal antibodies specific for anchoring fibrils (16, 17).

are major components of the lamina lucida, these results correlate well with the ultrastructural observations showing that the human skin chymotrypsin-like proteinase produces epidermal-dermal separation in a plane confined to the lamina lucida.

In skin treated with human leukocyte elastase, alterations in antibody localization were observed by 4 h incubation with active enzyme when epidermal-dermal separation was evident in most samples. As expected from the ultrastructural studies, the changes in antibody distribution and appearance were more extensive than observed with the human skin chymotrypsin-like proteinase. These changes are described in Table I. No immunofluorescent staining was observed for

laminin, type IV collagen (Fig. 8, a and b), KF1 antigen, and EBA antigen, indicating profound dissolution of the lamina lucida and lamina densa. The bullous pemphigoid antigen was clearly present, and again localized in a somewhat discontinuous pattern on the epidermal side of the separation plane. The intensity of its staining is comparable to controls and remains constant in longer incubations, suggesting that bullous pemphigoid antigen is not degraded by elastase. Immunofluorescent staining was also observed with the anchoring fibril antibodies, AF1 and AF2, on the dermal side of the separation plane (Fig. 8, c and d). The staining, however, was not uniformly linear as in the controls. Instead, there was significant speckling and scattering along the staining band, which probably reflects the disorganized pattern observed for anchoring fibrils in the ultrastructural studies.

Discussion

Freshly obtained human skin was degraded with relatively low concentrations (20–700 nM) of proteinases having chymotryptic, elastolytic, and tryptic specificities. The proteinases with chymotryptic and elastolytic specificities were isolated from human leukocytes and human skin, and the proteinase with tryptic specificity was purified from bovine pancreatic trypsin. In all cases the primary degradation observed was at the epidermal-dermal junction, and the ultimate result was epidermal-dermal separation. These studies clearly show that the epidermal-dermal junction is highly susceptible to proteolytic damage. Although all pro-
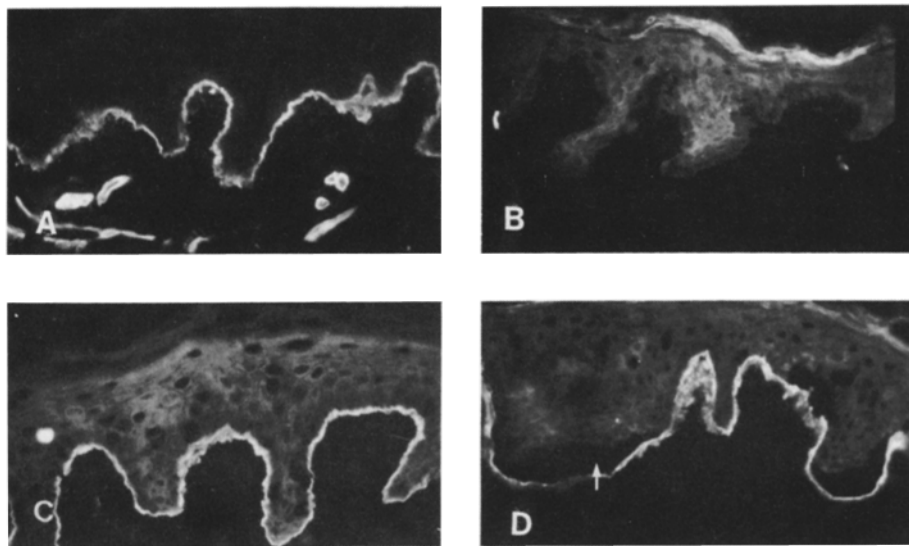

FIGURE 8.   Distribution of antibodies to specific basement membrane components in normal human skin treated for 4 h with active leukocyte elastase (B, D) and elastase inhibited with phenylmethylsulfonyl fluoride (A, C). A and B show antibody type IV procollagen antibody localization. Absence of antibody staining is seen in active elastase-treated skin (B). C and D show localization of monoclonal antibodies to anchoring fibrils (AF1 and AF2). Antibody staining is seen in active elastase-treated skin on the dermal side of the epidermal-dermal separation (arrow).

teinases altered the epidermal-dermal junction, the extent and type of degradation were proteinase specific.

The human skin chymotrypsin-like proteinase and cathepsin G, both proteinases with chymotrypsin-like specificity, degrade the epidermal-dermal junction in the lamina lucida, producing epidermal-dermal separation and little other apparent morphological damage. Long incubations (6–8 h) with the human skin chymotrypsin-like proteinase resulted in extensive epidermal-dermal separation while similar incubations with high concentrations of cathepsin G only resulted in focal damage. The junctional proteins degraded by the human skin chymotrypsin-like proteinase were not identified by immunofluorescent staining using antisera to a variety of basement membrane proteins, including laminin and type IV collagen. After epidermal-dermal separation, however, laminin localized on the dermal side of the split while the bullous pemphigoid antigen remained on the epidermal side, still apparently associated with an intact basal cell membrane. The intensity of staining of both these antigens and their persistence in incubations of long duration and high proteinase concentration suggest they were not the direct targets of proteolytic hydrolysis. Laminin is a high molecular weight protein that is believed to span the lamina lucida and to mediate the attachment of epithelial cells to type IV collagen (31). In view of this, the protein(s) degraded by the human skin chymotrypsin-like proteinase and cathepsin G may be those that help provide a linkage between laminin and the basal cell membrane. Terranova et al. (32) has recently characterized such a protein from human breast carcinoma cells.

The degradation of isolated basement membrane proteins with proteinases of chymotrypsin-like specificity has been reported. Rao et al. (33) showed that isolated laminin is readily degraded by bovine pancreatic chymotrypsin and cathepsin G. The immunohistochemical studies presented here indicate that laminin in its native environment is not highly susceptible to either cathepsin G or the human skin chymotrypsin-like proteinase, even though the plane of epidermal-dermal separation was in the lamina lucida. It has also been shown (34–36) that a chymotrypsin-like proteinase isolated from rat peritoneal mast cells, rat mast cell proteinase I, is capable of partially degrading isolated Type IV and V collagen. The human skin chymotrypsin-like proteinase, which may be the human analogue to this proteinase, did not appear to degrade basement membrane collagen; the lamina densa remained intact even after 8-h incubations, and the presence of type IV collagen was documented by immunofluorescence. This suggests that there may be significant differences in the proteolytic susceptibility of isolated basement membrane proteins as compared with the same proteins in their native environments.

Of the proteinases characterized, human leukocyte elastase produced the most widespread damage to the epidermal-dermal junction. Degradation of the lamina densa is consistent with the in vitro studies demonstrating that type IV and V collagens are good substrates for elastases of leukocyte or pancreatic origin (37, 38). Elastase digestion also resulted in the disappearance of laminin, the KF1 antigen, and the EBA antigen. Whether these proteins are removed by direct degradation or they are released because of the dissolution of type IV collagen cannot be determined from our studies. The only epidermal-dermal components

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that persisted after elastase digestion are the bullous pemphigoid antigen, micro-filament bundles, anchoring fibrils, and collagen fibers. Although microfilament bundles and anchoring fibrils remain, they appear scattered and disoriented, as a result of the loss of their attachment sites to the lamina densa. The extensive degradation of the epidermal-dermal junction by elastase is significant for it demonstrates that human leukocyte elastase alone is capable of dissolving the epidermal-dermal junction.

The proteinases used for these studies were isolated from human skin and from human leukocytes. Conditions may exist where all or some of these enzymes are released into the skin. The concentration of the human skin chymotrypsin-like proteinase in skin is ~200 nM, which is above the lowest concentration used to produce epidermal-dermal separation. The distinctive degradation patterns described here could help to implicate and identify these or similar proteinases as mediators in skin diseases where degradation of the epidermal-dermal junction occurs.

Summary

The degradation of normal human skin by the human polymorphonuclear leukocyte proteinases cathepsin G and elastase, and by a human skin chymotryp-sin-like proteinase that appears to be a mast cell constituent, was examined. Enzymes were incubated with fresh, split-thickness skin for up to 8 h; the tissue was examined ultrastructurally and immunohistochemically using antibodies to known basement membrane constituents. In all cases, the primary damage observed was at the epidermal-dermal junction. Elastase degraded the lamina densa leaving scattered and disorganized anchoring fibrils, dermal microfibril bundles, and normal-appearing collagen fibers. Immunohistochemically, type IV collagen, laminin, KF1 antigen, and EBA antigen were absent. The bullous pemphigoid antigen was present and localized on the basal cells.

Epidermal-dermal separation produced by the chymotrypsin-like proteinases, cathepsin G, and the human skin proteinase, was confined to the lamina lucida. The lamina densa and sub-lamina densa fibrillar network remained intact. The human skin chymotrypsin-like proteinase produced extensive epidermal-dermal separation, while cathepsin G, at comparable concentrations, produced only focal separations. Immunohistochemically, all antigens were present after incubation with enzyme. The bullous pemphigoid antigen, however, was found on the epidermal side of the split, while laminin was found on the dermal side.

These results show that the epidermal-dermal junction is highly susceptible to neutral serine proteinases located in mast cells and polymorphonuclear leuko-cytes. Although all the proteinases produce epidermal-dermal separation, the patterns and extent of degradation are different. The distinctive patterns of degradation may provide a clue to the involvement of these proteinases in skin diseases.

References

1. Fraki, J., G. S. Lazarus, and V. Hopsu-Havu. 1983. Protein catabolism in the skin. *In* Biochemistry and Physiology of the Skin. L. A. Goldsmith, editor. Oxford University Press, New York. 338.

2. Briggaman, R. A., F. G. Dalldorf, and C. E. Wheeler, Jr. 1971. Formation and origin of basal lamina and anchoring fibrils in adult human skin. *J. Cell Biol.* 51:384.

3. Fukuyama, K., M. Black, and W. Epstein. 1974. Ultrastructural studies of newborn rat epidermis after trypsinization. *J. Ultrastruct. Res.* 46:219.

4. Kahl, F., and R. Pearson. 1967. Ultrastructural studies of experimental vesiculation. II. Collagenase. *J. Invest. Dermatol.* 49:616.

5. Scaletta, L., and D. McCallum. 1974. A fine structural study of human oral epithelium separated from the lamina propria by enzymatic action. *Am. J. Anat.* 140:383.

6. Omar, A., and A. Krebs. 1975. Mode of dermal-epidermal adhesion. *Dermatologica (Basel).* 150:321.

7. Fraki, J. E., and V. Hopsu-Havu. 1975. Human skin proteases. Separation and characterization of two alkaline proteases, one splitting trypsin and other chymotrypsin substrates. *Arch. Dermatol. Res.* 253:261.

8. Schechter, N. M., J. E. Fraki, J. C. Geesin, and G. S. Lazarus. 1983. Human skin chymotryptic proteinase. Isolation and relation to cathepsin G and rat mast cell proteinase I. *J. Biol. Chem.* 258:2973.

9. Schmidt, W., and K. Havemann. 1974. Isolation of elastase-like and chymotrypsin-like neutral proteases from human granulocytes. *Hoppe-Seyler's Z. Physiol. Chem.* 355:1077.

10. Baugh, R. J., and J. Travis. 1976. Human leukocyte granule elastase: rapid isolation and characterization. *Biochemistry.* 15:836.

11. Olsson, I., and P. Venge. 1980. The role of the human neutrophil in the inflammatory reaction. *Allergy (Copenh).* 35:1.

12. Travis, J., P. J. Giles, L. Porcelli, C. F. Reilly, R. Baugh, and J. Powers. 1980. Human leukocyte elastase and cathepsin G: structural and functional characteristics. *In* Protein Degradation in Health and Disease. *Ciba Foundation Symp.* 75:51.

13. Starkey, P. M., and A. Barrett. 1976. Neutral proteinases of human spleen. *Biochem. J.* 155:255.

14. Nikajimi, K., J. C. Powers, B. M. Ashe, and M. Zimmermann. 1979. Mapping the extended substrate binding site of cathepsin G and human leukocyte elastase. *J. Biol. Chem.* 254:4027.

15. Anderson, C. W., P. R. Baum, and R. F. Gesteland. 1973. Processing of adenovirus 2-induced proteins. *J. Virol.* 12:241.

16. Goldsmith, L. A., and R. A. Briggaman. 1983. Monoclonal antibodies to normal and abnormal epithelial antigens. *In* International Symposium on Normal and Abnormal Epidermal Differentiation. University of Tokyo. 1–23.

17. Goldsmith, L. A., and R. A. Briggaman. 1983. Monoclonal antibodies to anchoring fibrils for the diagnosis of epidermolysis bullosa. *J. Invest. Dermatol.* 81:464.

18. Breathrach, S., P. Fox, G. Neises, J. Stanley, and S. Katz. 1983. A unique epithelial basement membrane antigen defined by a monoclonal antibody (KF-1). *J. Invest. Dermatol.* 80:392.

19. Beutner, E. H., and R. J. Nisengard. 1973. Defined immunofluorescence in clinical immunopathology. *In* Immunopathology of the Skin: Labeled Antibody Studies. E. H. Beutner, T. P. Chorzelski, S. Bean, and R. Jordan, editors. Dowden, Hutchinson & Ross, Inc., Stroudsburg, PA. 197.

20. Briggaman, R. A., and C. E. Wheeler, Jr. 1975. The epidermal-dermal junction. *J. Invest. Dermatol.* 65:71.

21. Daroczy, J., J. Feldman, and K. Kiraly. 1979. Human epidermal basal lamina: its structure, connections and functions. *Front. Matrix Biol.* 7:208.

22. Kobayasi, T. 1977. Anchoring of basal lamina to elastic fibers by elastic fibrils. *J. Invest. Dermatol.* 68:389.

23. Schaumburg-Lever, G., A. Rule, B. Schmidt-Ullrich, and W. F. Lever. 1975. Ultrastructural localization of in vivo bound immunoglobulins in bullous pemphigoid—a preliminary report. *J. Invest. Dermatol.* 64:47.

24. Holubar, K., K. Wolff, K. Konrad, and E. H. Beutner. 1975. Ultrastructural localization of immunoglobulins in bullous pemphigoid skin. *J. Invest. Dermatol.* 64:220.

25. Stanley, J. R., P. Hawley-Nelson, S. H. Yuspa, E. M. Shevach, and S. I. Katz. 1981. Characterization of bullous pemphigoid antigen—a unique basement membrane protein of stratified squamous epithelia. *Cell.* 24:897.

26. Foidart, J. M., E. Bere, M. Yaar, S. Rennard, M. Gullino, G. Martin, and S. Katz. 1980. Distribution and immunoelectron microscopic localization of laminin, a noncollagenous basement membrane glycoprotein. *Lab. Invest.* 42:336.

27. Yaiota, H., J. Foidart, and S. Katz. 1978. Localization of the collagenous component in skin basement membrane. *J. Invest. Dermatol.* 70:191.

28. Nieboer, C., D. M. Boorsma, M. J. Woerdeman, and G. L. Kalsbeek. 1980. Epidermolysis bullosa acquisita: immunofluorescence, electron microscopic and immunoelectron microscopic studies in four patients. *Br. J. Dermatol.* 102:383.

29. Yaoita, H., R. A. Briggaman, T. J. Lawley, T. T. Provost, and S. Katz. 1981. Epidermolysis bullosa acquisita: ultrastructural and immunological studies. *J. Invest. Dermatol.* 76:288.

30. Woodley, D. T., R. A. Briggaman, E. J. O'Keefe, A. O. Inman, III, L. L. Queen, and W. R. Gammon. Identification of epidermolysis bullosa acquisita antigen—a normal component of human skin basement membrane. *N. Engl. J. Med.* In press.

31. Timpl, R., J. Engel, and G. Martin. 1983. Laminin—a multifunctional protein of basement membranes. *Trends Biol. Sci.* 8:207.

32. Terranova, V. P., C. N. Rao, T. Kalebic, I. M. Margulies, and L. A. Liotta. 1983. Laminin receptor on human breast carcinoma cells. *Proc. Natl. Acad. Sci. USA.* 80:444.

33. Rao, C. N., I. M. Margulies, R. H. Goldfarb, J. Madri, D. T. Woodley, and L. A. Liotta. 1982. Differential proteolytic susceptibility of laminin alpha and beta subunits. *Arch. Biochem. Biophys.* 219:65.

34. Sage, H., R. G. Woodbury, and P. Bornstein. 1979. Structural studies on human type IV collagen. *J. Biol. Chem.* 254:9893.

35. Crouch, E., H. Sage, and P. Bornstein. 1980. Structural basis for apparent heterogeneity of collagens in human basement membranes: type IV procollagen contains two distinct chains. *Proc. Natl. Acad. Sci. USA.* 77:745.

36. Woodbury, R. G., and H. Neurath. 1980. Structure, specificity and localization of the serine proteases of connective tissue. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 114:189.

37. Mainardi, C. L., S. N. Dixit, and A. H. Kang. 1980. Degradation of type IV (basement membrane) collagen by a proteinase isolated from human polymorphonuclear leukocyte granules. *J. Biol. Chem.* 255:5435.

38. Sage, H., P. Pritzl, and P. Bornstein. 1981. Susceptibility of type V collagen to neutral proteases: evidence that the major molecular species is a thrombin-sensitive heteropolymer, [1 (V)]$_2$ 2(V). *Biochemistry.* 20:3778.