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Ultrastructural Changes of the Skin Induced by Human Leukocyte Elastase and Cathepsin G

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Human neutrophil elastase and cathepsin G at a concentration of 10^{-6} M were found to attack various substrates when normal skin biopsy specimens were incubated at 37°C for 1 h with either of these enzymes. Elastase damaged primarily hemidesmosomes, leading to the epidermal cleavage from the dermis, whereas cathepsin G damaged the membrane structures. Both these neutral proteinases were highly specific to basal lamina of blood vessels. This indicates that neutrophil elastase and neutrophil cathepsin G may play different roles in various skin diseases related to enhanced activity and infiltration of neutrophils. ***Key words: Electron microscopy; Neutrophil; Skin destruction.***

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Neutrophil elastase is known to be responsible for lung and liver injury in humans with a deficiency of α_1-proteinase inhibitor (1–3). The latter is known to be the main serum factor neutralizing neutral proteinases in tissue (3, 4). The in vivo activity of neutral serine proteinases released from human neutrophils has been suggested to play an important role in the pathogenesis of certain skin diseases.

In psoriasis, gap formation in the basement membrane and basal keratinocyte herniations through these openings were found to be related to the number of neutrophils in dermal infiltrate as well as to the activity of psoriatic lesions (5–7). Similarly, the possible role of leukocytes and their proteolytic enzymes was indicated in blister formation within the lamina lucida (LL) zone in epidermolysis bullosa acquisita (EBA), although the immunological reaction with type VII collagen occurred below the lamina densa (LD) (8). Moreover, insufficient inactivation of neutrophil enzymes underlies the pathomechanism of panniculitis in individuals having a genetic deficiency of α_1-proteinase inhibitor (9).

There are only a few published studies (e.g. 10) on the effect of purified neutrophil elastase and cathepsin G on human skin. Such investigations have concentrated predominantly on the epidermal-dermal junction (EDJ). Prolonged incubation with both neutral serine proteinases was shown to induce very severe damage (10).

The purpose of the present study was to determine by ultrastructural investigation the early changes in human epidermis and dermis induced in vitro by elastase and cathepsin G. We hoped to identify possible specific substrates for these enzyme activities within the skin and principal targets for neutrophil-mediated tissue damage. Elastase and cathepsin G were found to be directed against specific substrates (11, 12). Thus, they might play differing roles in the neutrophil-induced skin pathology.

MATERIAL AND METHODS

Skin specimens

Fresh skin biopsy specimens from normal healthy volunteers, approximately 1.5 mm × 1.5 mm in size, were placed in 1 ml of phosphate-buffered saline (PBS), pH 7.4, supplemented with 10 mM calcium chloride and 10 mM magnesium chloride, and incubated at 37°C for 1 h in medium alone, 1 mM phenyl-methyl-sulfonyl-fluoride (PMSF), 10^{-6} M of human neutrophil elastase (HNE), 10^{-6} M of HNE + 1 mM PMSF, 10^{-6} M of human neutrophil cathepsin G (HNCG), and 10^{-6} M of HNCG + 1 mM PMSF.

Samples were removed and rinsed in cold PBS, and fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, of osmolality about 530 mOsm/kg H_2O at 4°C for transmission electronmicroscopy. Tissue was postfixed with 1% osmium tetroxide.

Separation and purification of HNE and HNCG

Human neutrophil proteinases were separated from peripheral blood leukocytes by own modification of the method described by Briggaman et al. (10). Briefly, buffy coat cell suspension from heparinized blood was mixed with an equal volume of 3% Dextran T-500 in 0.15 M NaCl, and sedimented at 1xg and room temperature for 20 min. Supernate was layered on Ficoll-Hypaque solution, specific gravity of 1.077, and centrifugated at 400xg for 40 min. The leukocyte pellet contaminated with erythrocytes was re-

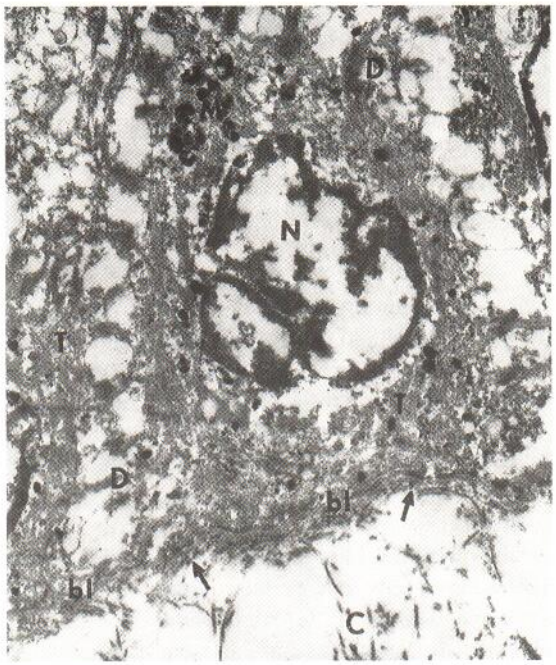

Fig. 1. Tissue preincubated with neutrophil cathepsin G. Epidermal-dermal junction. Adjoining keratinocytes are connected by preserved desmosomes *(D)*. The cell membranes are completely destroyed. There is homogenization of the cytoplasm, damaged tonofilaments *(T)*. No organelles survived besides the melanosomes *(M)*. The nuclei *(N)* are largely destroyed, but fragments of nuclear envelope remain. The basal lamina *(bl)*, slightly less electron-dense than usual, is very well preserved. Hemidesmosomes are intact *(arrows)*. C-collagen fibres. ×12,250.

suspended in 0.2% NaCl for 20 s, when an equal volume of 1.6% NaCl was added. Cells were washed three times with PBS at 200x*g* for 7 min.

The cell pellet was resuspended at 0°C in 1.0 M NaCl with addition of 10 mM sodium phosphate, 1 mM EDTA and 0.1% Brij 30, sonicated and centrifugated at 20,000 rpm for 30 min at 4°C in a Beckman ultracentrifuge. The supernatant containing leukocyte enzymes was adjusted to pH 8.0 by adding 1.0 M Tris-HCl, and layered on the Trasylol-Sepharose 4B affinity column. A 10 ml column packed with 3.0 g of swollen resin was prepared according to the Pharmacia Fine Chemicals handbook. Then the affinity column was washed with 0.05 M Tris buffer, 0.4 M NaCl, pH 8.0 for 2 h and 0.05 M Tris buffer, pH 8.0 for 30 min. Resin-bound HNE was eluted from the column with 10 mM MES [2-(*n*-morpholino)-ethanesulfonic acid) buffer, pH 5.5, while HNCG was eluted next with 0.1 M acetate buffer, pH 4.0. Fractions containing elastase or cathepsin G were concentrated separately using vacuum and 10 kD pore membrane up to about 0.6 mg protein per 1 ml and kept frozen at −22°C.

Purity of elastase was checked by the absence of hydrolysis of 1 mM Suc-Ala2-Pro-Phe-pNA (Sigma), a substrate for HNCG, and purity of cathepsin G respectively by absence of hydrolysis of 1 mM Suc-Ala3-pNA, a specific elastase substrate. The enzyme concentration was determined using their extinction coefficient of 9.85 (280 nm, 1% solution, 1 cm path length) for HNE, and 6.67 for HNCG (13). In addition, kinetic data for hydrolysis of these substrates by HNE and HNCG were obtained to verify the estimations using K_{cat} and V_{max} reported elsewhere (11).

Recovery of both enzymes was about 50% of the expected enzyme content in neutrophils.

RESULTS

The tissue injury was found to be dependent on the specific activity of HNE or HNCG, since no changes were observed in the epidermis or corium of skin samples incubated with enzymes in the presence of the PMSF, an inhibitor of serine proteinases.

In tissue samples preincubated with HNCG the most striking ultrastructural feature was the total loss of cell membranes from the keratinocytes, yet good preservation of desmosomes and hemidesmosomes (Fig. 1), of the endothelial cells and of other dermal cells (fibrocytes, mast cells, etc.) (Fig.2). However, membranes surrounding excretory granules in mast cells appeared to be more resistant to proteolysis and were found to be relatively well preserved (Fig. 2, inset A).

Less damage was seen in those nuclear envelopes which had shown partial dissolution in some parts of the nuclear circumference but had remained seemingly unharmed in others (Figs 1, 2). The nuclei of epidermal cells were partly destroyed and appeared as hollow structures with peripheral chromatin condensation (Fig. 1).

The keratinocyte cytoplasm was largely homogenized, with disappearance of all cell organelles except melanosomes and relatively well preserved tonofilaments, which were also varyingly damaged (Fig. 1). The extent of the epidermal damage was inversely proportional to the distance from the EDJ.

Apart from slight dissolution seen in some sections, the basal lamina at the EDJ was well preserved and hemidesmosomes remained intact (Fig. 1). By contrast, the basal lamina surrounding dermal blood vessels was almost completely destroyed (Fig. 2). In their vicinity, damaged collagen fibres presenting as long-spaced wide, low-density fibres could be observed (Fig. 2, inset B).

The morphology of the samples exposed to HNE showed only minor alterations. The epidermal structure was normal with the exception of a moderate

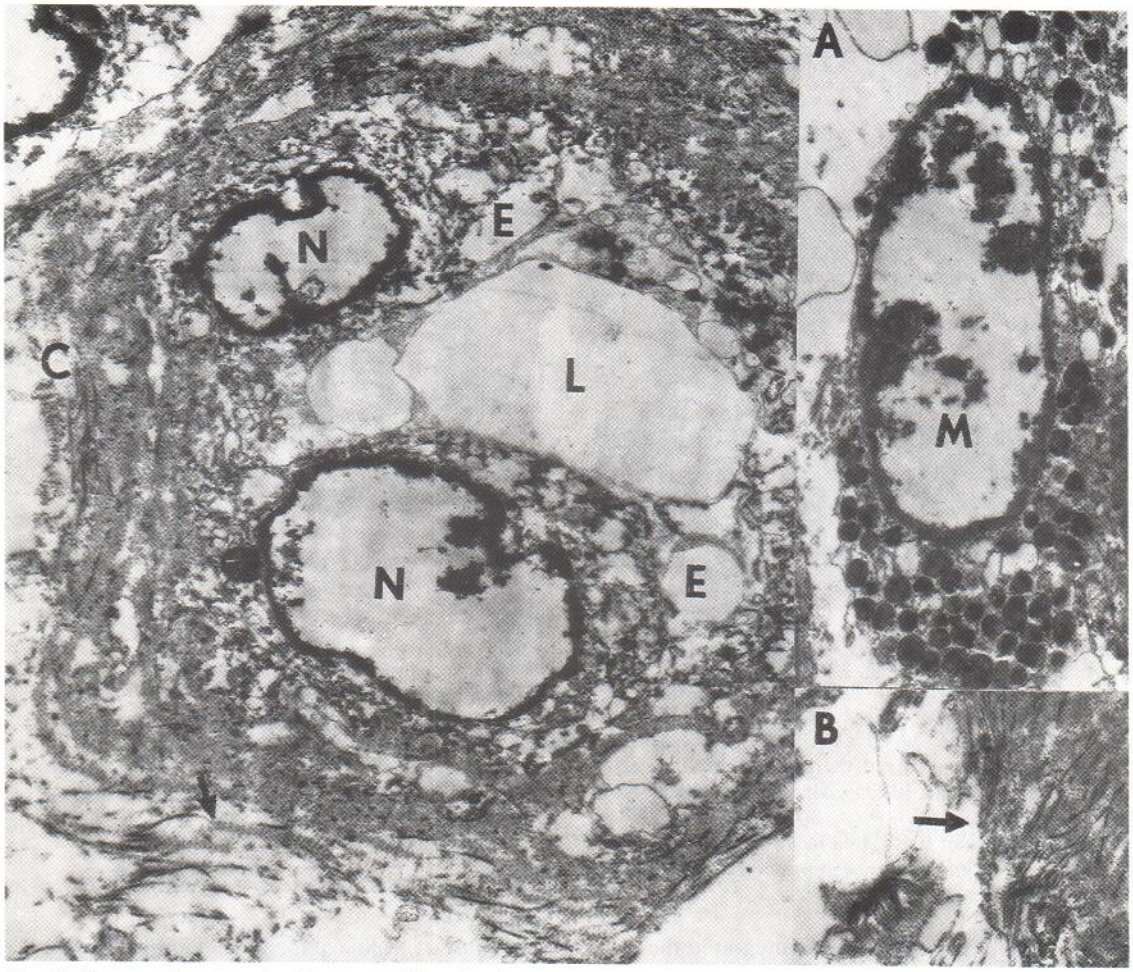

Fig. 2. Tissue preincubated with neutrophil cathepsin G. A dermal vessel shows damaged endothelial cells *(E)* with hollowed out nuclei *(N)* devoid of membranous structures. The basal lamina is absent. ×9,800. *Inset A.* The mast cell *(M)* granules are very well preserved. ×7,350. *Inset B.* In dermis, abnormal long-spaced, wide collagen fibres *(arrows)* intermingle with occasional undamaged collagen fibrils can be seen surrounding the vessel. ×7,350.

widening of intercellular spaces (Fig. 3). The most prominent abnormality was observed at the EDJ.

It started presumably as a widening (dissolution) of the LL, with progressive loss of hemidesmosomes, leading eventually to the formation of gaps (Fig. 3) and to the separation of the epidermis from the apparently intact LD (Fig. 3, inset A).

The corium was well preserved and the only abnormality was the disappearance of the basal lamina from the dermal blood vessels (Fig. 4).

DISCUSSION

HNE and HNCG belong to the neutral serine proteinases or serpin group, share many similarities including molecular weight and localization in azurophil granules of neutrophils, and are released simultaneously upon neutrophil activation (2). Despite this analogy, a short period of in vitro incubation of normal human epidermis with these enzymes revealed their distinct tissue specificities, summarized in Table I.

HNE hydrolysed mainly proteins of hemidesmosomes, widened the intracellular spaces, and damaged the basal lamina of dermal vessels. HNCG destroyed the continguity of both cell and nuclear membranes, allowing the enzyme to penetrate the cell and there to damage the nuclear proteins. Interestingly, HNCG did not destroy the mast cell

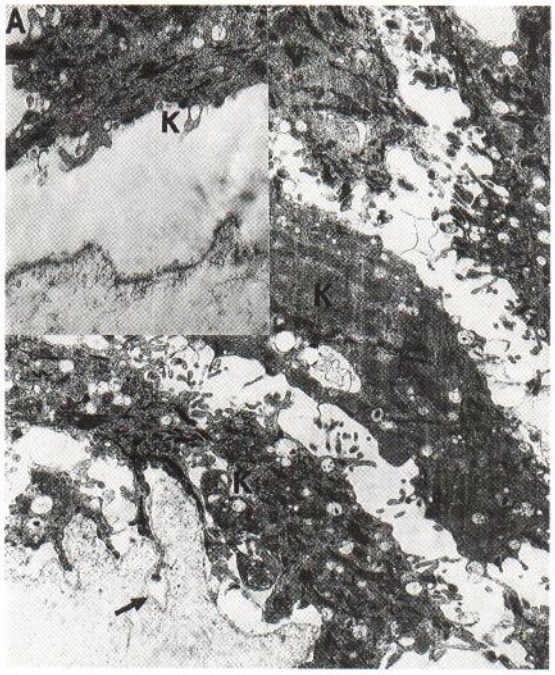

Fig. 3. Tissue preincubated with neutrophil elastase. Epidermal-dermal junction. The basal and suprabasal keratinocytes *(K)* display widened intercellular spaces. The lamina densa is unchanged but there is a considerable dissolution of the lamina lucida giving rise to subepidermal gaps *(arrows)*. ×9,800. *Inset A*. Subepidermal blister due to the dissolution of the lamina lucida. The lamina densa and the anchoring fibrils are well preserved. ×14,700.

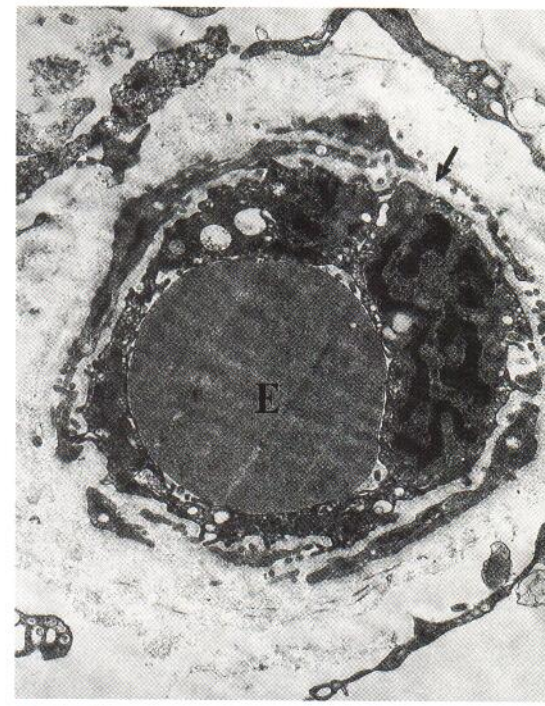

Fig. 4. Tissue preincubated with neutrophil elastase. Dermal vessel. Two endothelial cells of normal appearance having an erythrocyte *(E)* in the vessel lumen. The basal lamina is markedly destroyed and can be traced only in limited areas *(arrow)*. ×12,250.

granules. Both enzymes also disintegrated the basal lamina of blood vessels.

These findings differ somewhat from the data published by others (10). Prolonged exposure to weaker concentrations of HNE ($2–6 \times 10^{-7}$ M) was found within 8 h to damage the EDJ, with consequent complete disappearance of constituents of the basement membrane such as collagen IV, laminin, EBA antigen, and KF1 antigen reactive with the LD zone. However, the bullous pemphigoid antigen was still present on the basal layer cells, and AF1 and AF2 monoclonal antibodies were able to react with preserved anchoring fibres.

Our data did not confirm the finding of these authors (10) that HNCG induced focal epidermal-dermal separation in the LL with bullous pemphigoid antigen on the epidermal side, laminin on the dermal side and all EDJ zone antigens, mentioned above with respect to the HNE studies, present. For this type of destruction to occur there may require prolonged incubation of the skin with 10^{-7} M of HNCG for 4–8 h, whereas it does not occur at a HNCG concentration of 10^{-6} M for 1 h.

In contrast, we have made an additional observation that such a high HNCG concentration inflicted profound damage on the cell and nuclear membranes as well as on basal lamina of dermal vessels. The LD and sub-LD fibrillar network remained intact.

The HNE and HNCG induced in vivo hydrolysis is controlled by the presence in the skin of neutral serine proteinase inhibitors (3, 4). The tissue damage could take place only in a situation of either inhibitor deficiency or extensive infiltration of the skin with neutrophils. The damage to skin proteins is to be expected in close vicinity of migrating neutrophils, for example in psoriatic skin (5–7). The short-term in vitro incubation of the human skin with relatively high concentrations of either HNE or HNCG seems to reflect better the in vivo conditions than does prolonged exposure at low enzyme concentrations, because only a high concentration of enzymes can overcome the effect of physiological inhibitors. Since one neutrophil contains about 3 pg

Table I. *Divergent effect of human neutrophil elastase and cathepsin G on the human skin.*

	Elastase	Cathepsin G
Epidermis		
Keratinocyte membrane	Widening of intercellular spaces	Lysis of cell membrane
Desmosomes	–	–
Nucleus	–	Partial destruction of chromatin, damage to nuclear envelope
Cytoplasm	–	Homogenization, destruction of organelles (except melanosomes) & damage to tonofilaments
Epidermal-dermal junction		
Hemidesmosomes	Destruction	–
Lamina lucida	Split	–
Lamina densa	–	Slight dissolution, but otherwise intact
Anchoring fibrils	–	–
Capillaries		
Basal lamina	Destruction	Destruction
Nucleus	–	Damage to chromatin
Collagen	–	Dispersion & widening of fibres
Mast cells	–	Intact granules, nuclear damage, lysis of cell membrane

of HNE and 1.5 pg of HNCG (2), this proteinase concentration of 10^{-6} M in the skin can easily be reached around stimulated neutrophils undergoing degranulation.

One can speculate on the role of HNE and HNCG in the skin pathology. The damage to hemidesmosomes caused by HNE in psoriasis could represent a sufficient trigger for basal keratinocytes in psoriasis to respond to this trauma by activating repair mechanisms, resulting in the regenerative pathway of keratinization in psoriatic epidermis (14).

HNE may be involved in blister formation sites in EBA, since in almost all patients there were neutrophil infiltrates in the lesions (8). The cleavage occurred in the LL, whereas deposits of autoantibodies in EBA were found in the sub LD. Our data suggest that the LL seems to be a target for neutrophil enzymes, predominantly HNE, which were released from neutrophils attracted by immune complexes in the sub-LD zone.

Both HNE and HNCG may be of importance in panniculitis related to α_1-proteinase inhibitor deficiency (9). The damage to basal lamina of vessels and to endothelial cells caused by these enzymes, as was demonstrated in this study, might be critical in the induction of progressive liquefactive panniculitis.

These enzymes could be responsible for a variety of effects, as their tissue specificities differed. Our study has shown that hemidesmosomes are the most sensitive substrate for HNE, and the membrane structures for HNCG. The specific role of HNE and HNCG in skin pathology, e.g. vasculitis, requires further study.

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