

## Investigation of Intercellular Matrix Macromolecules Involved in Lichen sclerosus

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Dermal changes of the vulva in lichen sclerosus were compared with control vulvar samples using ultrastructural and immunofluorescence techniques. Collagen degeneration and regeneration were observed ultrastructurally in the superficial dermis of lichen sclerosus with increased amounts of ground substance. These processes appeared to alter the affinity of collagen fibres for the anticollagen antisera types I, III, IV. A decrease in elastin content was observed by electron microscopy. A loss of fibronectin was discovered at the dermo-epidermal junction, which looked normal ultrastructurally. The linear laminin pattern at the dermo-epidermal junction was also altered. These results suggested an enzymatic process in the pathogenesis of lichen sclerosus. Amidase activity could be determined in 6 normal and 6 pathological biopsies, though higher in the pathological samples ( $p < 0.01$ ). *Key words:* Lichen sclerosus; Collagen; Fibronectin; Laminin; Elastin; Elastase. (Received April 13, 1983.)

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Lichen sclerosus (LS) is considered to be a clinical and histological entity of unknown aetiology. The striking pathological change is the appearance of a band of hyalinization in the superficial dermis. Later a lymphohistiocytic infiltration is seen below the hyalinized area. The epidermis shows hyperkeratosis and atrophy.

As far as we know, the vulvar dermal changes have only been studied by light and electron microscopy. The aim of this work was to study the dermal macromolecular abnormalities using ultrastructural and immunofluorescence techniques. Recently, we postulated the involvement of an elastase type protease isolated from normal human vulvar fibroblasts in the pathogenesis of the disease (4). In order to check this hypothesis, we extracted elastase-type activities from pathological vulvar samples, which were compared with those extracted from control samples.

### MATERIAL AND METHODS

#### *Vulvar samples*

Mucosal vulvar biopsies from women suffering from a histologically proven LS were done prior to any treatment. The average age of the patients was 45 years (20-75). For control purposes, we took similar biopsy samples during surgical operations on women suffering from genital prolapsus. The average age of the control patients was 48 years (34-73).

### Electron microscopy

Four control and four LS samples were used for electron microscopy. Immediately after removal, the specimens were fixed in 2.5% glutaraldehyde in pH 7.4 cacodylate buffer for 6 hours at 4°C. This was followed by an osmium tetroxide fixation, dehydration in a series of ethanol solution and embedding in epoxy resin. The ultrathin sections were contrasted with lead citrate-uranyl acetate and observed in a Philips EM 300 electron microscope.

### Immunofluorescence studies

*Antisera against fibronectin, laminin, collagen types I, III, IV.* Antifibronectin serum was produced in rabbits, using a highly purified human plasma cold-insoluble globulin preparation obtained by the gelatin affinity chromatography procedure (15). The IgG fraction of the antisera was further purified by affinity chromatography on plasma proteins devoid of fibronectin, linked to a CNBr-Sepharose column followed by passage on a gelatin Sepharose column.

Antilaminin was obtained from Dr G. R. Martin (Laboratory of Developmental Biology and Diseases, N.I.D.H., N.I.H., Bethesda Md, USA). Collagens type I, III, IV were prepared from fibrotic human liver as described by Rhodes and Miller (11), modified for human liver (1). The purity of the collagen fractions was controlled by SDS-PAGE electrophoresis. Antibodies raised in New Zealand white rabbits were submitted to an immuno-adsorption procedure according to Timpl et al. (13). Native collagens are bound on CNBr-activated Sepharose. Antibodies cross-reacting with common determinants of the different collagen types were eliminated by absorption after repeated passages on the different collagen types bound to CNBr-activated Sepharose. Finally, purified antibodies were obtained by immuno-adsorption elution procedure against the required collagen types, using chromatographically purified collagens (14). Purified antibodies are tested by ELISA microassay before use. For purified anti-basement membrane collagens, no cross-reaction was detectable with laminin, using a double immunodiffusion test.

*Indirect immunofluorescence techniques.* Vulvar samples of 4 patients and 4 controls were cut in a cryostat at 4 µm and stained with a "sandwich technique" essentially according to Jablonska et al. (5). As the first layers the absorbed specific antisera were used: antifibronectin serum diluted 1/20 with phosphate-buffered saline (PBS), pH 7.3, antilaminin serum diluted 1/12 with PBS, anticollagen type I, III, IV, all diluted 1/8 with PBS.

The staining time of the first layer was 30 min. At the second layer FLTC-conjugated sheep antirabbit (Institut Pasteur Laboratory, Paris) diluted 1/40 in PBS was soaked on the sections, which reacted earlier with the antifibronectin, antilaminin, anticollagen type I, IV sera, for 30 min at room temperature. For the sections which reacted earlier with the anticollagen type III, a FITC-conjugated rabbit antigoat serum (Institut Pasteur, Paris) was used. After 10-min washes with PBS, the preparations are mounted using a solution of Veronal-buffered glycerol. The specificity of the immunofluorescence was tested by blocking with purified human plasma fibronectin, collagen types I, III, IV, used before the specific antisera, demonstrating a total loss of immunofluorescence. Sections with PBS at the first layer were used as controls. The sections were observed immediately with UV epicondenser, Zeiss III.

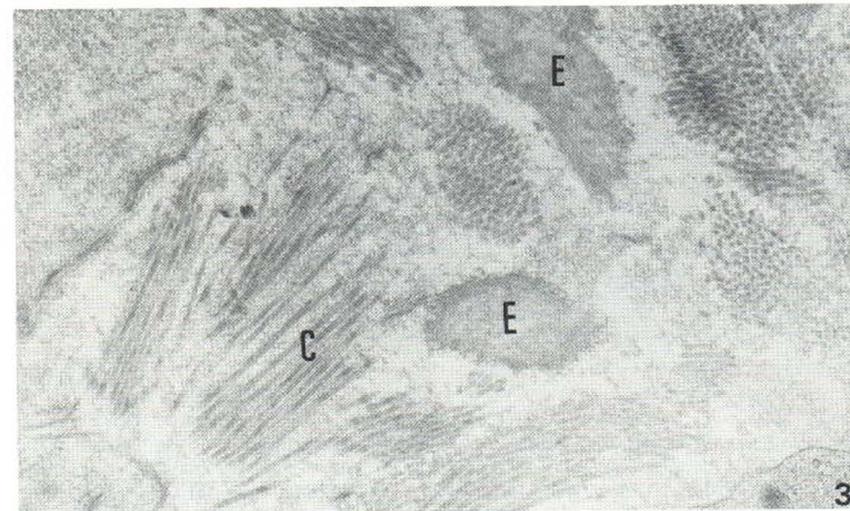
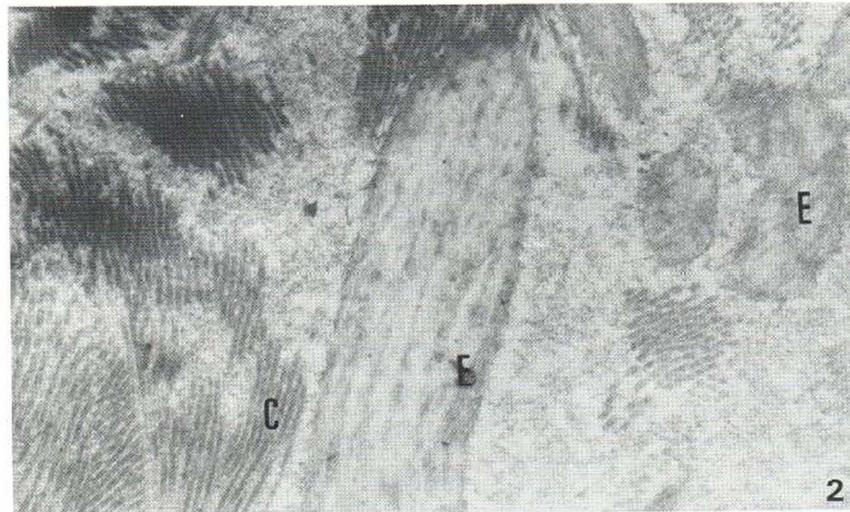
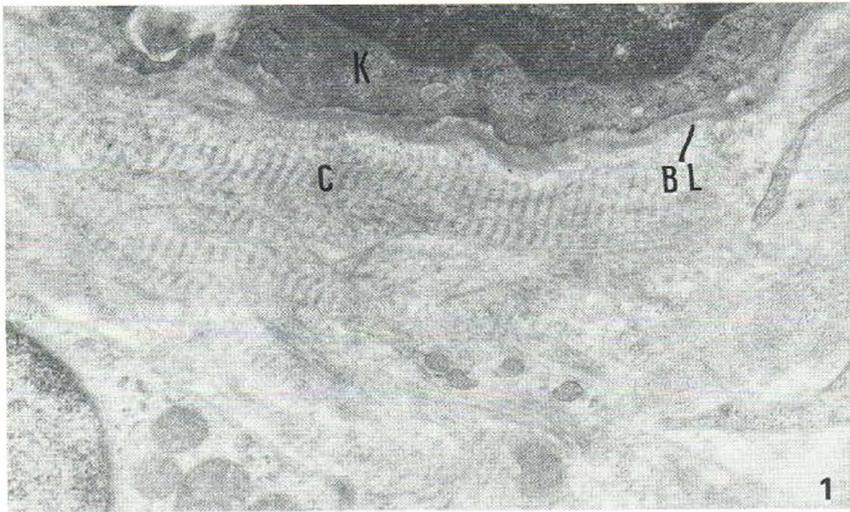
### Extraction and quantitation of an elastase-type protease activity from vulvar samples

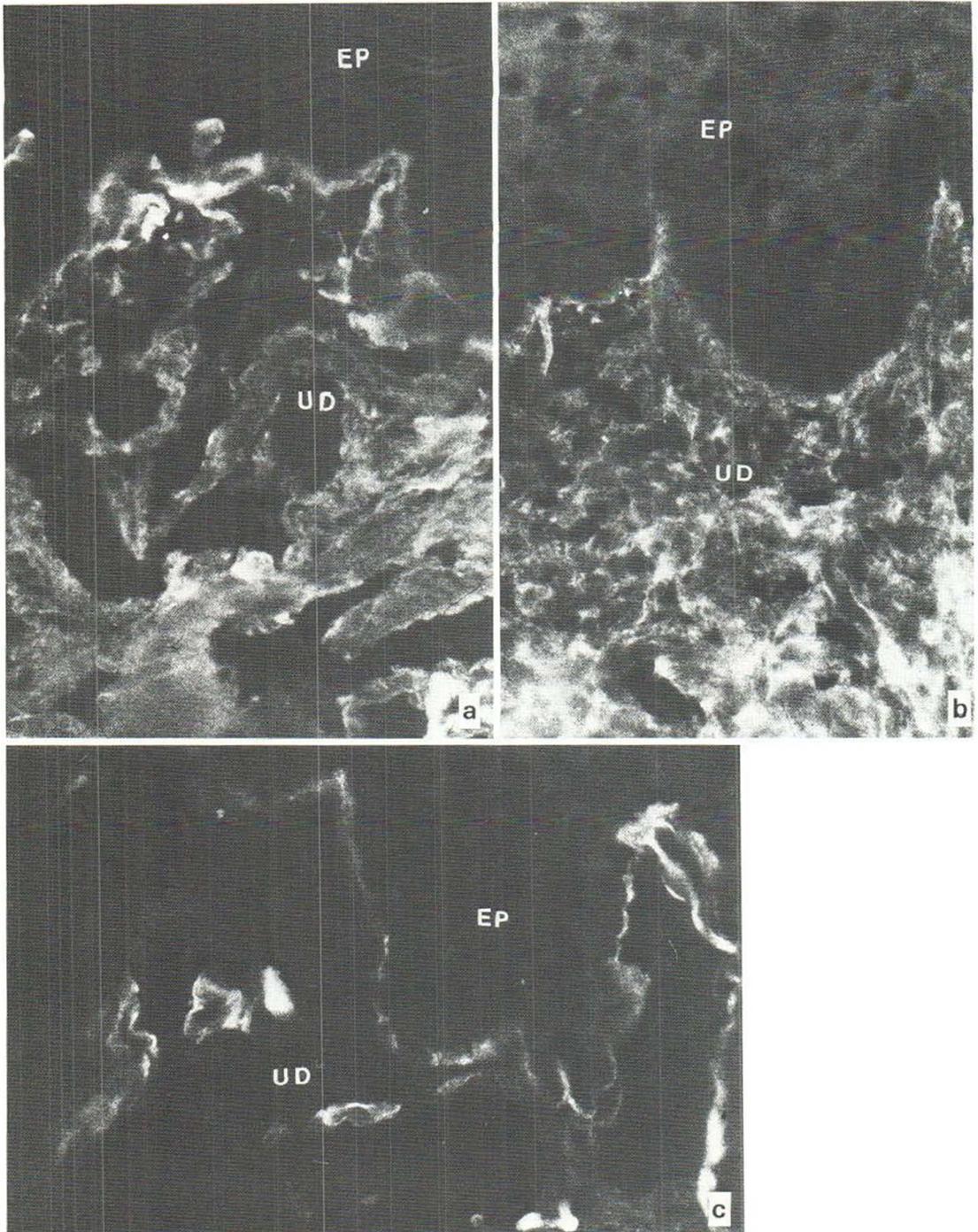
Protease activity was determined in the whole individual biopsies of 12 patients (6 L. S., 6 controls). After washing, the biopsies were finely minced with razor blades and extracted with 1 ml of 100 mM Tris-HCl, 5 mM CaCl<sub>2</sub>, 0.1% Triton X-100, 0.2% sodium azide, pH 8. The tissues were pulverized mechanically with this solution. Elastase-type activities were determined on the synthetic substrate *N*-succinyl-trialanyl-paranitroanilide (Suc(Ala)<sub>3</sub>NA). 20 µl of a 125 mM solution of Suc(Ala)<sub>3</sub>NA in *N*-methyl-pyrrolidone were added to 1 ml of enzyme solution in 0.1 M Tris-HCl buffer, 5 mM CaCl<sub>2</sub>,

*Fig. 1.* Lichen sclerosus. A large diameter collagen fibre lying beneath the dermo-epidermal junction, dividing into two branches and ending in thin fibrils. The basal lamina appears normal. *K*, keratinocyte; *BL*, basal lamina; *C*, collagen. Electron micrograph, ×24 300.

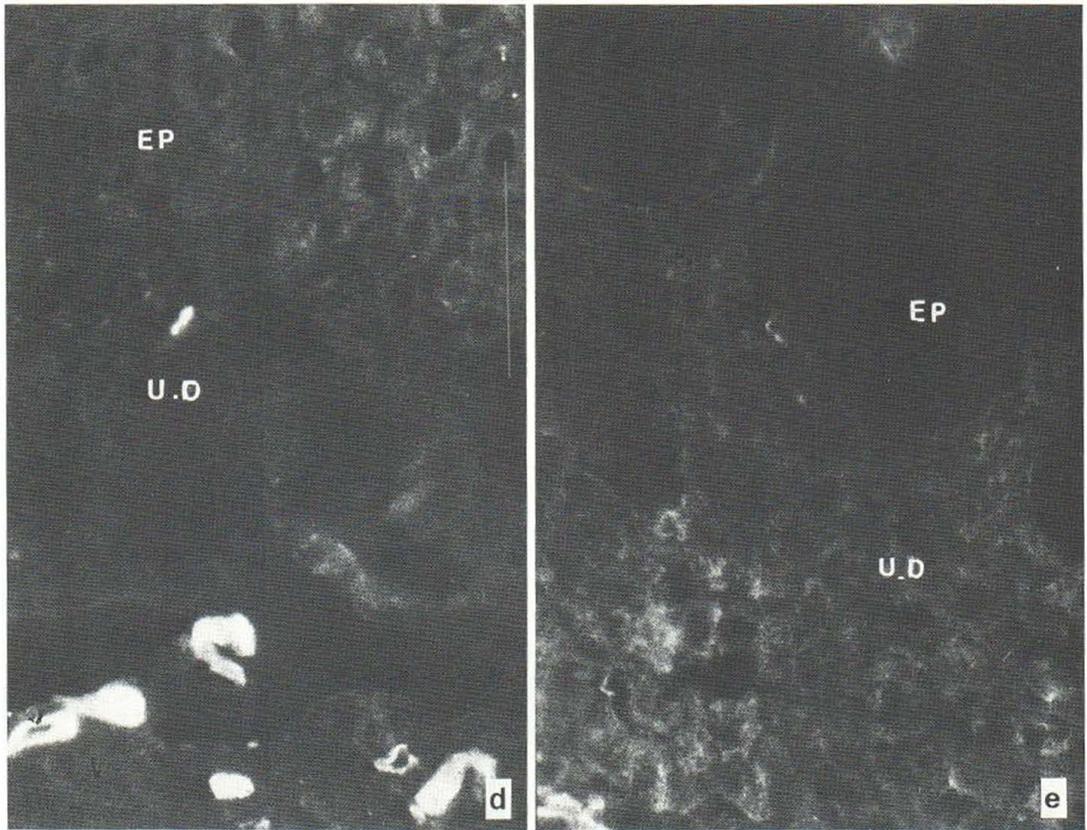
*Fig. 2.* Lichen sclerosus. Abnormal elastic fibres in lichen sclerosus: the microfibrillar dense zones are enlarged, irregular; elastin appears finely granular. *C*, collagen; *E*, elastic fibre. Electron micrograph, ×24 000.

*Fig. 3.* Lichen sclerosus. Collagen and elastic fibres are scattered in an abundant and granulofilamentous ground substance. *C*, collagen; *E*, elastic fibre. Electron micrograph, ×28 500.





*Fig. 4.* Immunofluorescent microphotographs using the anticollagen (I, III, IV) sera. *EP*, epidermis; *UD*, upper dermis. (a) Normal vulva with the anticollagen I serum: immunofluorescence of the thick collagen bundles of the upper dermis ( $\times 400$ ). (b) Normal vulva with the anticollagen III serum: immunofluorescence of the upper dermis fine structure ( $\times 400$ ). (c) Normal vulva with the anticollagen IV serum: visualization of the basal lamina and of the vessels ( $\times 400$ ). (d) Lichen sclerosus with the anticollagen IV serum: the basal lamina is negative, only the vascular basal lamina are marked ( $\times 400$ ). (e) Lichen sclerosus with the anticollagen I: no specific immunofluorescence in the upper dermis ( $\times 400$ ). Identical results were obtained with anticollagen III sera.



0.02%  $\text{NaN}_3$ , 0.1% Brij 35, pH 8.0. The variations in optical density at 410 nm were recorded as a function of time at 20°C in a spectrophotometer, Beckman type Acta C III. The results are expressed as nanomoles of  $\text{Suc(Ala)}_3\text{NA}$  released during one hour per mg of tissue weight.

The activity of the extracts was also tested on alkali-purified ligamentum nuchae elastin, as previously described (4).

## RESULTS

### *Electron microscopy*

The electron-microscopic changes found in the superficial dermis were similar in all the LS biopsies, varying only in degree. The collagen fibres exhibited highly variable diameters (200–1400 Å) and were quite sparse. Fibres of 200–400 Å were frequently seen with fine fibrils. Normal cross-striations were present in thicker fibres. Some fibres presented gross alteration in their alignment. Others varied greatly in their diameter (Fig. 1). A dense network of elastic fibres was found in the control specimens. The number of elastic fibres was appreciably reduced in the LS samples. Dense zones rich in microfibrils are often enlarged, lobular or irregular, as usually encountered in ageing elastic fibres (Fig. 2).

Collagen and elastic fibres are scattered in an abundant granulo-filamentous ground substance (Fig. 3). Only a few fibroblasts could be seen. Their dense cytoplasm was sometimes filled with thin filaments which also appeared in the extracellular space. Blood

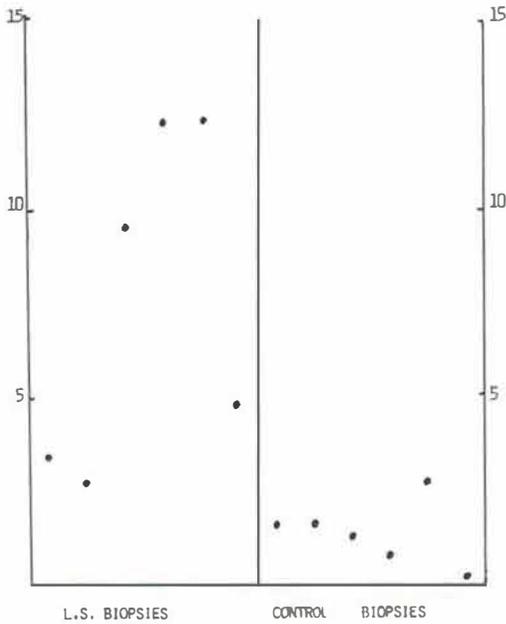


Fig. 5. Amidase activity on Suc(Ala)<sub>3</sub>NA in LS and control biopsies. The results are expressed in nanomoles of Suc(Ala)<sub>3</sub>NA released during one hour per mg of tissue weight.

and lymph vessels were dilated. The dermo-epidermal junction appeared normal, without breaking or duplication. No collagen fibres were seen in the space between the keratinocytes.

#### Immunofluorescence studies

In normal human vulva, fibronectin was found mainly in the papillary dermis in areas below the basement membrane, in a reticular distribution. In deeper dermis, the immunofluorescence staining specific for fibronectin was less pronounced and showed a fibrillar pattern surrounding collagen and elastic fibres.

The anticollagen type I reacted with fibres located in the papillar and reticular dermis. The anticollagen type III visualized collagen fibres predominantly in the upper dermis. The basement membranes of dermo-epidermal junction and vessels were labelled by the immunofluorescence staining with the anticollagen type IV and antilaminin sera. All these immunofluorescence observations are similar to those usually described in normal skin.

No fluorescence was observed in the upper, diseased dermis, with any of the antisera used (anticollagen types I, III, or antifibronectin). In the dermoepidermal junction, the linear laminin and collagen type IV pattern of normal samples was altered. The immunofluorescent band became intermittent and disappeared completely in the advanced lesions (Fig. 4).

#### Biochemical studies

No elastase activity on insoluble ligamentum nuchae elastin could be detected from pathological and normal samples. In contrast, an amidase activity on Suc(Ala)<sub>3</sub>NA was detected in both groups. This activity was significantly higher in the pathological samples ( $p < 0.01$ ) (Fig. 5).

## DISCUSSION

Dermis is a differentiated form of connective tissue made up of four major types of macromolecules: collagen, elastin proteoglycans and structural glycoproteins. As a result

of our investigations it appears that all are involved in the pathological modifications observed in lichen sclerosus. The ultrastructural abnormalities of collagen are similar to those observed by other authors (2, 6, 8). Sclerosis is usually defined as a densification of collagen fibres. Although the disease is called 'sclerosis', no real sclerosis is observed. Collagen is destroyed and regenerated, as suggested by the presence of fine fibrils, fibres of irregular diameter and the occurrence of intracellular fibres. These processes appeared to alter the affinity of collagen fibres for the anticollagen antibodies used.

On the basis of our ultrastructural and previous biochemical findings (4), we confirmed the disappearance of elastin in the vulva of patients suffering from LS. Mann & Cowan described large clumps of elastin, present throughout the diseased dermis and concluded that elastin was seen in larger amounts than in normal skin (8). Our results, in agreement with those of other authors, are at variance with the above statement (2, 6).

The selective disappearance of fibronectin was demonstrated by indirect immunofluorescence. In recent years, interest in this polymorphic glycoprotein has increased (7, 9, 10, 12). Its biological role is still incompletely understood. It has been suggested that it is involved in the morphogenesis of the connective tissues. Its disappearance could be related to the altered alignment of collagen fibres.

Proteoglycans were not investigated in the present study. A few years ago, changes of dermal and urinary acidic glycosaminoglycans in LS were investigated by Yoshima with the following results. Hyaluronic acid was excreted in urine of a patient with LS and involved dermal tissue contained about 50% of hyaluronic acid as compared with the skin of a healthy adult (16). In fact, the pseudosclerotic material in the upper dermis of LS consisted of degenerated connective tissue and of some newly formed elements.

The dermo-epidermal junction appeared normal in our ultrastructural study. In contrast, other authors mentioned thickening of the basal lamina, the appearance of holes, its absence in places and its duplication in other areas (3, 8). These findings correlate well with our immunological observations. The irregular labelling of the dermo-epidermal junction by the immunofluorescence staining with the antilaminin or anticollagen type IV antisera appears to precede the onset of the above-mentioned ultrastructural abnormalities which were not yet noticeable in our cases.

Connective tissue destruction is believed to be the result of enzymatic degradative processes. We demonstrated a higher elastase-type activity in pathological vulvar biopsies as compared with normal control biopsies, using a synthetic substrate Suc(Ala)<sub>3</sub>NA. This elastase-type protease is incapable of solubilizing bovine ligamentum nuchae elastin at an observable rate. Its ability to degrade dermal vulvar macromolecules remains to be elucidated. The elastase-type enzyme isolated from normal vulvar fibroblasts was found also to be inactive towards <sup>3</sup>H ligamentum nuchae insoluble elastin; in contrast, it was able to hydrolyse insoluble elastin from human aortas and to degrade the human skin elastic fibre system (in preparation). These findings support the hypothesis that enzyme activity found in LS samples may be related to the elastase-type protease previously isolated from normal human vulvar fibroblasts (4).

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