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Possible Formation of Cutaneous Amyloid from Degenerative Collagen Fibers

Ultrastructural Collagen Changes and the Immunoreactivity of Cutaneous Amyloidosis Employing Anti-type I, III, IV, V Collagen Antibodies

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The origins of primary cutaneous amyloid have been investigated and several possibilities are proposed. We investigated here several cases by electron microscopy and found in all cases characteristic filamentous changes which were identical with amyloid structures in the collagen bundles. These observations suggested a possible pathway of cutaneous amyloid formation from degenerative collagen fibers. Further studies were performed using immunoelectron microscopical and immunohistochemical methods employing a panel of anti-collagen antibodies to examine the reactivity of amyloid to them. Our results were much more suggestive of the collagen origin of this kind of amyloid. ***Key word: Origin of amyloid.***

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It has been thought that cutaneous fibroblasts are the most likely source of amyloid in the primary cutaneous amyloidosis (1–5). However, more recently a new concept has been proposed, that cutaneous amyloid in primary cutaneous amyloidoses originates from necrotizing keratinocytes (6–10). This was first reported by Black & Wilson Jones in 1970 (6), using light microscopy. In 1979, this view was re-affirmed by Hashimoto & Kumakiri (7) with the first report of 'colloid–amyloid body' which was considered to be the precursor of amyloid.

Further support for this derived from immunohistological and immunoelectron microscopical observations. These studies showed a positive reaction to amyloid (8, 9). Monoclonal anti-keratin antibodies were also employed but only one antibody reacted with the cutaneous amyloid (10). There are, however, several unresolved questions concerning this theory. In the monoclonal experiments, it has not been explained why only one antibody reacted with amyloid and why the antibodies that reacted with whole epidermis did not react with amyloid. Nor was it clear how 'colloid–amyloid body' reacted with antibodies positive for cytoid bodies and negative for amyloid. In addition, it is difficult to explain why many cytoid bodies, which appear in the cutaneous disorders, e.g. lichen planus and discoid lupus erythematosus, are not converted to amyloid masses if the amyloid derives from degenerating keratinocytes. In a recent report of an unusual case of cutaneous amyloidosis, in which numerous cytoid bodies were found in the epidermis, there was no evidence of their contribution to amyloid formation (11). One more recent report on the reactivity of monoclonal anti-keratin antibodies did not indicate a positive reaction to either cutaneous amyloid or the overlying epidermis (12). In addition, both Noren et al. and we (13, 14) have reported that in some instances amyloids are anti-keratin negative.

For these reasons, it is suggested that the keratinocyte origin theory of cutaneous amyloid is still tentative and not conclusive. In 1981, we reported unusual changes in collagen islands in a case of macular amyloidosis and we postulated that in this case, amyloid was derived from these changed collagen fibres (15). For more quantitative and precise estimations of the role of these collagen changes in the pathogenesis of cutaneous amyloid formation, we have examined a number of cases of macular and papular amyloidosis by conventional electron microscopy. Furthermore, we have examined their antigenic properties by employing type-specific poly- and monoclonal anti-collagen antibodies of types I, III, IV, V.

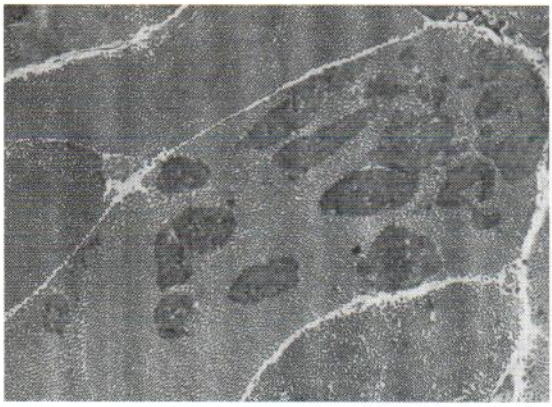

Fig. 1. A mottled collagen island containing many filamentous changes. ×3 500.

PATIENTS AND METHODS

Nine cases of macular amyloidosis and 4 cases of lichen amyloidosus have been examined by conventional electron microscopy. Seven cases of macular amyloidosis and 4 of lichen amyloidosus have also examined by immunofluorescence, immunoperoxidase and immunoelectron microscopic methods with anti-type I, III, IV, V collagen antibodies. The patients were diagnosed as having amyloidosis on the basis of clinical appearance and positive histology with thioflavin-T and Dylon multi-dye no. 9 in the biopsied specimens.

Conventional electron microscopic study

Biopsied samples were divided equally and prefixed in a solution of 2.5% glutaraldehyde buffered with phosphate and post-fixed after washing in 2% unbuffered solution of osmium tetroxide at pH 7.4. The samples were dehydrated with a graded series of alcohols and then embedded in Epon 812. Thin sections were stained first with uranyl acetate and then lead citrate. Observation was done in a Hitachi HS-9 electron microscope.

Immunohistochemical studies

Antigen and antibody: Production methods, purity and specificity of the antigen and antibodies used were described in detail in our recent reports (16, 17) and we merely summarize them here. Antigens were prepared by treating human placenta with pepsin-containing acetic acid, extracting type I, III, IV, V collagens by the differential salt precipitation technique, and purifying the extract by DEAE cellulose chromatography. The purity of these antigens was assessed by SDS–polyacrylamide gel electrophoresis. The anti-type I, III, IV, V collagen antibodies were obtained by immunizing rabbits with these antigens. The specificity of the antibodies was determined by immunoblotting techniques and their titers were determined by enzyme-linked immunosorbent assay (ELISA). These antibodies had no cross-reactivity with other types of collagen (Type I, III, IV, V), laminin or fibronectin.

Monoclonal anti-type III collagen antibody: Monoclonal anti-type III collagen antibody was prepared by the method we previously described (16). This monoclonal antibody also reacted with only α1(III) chain by immunoblotting.

Indirect immunofluorescence staining: Cryostat sections were treated for 30 min with the primary antibodies and

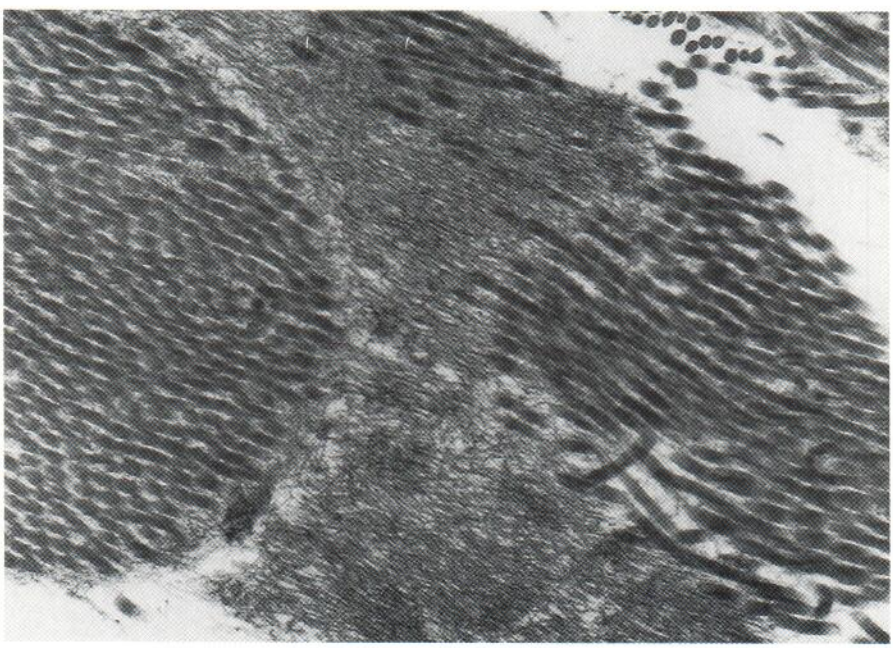

Fig. 2. Filamentous changes are connected originally to the adjacent collagen fibers. ×16 000.

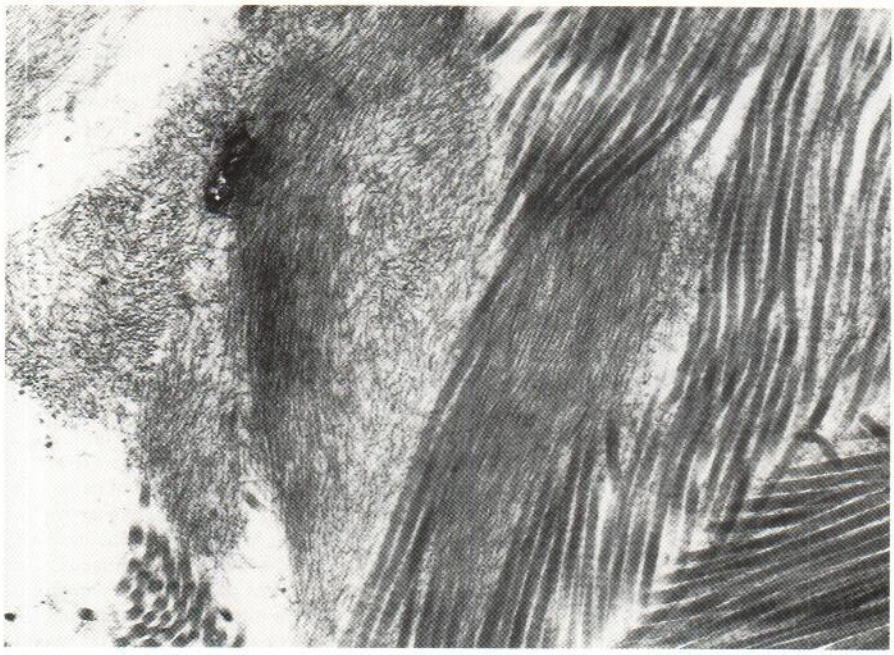

Fig. 3. Characteristic parallel filaments are observed near the region where collagen and fine filaments were connected. ×16 000.

washed with PBS. Following 30 min incubation with fluorescein-conjugated affinity-purified goat $F(ab')_2$ anti-rabbit IgG (TAGO), or goat $F(ab')_2$ anti-mouse IgG (TAGO), specimens were washed in PBS, mounted in 50% glycerine in PBS and examined with a Nikon VFD-R fluorescence microscope. In control experiments, specific antibodies were replaced by normal rabbit serum, normal mouse serum, or PBS.

Immunoperoxidase stain and immunoelectron microscopy: For the indirect immunoperoxidase technique, the sections were treated with 0.3% hydrogen peroxide in methanol to block endogenous peroxidase activity. The secondary anti-

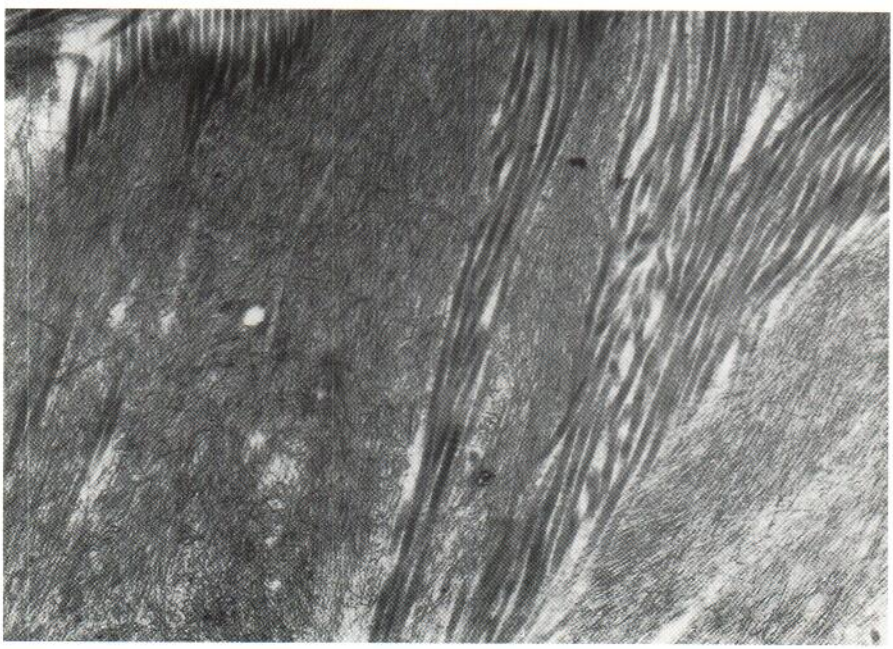

Fig. 4. Three main changes, viz. parallel, somewhat randomly orientated, randomly orientated filaments are seen ina same area. ×13 500.

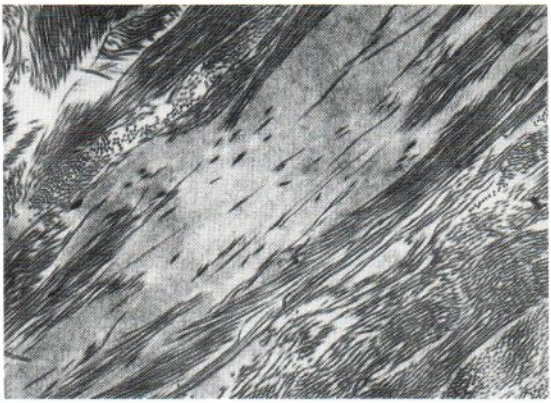

Fig. 5. A large collagen bundle also shows dissociated filamentous structures. ×6500.

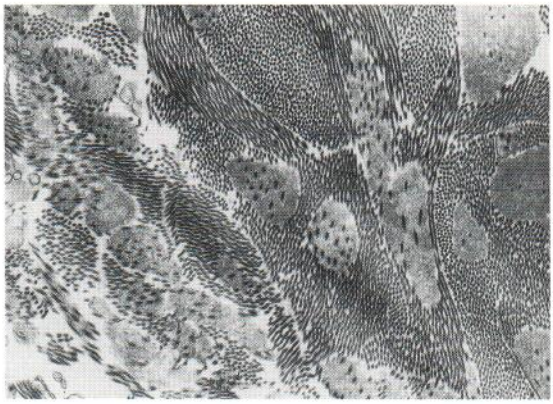

Fig. 6. small segmented collagen residues display an orientation identical with each fiber of the surrounding collagen bundle. ×6000.

body was 1 : 10-diluted peroxidase-conjugated goat $F(ab')_2$ anti-rabbit IgG (TAGO) or peroxidase-conjugated goat $F(ab')_2$ anti-mouse IgG (TAGO) for monoclonal examination. After the diaminobenzidine reaction in the presence of hydrogen peroxide, sections were fixed with 2.5% glutaraldehyde and post-fixed with 1% osmium tetroxide and embedded in Epon 812. Thin sections were examined without an electron stain. For the control study, primary antibodies were replaced by normal rabbit serum, mouse serum, or PBS.

RESULTS

Conventional electron microscopy

Conventional electron microscopy showed all specimens to have similar changes to those of the first case we reported. In the upper dermis, there were many amyloid islands in which unusual mottled collagen islands were scattered. These contained filamentous changes of varying size (Fig. 1). The boundary between these filaments and adjacent collagen fibers was carefully observed to decide whether they were connected with each other. The many observations showed that these filamentous structures had originally been connected to the adjacent collagen fibers (Fig. 2). The possibility of their chance superimposition was unlikely, as frequently we saw their obvious continuity. Near the regions where they were connected, the fine filaments showed a characteristic parallel structure which was repeatedly observed in areas of randomly orientated filamentous changes (Fig. 3). Large areas of the filamentous structures showed three main changes, viz., parallel, somewhat randomly orientated, randomly orientated filaments (Fig. 4). All of these had a similar diameter of 7–10 nm and were thought to be different phases of changes of the same material derived from the surrounding collagen fibers. Randomly-orientated filaments had precisely the same electronmicroscopical characteristics as the amyloid filaments. Not only the small collagen bundles but also the large ones were highly dissociated and demonstrated filamentous structures (Fig. 5). In the patches of filamentous degeneration of the collagen islands, small segmented collagen residues were observed, which were orientated and displayed, as a group, the same orientation as each fiber of the surrounding collagen bundle (Fig. 6). This observation supported the opinion that small collagen fragments were not intermingled from the outside of the collagen islands and that the filamentous changes also occurred *in situ*. Sometimes, in the upper dermis, fragmented small pieces of basement membrane and anchoring fibrils were incorporated

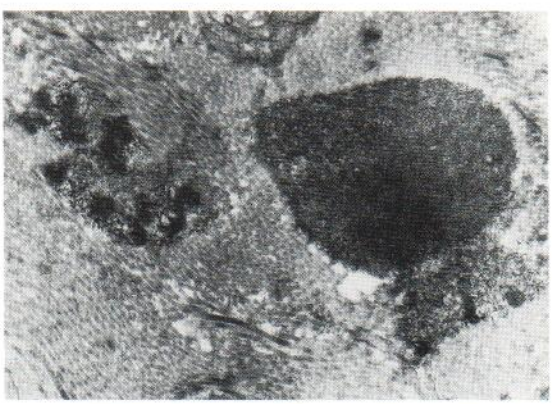

Fig. 7. Fragmented small pieces of basement membrane and anchoring fibrils were incorporated in the amyloid islands. ×11500.

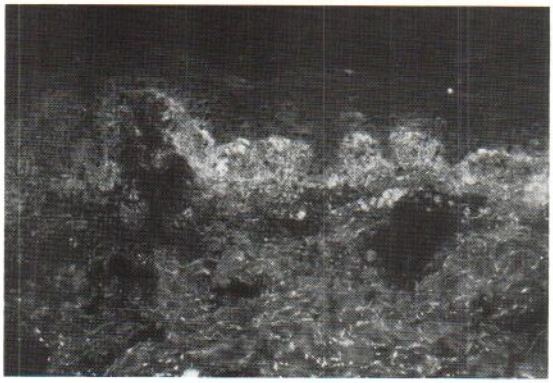

Fig. 8. Immunofluorescence-positive amyloid is seen in the papillary dermis, with anti-type I polyclonal antibody.

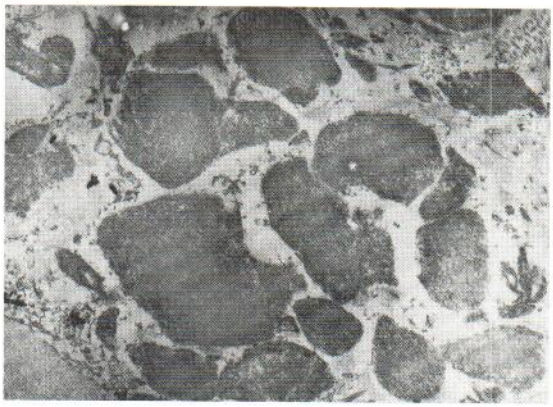

Fig. 10. An immunoelectron micrograph showing reaction products on the amyloid islands. Anti-type III monoclonal antibody. ×2500. No electron stain.

in the amyloid islands and became one of the constituents of the amyloid mass (Fig. 7).

Immunofluorescence and immunoperoxidase methods

Immunofluorescence and immunoperoxidase examination showed positive results in 9 cases of amyloid deposition while 2 cases were negative. In positive specimens, anti-type I, III, V collagen antibodies, both polyclonal and monoclonal, reacted in the same way as deposited amyloid, displaying a greenish fluorescence. Since dermal collagens of type I, III, V also reacted with these antibodies, the fluorescence of amyloid was not much stronger than that of surrounding collagens, though it was easily recognizable (Fig. 8). To support this, all specimens were stained with Dylon dye after the fluorescence observation in order to confirm the existence of amyloid deposit in the corresponding areas where we postulated the existence of positive amyloid fluorescence and, in fact, they all corrresponded.

Anti-type IV collagen antibody only partly reacted with amyloid deposits and fully reacted with the basement membranes of subepidermal or vessels (Fig. 9). In the cases of immunoreaction-negative amyloid deposition, greenish fluorescence was absent and darker patches than the surrounding collagen fluorescence were observed. In these negative cases, amyloid deposits were found in the deeper dermis than in the other positive cases and there were few deposits in the papillary dermis.

Immunoperoxidase examination gave results similar to those of fluorescence study, showing brownish reaction products instead of the greenish fluorescence. In the positive cases, immunoelectron micros-

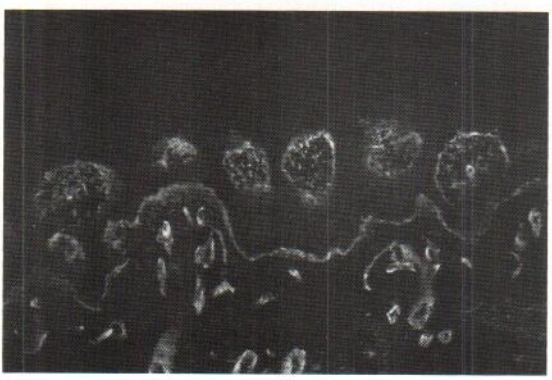

Fig. 9. Only some small parts of the amyloid are immunopositive in the papillary dermis, with anti-type IV polyclonal antibody.

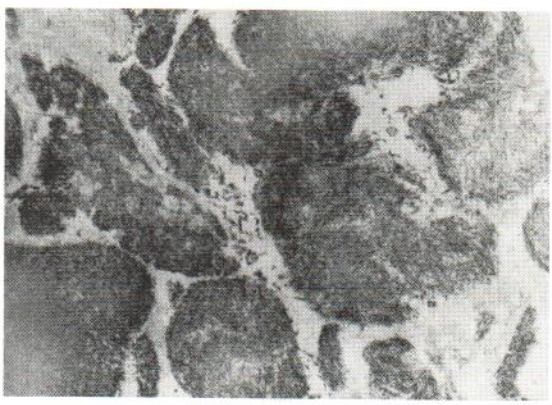

Fig. 11. High-power view showing that these dense masses consisted of positively reacted fine filaments of amyloid. Anti-type III monoclonal antibody. ×6000. No electron stain.

copy revealed the more precise structure of the immunoreaction of amyloid deposits with these antibodies. In this examination, using anti-type I, III, V collagen antibodies, amyloid deposits were revealed as immunoreaction products and were seen as electron-dense masses in unstained ultrathin sections (Fig. 10). In the high-power view, these dense masses consisted of positively reacted fine filaments of amyloid (Fig. 11). When the anti-type IV collagen antibody was applied, the reaction products were mainly in the basement membrane zones, but they were fragmented and scattered below the basal lamina. Some parts were intermingled in the amyloid deposits and formed part of the amyloid masses, but never appeared as the main part.

DISCUSSION

Collagen changes observed in cutaneous amyloidosis had not been noted before our report of peculiar patchy filamentous changes of collagen in a case of macular amyloidosis (15). Previously, only one report had shown the ultrastructural filamentous changes of collagen fibers in a case of lichen amyloidosus (1). Although similar filamentous changes in the collagen fibers were partly demonstrated in this report, the author interpreted this as the phase of collagen synthesis rather than its degeneration.

We saw the same filamentous changes of collagen bundles in all cases of primary cutaneous amyloidosis, and the three kinds of filamentous change which are probably sequential phases of changing collagen islands were identified in many cases. It seems that parallel filaments are changing into randomly orientated amyloid-like filaments through the intermediate phase of wavy or somewhat randomly orientated filaments. Parallel filaments were presumably connected to surrounding collagen fibers. In addition, small collagen residues in the filamentous patches were similarly oriented to surrounding collagen fibers, which suggests that in these patches, dynamic or drastic movements were not seen and only static changes were occurring inside the patches. Taking these observations into consideration, it seemed that patchy filamentous changes appeared *in situ* and did not come from outside the surrounding collagen bundles. Although some parts of basement membrane were intermingled in the amyloid islands or in the patches of collagen degeneration, this seemed to be by chance and they did not form the main substance of the amyloid islands. In the micro-dermal papilla, small amounts of amyloid were found and consecutive sections showed no other structures such as large masses of amyloid or cytoid bodies, which indicated that these small amounts of amyloid were not derived from cytoid bodies. From these considerations, the degenerative collagen origin of cutaneous amyloid in primary cutaneous amyloidosis was suggested by conventional electron microscopy.

Further observations using anti-collagen antibodies to examine the antigenic properties of amyloid revealed interesting results. In 9 cases out of 11, a greenish immunofluorescence and brownish reaction products of immunoperoxidase were positive in the amyloid deposits as well as in the collagen bundles, with the use of anti-type I, III, V collagen antibodies. This observation was confirmed by immunoelectronmicroscopy and positively stained electron-dense amyloid deposits were observed. Higher magnification revealed that every amyloid filament was positive for these antibodies and this suggested the antigenic identity of amyloid filaments with these collagen fibers. Since type I, III and V collagens of dermis are widely distributed in the dermis in the same manner and every collagen fiber was positive for all these antibodies, the immunoreaction of amyloid with these antibodies was compatible with the reaction of collagen fibers, if these amyloid deposits were derived from degenerated collagen fibers which preserved their original antigenic properties. Although 2 cases, one of the macular type and the other of the lichenoid type, were negative, there were some common histological features in these cases, i.e., there are few amyloid deposits in the papillary dermis and the negative amyloid masses existed relatively deeply in the dermis.

Considering the positive reaction of amyloid in the papillary and upper dermis, this rare negative reaction of amyloid in the deeper dermis may represent the antigenic denaturalization of amyloid-orientated changed collagen by unknown factors. The immunohistological reaction of amyloid with anti-type IV collagen antibody was only partly seen and because the reaction products were thought to be fragmented basal lamina intermingled with amyloid they could not be the main filamentous substance of amyloid.

A variety of cells and dermal components have been thought to be the origin of cutaneous amyloidosis (3, 5, 6, 15, 18–21). In nodular amyloidosis, plasma cells are probably the most important (22). In other types of macular and lichenoid amyloidosis,

no reliable results have yet been obtained. It is said that keratinocytes are converted into amyloid via filamentous changes as colloid body and this theory is supported by the immunohistological positive reaction using anti-keratin antibody (8, 10). However, as we have discussed in the introductory section, the keratinocyte origin theory is still not conclusive.

Although fibroblasts were the most likely cells of producing amyloid production, such direct evidence have not been reported. Recently, Lo & Wong (23) reported the formation of amyloid-like filaments by cultured fibroblasts obtained from the amyloid lesions. Though it is unclear how this in vitro study would be interpretated in vivo, the study shows that abnormal collagen synthesis and sequential easy degradation of collagen may occur. In such a situation, it may be possible to form amyloid derived from degenerative or changed collagen. The possibility of the multi-origin of amyloid of these cutaneous amyloidosis may exist, but further investigation of the precise biochemical analysis of amyloid deposits itself is necessary.

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