Apolipoprotein E is Present in Primary Localized Cutaneous Amyloidosis

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Apolipoprotein E (apoE) is one of the amyloid associated proteins that is found in the amyloid plaque of Alzheimer's disease and systemic amyloidosis. ApoE might play an important part in the etiology of Alzheimer's disease by functioning as a "pathologic chaperone" to promote the formation of amyloid filaments. In this study, we investigated whether apoE is associated with amyloid deposits of primary localized cutaneous amyloidosis using immunohistochemistry, immunogold electron microscopy, and immunoblotting. The subjects consisted of 12 patients with lichen amyloidosus and one patient with macular amyloidosis. Light microscopically, amyloid deposits in the dermal papillae were round in shape and stained with Congo red. Immunohistochemically, apoE was detected in amyloid deposits in all the cases examined. Immunogold electron microscopy showed apoE immunoreactivity on the amyloid deposition. Immunoblots of amyloid-positive skin showed 35K and 14K proteins, which were taken to be apoE and its fragment, respectively. In normal skin extract, only the 35K protein was detected by the anti-human apoE. Moreover, the intensity of the amyloid-positive skin sample was stronger than that of the normal skin sample. Monoclonal anti-cytokeratin antibody reacted with the 45K protein of the amyloid-positive skin extract. These results indicate that apoE is a component of primary localized cutaneous amyloidosis, and that it might play an important role in primary localized cutaneous amyloidosis. ***Key words: immunoblotting/immunogold electron microscopy/immunohistochemistry. J Invest Dermatol 111:417–421, 1998***

Apolipoprotein E (apoE) is a protein of molecular weight of 34,200 (299 amino acid residues) and is a component of lipoproteins of VLDL, IDL, HDL, and chylomicron. ApoE interacts with the "remnant receptor" (apoE receptor) and the low-density lipoprotein (LDL) receptors (apoE/B receptor) of the liver and other organs to modulate the catabolism of triglyceride-rich lipoprotein particles (Poirier *et al*, 1993). The protein is also produced and secreted in the skin, and implicated to play roles in epidermal differentiation and proliferation (Barra *et al*, 1994). Localization of apoE in the normal skin has been demonstrated by immunohistochemistry (Miyauchi, 1991), and the relationship between the apoE allele and a skin disease has been shown (Furumoto *et al*, 1997). It has been reported that apoE is associated with the amyloid plaque of various amyloid-forming diseases (e.g., Alzheimer's disease, Creutzfeldt–Jakob disease, and Down's syndrome) (Namba *et al*, 1991). ApoE is found both in senile plaques and in amyloid deposits in the cerebral vessel associated with Alzheimer's disease (Namba *et al*, 1991; Wisniewski and Frangione, 1992; Strittmatter *et al*, 1993a). Although the biochemical function of apoE in amyloid has not been clarified, apoE may be related to the formation of amyloid fibrils.

Primary localized cutaneous amyloidosis (PLCA), lichen amyloidosus, and macular amyloidosis are characterized by the finding that the amyloid deposits are limited to the papillary dermis (Kumakiri and Hashimoto, 1979). There is a hypothesis that the precursor protein of PLCA might be keratin protein from epidermal keratinocytes (Hashimoto *et al*, 1990). The pathogenesis of PLCA, however, remains undetermined and the amyloid fibril protein has not been identified. Recently, Yamada *et al* (1994) reported that fragments of apoE were common constituents of amyloid fibrils in systemic amyloidosis, but there have been no reports on the relationship between apoE and PLCA. The aim of this study was to clarify the relationship between apoE and amyloid in PLCA using immunohistochemistry, electron microscopy, and immunoblotting. We also discuss the role of apoE in the formation of amyloid in PLCA.

MATERIALS AND METHODS

Immunohistochemistry

Tissue specimens Skin biopsies were obtained from 12 patients with lichen amyloidosus and one patient with macular amyloidosis (**Table I**). In the patients with lichen amyloidosus, the lesions were located on the legs or the back. In the patient with macular amyloidosis, the hyperpigmented lesion on the back was examined. Formalin-fixed, paraffin-embedded blocks were cut in 4 μm thick sections. The presence of amyloid was confirmed by staining serial sections with Congo red and demonstrating birefringence in polarized light. For the control specimens of immunohistochemical staining, histologically normal skins were investigated using anti-apoE antibody.

Immunoperoxidase staining Sections were deparaffinized in xylene and washed in 50 mM Tris-buffered saline (pH 7.6). Endogenous peroxidase activity was destroyed by incubating with 3% hydrogen peroxide in methanol at room temperature for 10 min, and the sections were incubated in normal swine serum (DAKO PAP, DAKO, Copenhagen, Denmark) for 30 min to reduce non-specific background staining. The sections were incubated in moist

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Abbreviations: ApoE, apolipoprotein E; GAG, glycosaminoglycans; PLCA, primary localized cutaneous amyloidosis.

Table I. Clinical data and immunohistochemical findings of primary localized cutaneous amyloidosis

No.	Type of amyloidosis[a]	Sex[b]	Age[c] (y)	Reactivity to anti-human apoE antibody
1	LA	M	47	+
2	LA	M	82	+
3	LA	F	67	+
4	LA	F	73	+
5	LA	M	40	+
6	LA	M	57	+
7	LA	M	67	+
8	LA	F	26	+
9	LA	M	57	+
10	LA	M	41	+
11	LA	M	65	+
12	LA	F	71	+
13	MA	F	84	+
Total				13/13 (100%)

[a] LA, lichen amyloidosus; MA, macular amyloidosis.
[b] M, male; F, female.
[c] Age at skin biopsy.

chambers with polyclonal rabbit anti-human apoE antibody (DAKO) diluted 1:50 at 37°C for 30 min and followed by incubating with swine immunoglobulins to rabbit immunoglobulins (DAKO) for 30 min. They were incubated with peroxidase rabbit anti-peroxidase complexes (DAKO) for 30 min. They were then incubated with 3′,3′-deamino-benzidine tetrahydrochloride in H_2O_2 for 5 min, followed by hematoxylin staining.

Electron microscopic immunocytochemistry

Tissue processing A case of lichen amyloidosus was examined. The biopsy was taken from the hyperpigmented papules on the back. The tissues were immediately cut into small pieces about 1 mm^3 and were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4). After an overnight rinse with 0.1 M cacodylate buffer, they were dehydrated in alcohol and embedded in Epon 812 for the immunogold electron microscopy. For conventional electron microscopy, they were postfixed in 1% osmium tetroxide for 1 h, dehydrated, and embedded in Epon 812.

Post-embedding colloidal gold labeling Thin sections were cut and collected on 150-mesh nickel grids coated with 2% Collodion solution in amyl acetate (Ohken, Tokyo, Japan) for improved section adhesion. Post-embedding immunocytochemistry was performed by techniques previously described (Phend *et al*, 1995; Takahashi *et al*, 1996). Briefly, grids were washed with 10 mM phosphate-buffered saline (PBS, pH 7.4) for 30 min. Then binding of non-specific antibodies was blocked with PBS containing 1% bovine serum albumin for an hour. The sections were incubated with rabbit anti-human apoE antibody (DAKO) diluted (1:200) with PBS containing 1% bovine serum albumin overnight. After several rinses with PBS, the sections were treated with PBS containing 1% bovine serum albumin for 1 h to block non-specific binding sites. The sections were then incubated with anti-rabbit IgG conjugated 20 nm colloidal gold particles (EY Laboratories, San Mateo, CA) diluted (1:10) with PBS containing 1% bovine serum albumin for 4 h. They were then washed with PBS three times and with distilled water (DW), and stained with uranyl acetate and lead citrate solution. Sections were examined in an H-800 electron microscope (Hitachi, Tokyo, Japan).

Extraction of amyloid proteins Amyloid-positive skin biopsy was obtained from a patient with lichen amyloidosus. Amyloid proteins were extracted according to the procedures of Kaplan *et al* (1993). Briefly, the sample of amyloid-containing tissue was dispersed in saline and incubated overnight at room temperature with a moderate shaking. The sample was centrifuged at 14,000 r.p.m. for 10 min. The supernatant was discarded, the pellet was redispersed in saline and centrifuged, and this procedure was repeated four times. The pellet was then washed with DW and homogenized in aqueous 20% acetonitrile solution containing 0.1% trifluoroacetic acid. The homogenate was incubated at room temperature for several hours with a moderate shaking and centrifuged for 10 min at 14,000 r.p.m. The supernatant was pooled and lyophilized. The pellet was redispersed in 20% acetonitrile solution containing 0.1% trifluoroacetic acid and the procedure was repeated four times. For the control, histologically normal skins were investigated for extraction of insoluble fractions and immunoblotting.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting of amyloid protein The samples were dissolved in 62.5 mM Tris-HCl pH 6.8 sample buffer containing 3% sodium dodecyl sulfate, 10% 2-mercaptoethanol, and 20% glycerol. The samples of amyloid proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel (15%T and 2.67%C) electrophoresis as described by Laemmli (1970). The proteins were electrotransfered from the gel to a polyvinylidene difluoride membrane (Immobilon, Millipore, Japan) using 3-[cyclohexylamino]-1-propanesulfonic acid (Sigma, St. Louis, MO) in methanol pH 11 (Matsudaira, 1987). The polyvinylidene difluoride membrane was stained with Coomassie Brilliant Blue R-250. Immunostaining was performed using a goat anti-human apoE (Phenotyping ApoE IEF system, Joko, Tokyo, Japan) at a dilution of 1:500 as the first antibody and alkaline phosphatase conjugated rabbit anti-goat IgG (Phenotyping ApoE IEF system, Joko) at a dilution of 1:500 as the second antibody, according to the procedure described by Blake *et al* (1984). In a further experiment, cytokeratins were also identified by a murine monoclonal anti-cytokeratin antibody (MNF-116 clone, DAKO) at a dilution of 1:500, followed by horse radish peroxidase-conjugated goat anti-mouse immunoglobulins (Cappel, Costa Mesa, CA) at a dilution of 1:500. The peroxidase reaction product was visualized using 4-chloro naphthol (Sakurai *et al*, 1986). For the positive controls of immunostaining, normal human serum and purified human keratin (Sigma) were investigated using anti-apoE antibody and anti-cytokeratin antibody, respectively. In principle, cytokeratin and intermediate filaments can be solubilized in high concentrations (9–10 M) of urea (Achtstaetter *et al*, 1986). The skin extracts were also solubilized in the buffer containing 9.5 M urea, 5% Nonidet P-40, and 5% 2-mercaptoethanol at room temperature for 30 min, to which equal volumes of the sample buffer were added. The protein solutions were then subjected to the immunoblotting. The samples loaded on the focusing gel were prepared in the same quantity of protein.

RESULTS

Amyloid deposits in PLCA react with anti-apoE Amyloid was detected in the lesions of all cases. Light microscopically, the deposits were small in size and were weakly eosinophilic materials in the dermal papillae on hematoxylin and eosin-stained preparations. The deposits were stained with Congo-red and showed emerald-green birefringence under polarized light. Most of the congophilic amorphous masses were in the form of nodules measuring about 10–30 μm in diameter (**Fig 1*A***).

Rabbit anti-apoE antibody reacted with epidermal cells, particularly basal cells, and strongly reacted with sebaceous glands. ApoE-immunoreactive amyloid deposits were detected in the dermis of sections from all the cases examined (**Table I**, **Fig 1*B***). The staining intensity of apoE was varied in each section, but the interstitium of the dermis showed negative staining with apoE.

Amyloid fibrils are observed in the papilla and reticular dermis using electron microscopy At low magnification, amyloid deposits were found as electron-dense round or oval individual islands. Amyloid deposits exhibited a nodular or granular form beneath the basement membrane or among the collagen fibers in the dermis (**Fig 2*A***). At higher magnification, amyloid fibrils were seen as irregular string-like structures of varying thickness and density along their length, measuring 7–10 nm in width. Morphologic changes of the dermo–epidermal junction were seen near the amyloid deposits. Some regions of basal lamina were destroyed and could not keep the structure. There was no amyloid deposit in the reticular dermis and the perivascular area.

Amyloid deposits are labeled with 20 nm gold particles to anti-apoE antibody After immunolabeling for apoE, amyloid deposits were positively labeled with 20 nm gold particles (**Fig 2*B***). Collagen fibers were not labeled with the gold particles.

ApoE and cytokeratin are present in the skin extracts Immunoblots of amyloid-positive skin showed 35K and 14K protein bands that were reacted with anti-apoE antibody (**Fig 3*A***). The 35K and 14K proteins were considered to be apoE and its fragment, respectively (Yamada *et al*, 1994). In normal skin extract, only 35K protein was detected by the anti-human apoE. The intensity of the 35K protein band of the amyloid-positive sample was stronger than that of the normal skin sample (**Fig 3*A***). The difference in the intensity of immunostaining paralleled the amount of protein. The 45K protein band was detected by the anti-cytokeratin antibody in an amyloid-positive case, but not in a normal case. In the skin samples solubilized

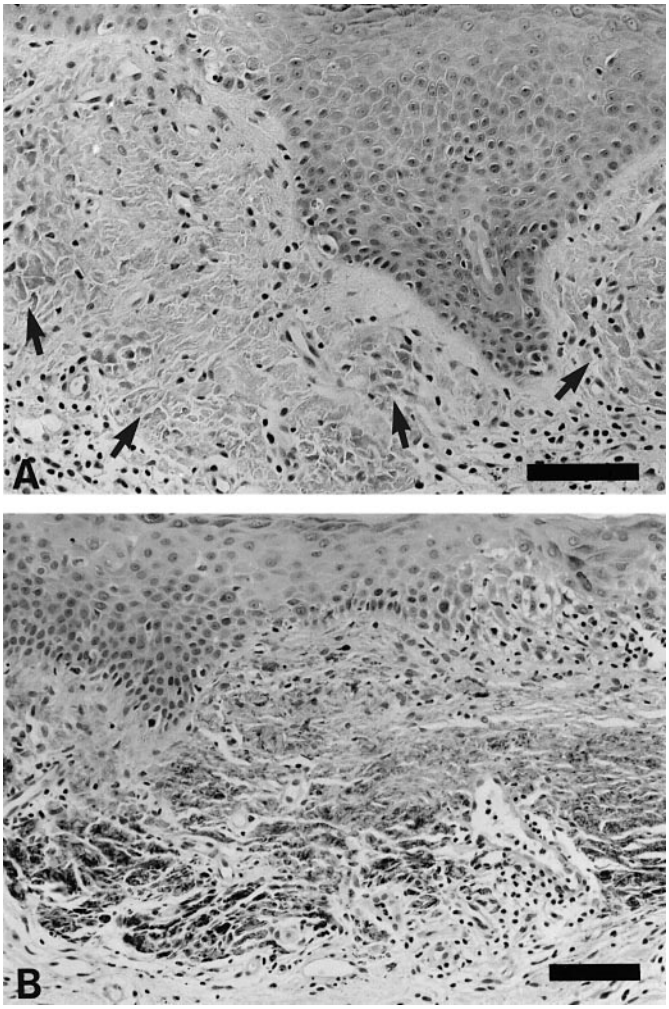

Figure 1. Lichen amyloidosus stained with Congo red (*A*) and anti-apoE serum (*B*). Congo red-positive amyloid deposits, indicated by *arrows*, react with anti-apoE anti-serum. *Scale bars*: 100 μm.

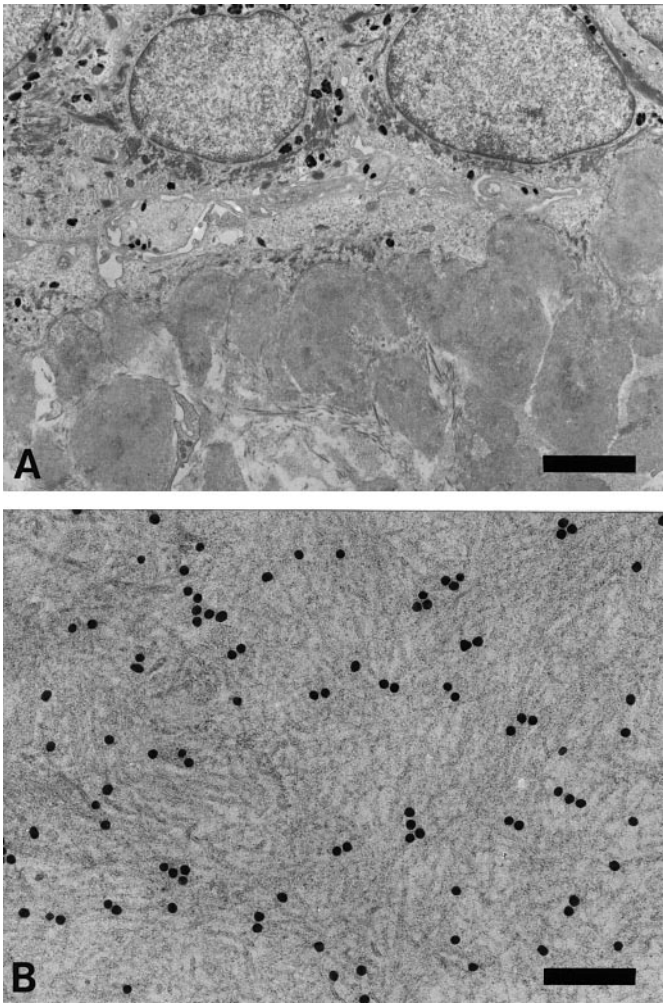

Figure 2. Electron microscopic findings of lichen amyloidosus. (*A*) At low magnification of conventional electron microscopy, amyloid deposits show nodular electron-dense individual islands in the upper dermis. (*B*) By postembedding immunogold electron microscopic procedure, 20 nm gold particles to anti-apoE anti-serurn label on amyloid deposits. *Scale bar*: (*A*) 3 μm; (*B*) 0.2 μm.

with 9.5 M of urea, the antibody reacted with higher molecular weight bands of cytokeratin, 54K in the amyloid positive case and 67K in the normal case (**Fig 3*B***).

DISCUSSION

In this study we demonstrated the presence of apoE in PLCA by three methods, immunohistochemistry, immunoelectron microscopy, and immunoblotting. In amyloid-positive skin extract, immunoblotting using polyclonal anti-apoE antibody demonstrated the existence of 35K and 14K proteins that were regarded as apoE and its fragment. The 35K protein band of normal skin sample is considered to be apoE naturally synthesized by the epidermal keratinocytes, and the intensity of the band was weaker than that of the amyloid-positive skin sample. In systemic amyloidosis, the small molecular weight (14K and 7K) bands, which corresponded to fragmented peptides of apoE, were detected by immunoblotting of AA fibril using monoclonal anti-apoE antibody (Yamada *et al*, 1994). The 14K band was detected in both PLCA and systemic amyloidosis but not in normal skin. The 14K fragmented peptide of apoE might play an important role in the pathogenesis of PLCA.

It has been suggested that, in immunogold electron microscopic preparations, the relevant antigenicity for trapped plasma protein is neither preserved by the tissue processing nor revealed by the etching procedure (Donini *et al*, 1989). Moreover, in the course of extraction of amyloid proteins, trapped plasma proteins were washed out during incubations in saline. Thus, it seems reasonable to assume that apoE and its product, which were detected in PLCA, were components of the amyloid.

ApoE is one of the amyloid-associated proteins that might play a key role in the etiology of Alzheimer's disease by functioning as a "pathologic chaperone" to promote the formation of amyloid filaments (Wisniewski and Frangione, 1992). Recent studies have implicated that apoE plays a role in the accumulation of proteins in the senile plaques and congophilic angiopathy of Alzheimer's disease (Strittmatter *et al*, 1993b). ApoE is also associated with all cerebral and systemic amyloids, apparently reflecting a more general affinity for the β-pleated sheet conformation characteristic of amyloid filaments (Ma *et al*, 1994); however, there have been no reports on the relationship between apoE and PLCA.

PLCA, lichen amyloidosus, or macular amyloidosis are characterized by the findings that the amyloid deposits are limited exclusively to the papillary dermis (Kumakiri *et al*, 1979). Hashimoto *et al* (1990) proposed the term amyloid-k disease for the skin-limited amyloidosis derived from keratin protein. In most skin-limited amyloidoses, "amyloid deposits" have been found using immunohistochemical techniques to contain keratin proteins or their derivatives. Some monoclonal anti-keratin antibodies reacted with amyloid deposits of the PLCA (Yoneda *et al*, 1989; Hashimoto *et al*, 1990). It is suggested that the amyloid substances come from a special degeneration of keratinocytes of the epidermis. In this study, immunoblots of amyloid extract using monoclonal anti-cytokeratin antibody showed the 45K protein band. Using the buffer containing 9.5 M urea, the anti-cytokeratin antibody reacted with 67K and 54K protein in the normal skin and amyloid-

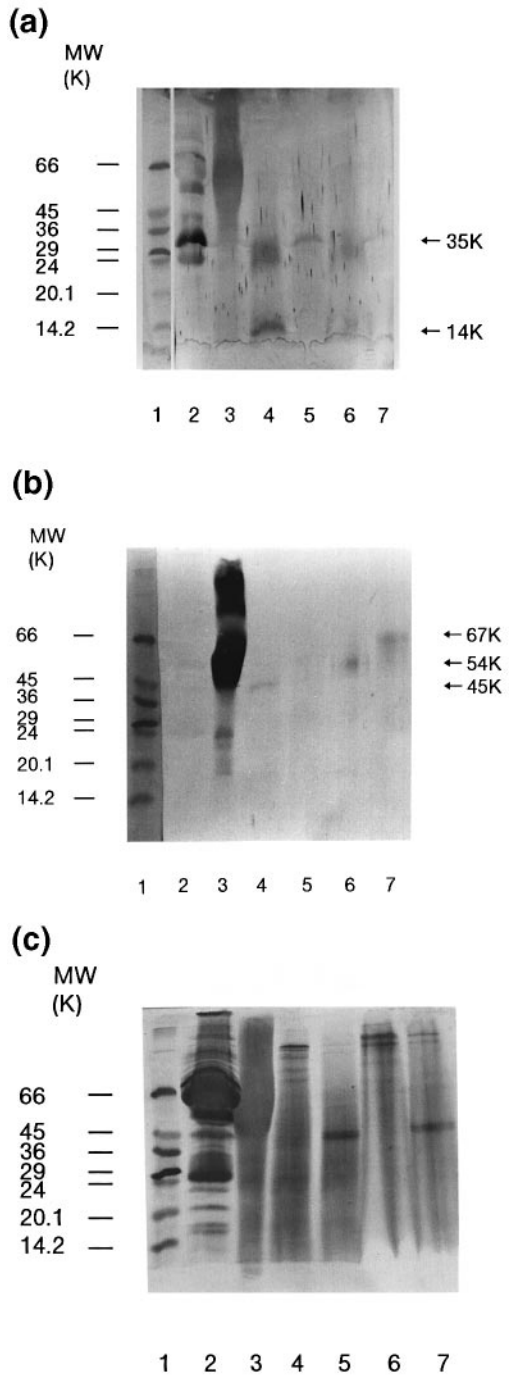

Figure 3. Separation of apoE and cytokeratin in skin extracts by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting. (*A*) Immunostaining using goat anti-human apoE antibody; (*B*) immunostaining using murine anti-cytokeratin antibody; (*C*) protein staining with Coomassie blue R-250. *Lane 1*, molecular weight standards; *lane 2*, normal human serum; *lane 3*, human keratin; *lane 4*, extracts of amyloid-positive skin; *lane 5*, extracts of normal skin; *lane 6*, extracts of amyloid-positive skin solubilized in buffer containing 9.5 M of urea; *lane 7*, extracts of normal skin solubilized in buffer containing 9.5 M of urea.

positive skin, respectively. Although the 45K and 54K proteins might be associated with amyloid deposits of PLCA, the significance for the formation of amyloid deposits in PLCA is uncertain.

In addition to the primary structure of amyloid precursor proteins, other elements, which confer a susceptibility to amyloid formation, may be important for the initiation, development, and persistence of amyloid deposits: proteoglycans, glycosaminoglycans (GAG), amyloid P component, apoE, vitronectin, and so on, most of which are normal constituents of basement membrane (Bellotti and Merlini, 1996; Dahlbäck *et al*, 1998). In order to clarify the role of apoE in the formation of amyloid deposits in PLCA, we have to investigate the binding of apoE to isolated keratin filaments as previously shown for serum amyloid P component and vitronectin (Hintner *et al*, 1988, 1989).

ApoE is synthesized by many organs, including the skin, and secreted into systemic circulation (Fenjves *et al*, 1989, 1994). ApoE in the amyloid deposits in PLCA might be derived from the epidermal keratinocytes. The localization of apoE in the normal skin has been revealed by immunohistochemical methods (Miyauchi, 1991). ApoE is observed intracellularly and also in the basement membrane of the dermo–epidermal junction. *In vitro*, apoE is secreted by basal cells in the culture of epidermal keratinocytes (Gordon *et al*, 1989; Barra *et al*, 1994). Moreover, in the animal model, apoE is physiologically secreted by epidermal keratinocytes and enters into systemic circulation (Fenjves *et al*, 1989, 1994). Thus, apoE secreted by the epidermal keratinocytes may play a part in the formation of amyloid filaments in the papillary dermis. Another amyloid-associated matrix protein, GAG, which is a constituent of almost all types of amyloid deposits, is also synthesized by the epidermal keratinocytes and is a composition of the basement membrane of the dermo–epidermal junction (Snow *et al*, 1987). There have been no reports on the localization of GAG in PLCA. In our cases, amyloid deposits were positively stained with sulfate alcian blue (data not shown), indicating the existence of GAG. ApoE has binding capacities to heparin sulfate (Mahley, 1988), which is thought to be a predominant composition of GAG in amyloid deposits (Norling *et al*, 1988). These data lead to a hypothesis that degenerating apoE, derived from apoptotic keratinocytes or basement membrane, may present in the amyloid deposits of PLCA in conjunction with other amyloid associated matrix proteins such as GAG.

ApoE is a polymorphic protein, and has the biologic functions related to the lipid metabolism. Three common alleles for apoE have been designated as $\varepsilon 4$, $\varepsilon 3$, and $\varepsilon 2$, and their gene products are E4, E3, and E2, respectively. Amino acid sequence analysis established that the two common variants of apoE, E4 and E2, differ from E3 by a single amino acid substitution. In E4, arginine is substituted for cysteine at residue 112, and in E2 cysteine for arginine at residue 158. The relationship between apoE2 and type III hyperlipoproteinemia has been reported (Mahley, 1988). Recently we have reported the association of apoE2 allele with psoriasis vulgaris in a Japanese population (Furumoto *et al*, 1997). On the other hand, apoE4 allele is genetically linked to the incidence of Alzheimer's disease (Poirier *et al*, 1993; Martinoli *et al*, 1995), and the amount of β-amyloid deposition in senile plaques and in the microvasculature of the patients with late-onset Alzheimer's disease, correlates with the E4 allele (Strittmatter *et al*, 1993a). Interestingly, apoE2 inhibits the onset of late-onset Alzheimer's disease (Corder *et al*, 1994). Further study will be required to document the phenotypic variation of apoE in cutaneous amyloidoses, including PLCA.

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