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Skin deposits in hereditary cystatin C amyloidosis

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Summary. Clinically normal skin from 47 individuals aged 9–70 years was investigated. Cystatin C amyloid deposits were found in various locations of the skin by light and/or electron microscopy, in all 12 patients with a clinical history of hereditary cystatin C amyloidosis (HCCA). Six asymptomatic individuals, who had the Alu 1 restriction fragment length polymorphism (RFLP) marker reported to cosegregate with the disease, also had cystatin C amyloid deposits in the skin. Three asymptomatic individuals (age 17–46) belonging to the HCCA families were without amyloid in the skin but had Alu 1 RFLP marker. Skin from 12 individuals who served as controls and skin from 14 close relatives of the patients was negative for amyloid. Punch biopsy of the skin is a simple procedure which is of value for the diagnosis of HCCA, even before the appearance of clinical symptoms. This method might also be of use in following progression of the disease.

Key words: Hereditary disease – Amyloidosis – Skin – Cysteine proteinase inhibitor – Immunohistochemistry

Introduction

Amyloid is composed of different proteins in different forms of the disease but all amyloid has a β-pleated structure and affinity for Congo red (congophilia) and when so stained gives green birefringence in polarized light. It has a characteristic fibrillar structure when viewed in the electron microscope (Glenner 1980).

Amyloid deposition in the skin is known to occur in primary systemic amyloidosis (Brownstein and Helwig 1970), secondary systemic amyloidosis (Lever and Schaumburg-Lever 1975), systemic amyloidosis with myeloma (Brownstein and Helwig 1970), primary localized cutaneous amyloidosis (Masu et al. 1981) and familial cutaneous amyloidosis (Wong 1987). Amyloid in the skin has also been described in association with some rare familial amyloid diseases (Brownstein and Helwig 1970), for example familial amyloidotic polyneuropathy, in which skin biopsy has been suggested as a reliable method of early diagnosis (Rubinow and Cohen 1981).

A hereditary cerebral haemorrhagic disease has been known to exist in Iceland since early this century (Árnason 1935). In 1972 amyloid deposits were found in the cerebral arteries of patients with this disease (Guómundsson et al. 1972) and amyloid fibrils were isolated from the leptomeninges of patients in 1983. A component protein was identified and sequenced, and was found to be a variant of the proteinase inhibitor cystatin C (gamma-trace). This variant has an amino acid substitution in position 68 of the normal cystatin C molecule and lacks the first ten amino-terminal residues (Ghiso et al. 1986). Later it was reported that patients with this disorder had an abnormally low concentration of cystatin C in their cerebrospinal fluid (CSF) and that this fact could be used for diagnosis of the disorder (Grubb et al. 1984). Immunohistochemical staining of the amyloid deposits, in the central nervous system and a lymph node, using a panel of antibodies including antibodies to known amyloid proteins showed only positive staining to cystatin C (Löfberg et al. 1987).

In 1988 DNA from patients with hereditary cystatin C amyloidosis was found to have an Alu 1 restriction fragment length polymorphism (RFLP) (Pálsdóttir et al. 1988). The loss of an Alu 1 restriction site is caused by a point mutation in the codon for leucin in position 68 of the normal protein sequence (Abrahamson 1988; Levy et al. 1989). In the cases examined the point mutation cosegregates with the disease (Pálsdóttir et al. 1988).

Amyloid deposits were previously believed to be confined to arteries in the central nervous system and the disease was therefore called hereditary cerebral haemorrhage with amyloidosis (Gudmundsson et al. 1972). Recently, however, amyloid has been found in various other organs including lymph nodes, spleen, adrenal

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gland, testis, ovary, appendix and peripheral nerves (Benedikz and Blöndal, unpublished observations). Therefore we believe the name hereditary cystatin C amyloidosis (HCCA) to be more appropriate for this condition (Blöndal et al. 1989).

In this paper we report amyloid deposits in the skin of 18 individuals with HCCA. The diagnostic potential of skin biopsies in this condition is discussed.

Materials and methods

Clinically normal skin from 47 people was investigated. The skin samples were obtained from 35 individuals, patients with HCCA and their close relatives (Table 1). Skin from 12 individuals, unrelated to the patients, served as controls (Table 2).

Skin was obtained at autopsy from 3 patients and 4 unrelated individuals without any known disease. Skin biopsies were taken from 32 patients and relatives, and from 8 unrelated individuals.

The skin biopsies were usually obtained from the upper arm using a 3-mm biopsy needle. In 2 cases skin samples were taken from the thigh, 1 from the abdomen, 1 from the back of the neck and one from the scalp. In a few cases, skin was obtained from more than one location from the same individual. Skin from controls was obtained from the breast in 4 cases, from the thigh in 2, from the chest in 2 and from the upper arm in 4.

The tissue specimens were fixed in formalin and/or Bouin's fluid and embedded in paraffin for light microscopic investigation. Sections were stained with haematoxylin and eosin (H&E) and Congo red and the Congo-stained sections viewed in polarized light. Paraffin sections were stained immunohistochemically using the avidin-biotin complex method (Hsu et al. 1981). Diaminobenzidine tetrahydrochloride was used to develop colour. A set of slides were pretreated with formic acid for 5 min to enhance the immunoreactivity (Kitamoto et al. 1987).

In most cases skin was also fixed in 2.5% glutaraldehyde and/or MacDowell's fluid for electron microscopy. For routine investigation specimens were post-fixed in osmium tetraoxide and embedded in Spurr resin. Specimens for immunogold labelling of cystatin C amyloid were not post-fixed and were embedded in L.R. White resin. Immunogold labelling using protein A-gold followed by incubation with 5% uranyl acetate for 15 min was used to visualize cystatin C in the electron microscope. The following antibodies were used: polyclonal rabbit antisera and monoclonal mouse antibodies against human cystatin C were a gift from Anders Grubb, Malmö, Sweden. Rabbit antibodies to human serum amyloid P component (SAP) and keratin were obtained from Dakopatts (Copenhagen, Denmark). Normal serum, secondary antibodies and

Table 1. Clinical and laboratory data on hereditary cystatin C amyloidosis (HCCA) patients and close relatives

Case	Sex	Age (years)	Symptoms at biopsy	Autopsy	Amyloid in skin	CSF cystatin C (mg/l)	Alu 1 RFLP marker
B 187	F	30	B	A	Yes	1.4	ND
B 287	F	29	B	A	Yes	1.8	ND
B 387	F	19	C	A	Yes	2.8	ND
B 487	F	21	D		Yes	2.0	ND
B 588	F	21	D		No	8.4	ND
B 688	F	22	D		No	7.6	ND
B 788	F	41	B		Yes	2.6	Yes
B 988	M	14	D		No	12.8	No
B 1188	F	28	C		Yes	2.1	Yes
B 1288	F	23	D		No	ND	No
B 1388	F	29	D		No	4.2	No
B 1488	F	24	C	A	Yes	4.6	ND
B 1588	F	39	D		No	ND	ND
B 1688	F	48	D		No	5.3	ND
B 1888	M	35	D		No	7.2	ND
B 2188	M	17	C		Yes	ND	Yes
B 2388	M	14	D		Yes	2.0	Yes
B 2488	M	45	D		No	5.6	No
B 2588	F	24	D		Yes	2.6	Yes
B 2688	M	23	D		No	7.6	ND
B 2788	F	38	D		No	ND	ND
B 2888	F	18	D		No	ND	Yes
B 289	F	14	D		No	ND	No
B 389	F	12	D		Yes	ND	Yes
B 489	M	9	D		No	ND	No
B 1589	F	28	D		No	2.5	Yes
B 1789	M	46	D		No	ND	Yes
B 2189	F	31	D		No	ND	No
B 190	M	19	C		Yes	ND	Yes
B 290	M	17	D		Yes	ND	Yes
B1390	M	24	D		Yes	ND	ND
B 9002	F	29	C	A	Yes	ND	ND
S 127	M	31	A	A	Yes	1.9	ND
S 258	M	30	A	A	Yes	1.8	ND
S 35	F	53	A	A	Yes	2.3	ND

A, Cause of death HCCA; B, suffered multiple haemorrhages and severely affected; C, suffered one or more haemorrhages and slightly affected; D, asymptomatic; ND, not done

Table 2. Clinical and laboratory data on control cases

Case	Sex	Age (years)	Illness or cause of death	Amyloid in skin
B 1088	M	19	Cerebral haemorrhage	No
B 2088	M	70	Cerebral haemorrhage[a]	No
B 2288	M	18	Cerebral haemorrhage	No
B 1989	M	39	Charcot-Marie-Tooth	No
H 288	F	65	Cancer	No
H 388	F	19	Cancer	No
H 488	F	34	Cancer	No
H 588	F	54	Cancer	No
H 688	F	19	Accident	No
H 788	M	25	Accident	No
H 888	M	27	Accident	No
H 988	F	27	Accident	No

[a] Cerebral amyloid angiopathy in the aged

the ABComplex kit were also obtained from Dakopatts. Colloidal gold labelled protein-A was obtained from Sigma (St. Louis, USA).

The level of cystatin C in CSF was estimated in 21 patients and close relatives by immunodiffusion (Löfberg and Grubb 1979).

Routine investigation for the HCCA DNA marker was performed as described (Pálsdóttir et al. 1988) on 20 cases.

Results

Investigation of the skin from the patients and their close relatives (35 individuals), revealed amyloid deposits in 18 cases. The diagnosis of HCCA was confirmed post mortem in 8 of 18 cases. The skin from the 12 controls showed no amyloid depositions of any kind. CSF from 13 of 18 patients was investigated and all except 1 had a low level (<3.0 mg/l) of cystatin C (Table 1). That individual had 4.6 mg/l of cystatin C in the CSF but the normal mean is 7.1 mg/l, range 4.0–13.6 mg/l (Löfberg et al. 1987). The disease was verified post mortem in this patient.

Routine investigation for the Alu 1 RFLP marker reported to cosegregate with the disease was done on 18 patients and their close relatives. Seven cases did not have the Alu 1 marker. Three who had the marker did not have any amyloid in skin biopsies, obtained on two different occasions. However, of 8 patients who were investigated out of the 18 with amyloid in the skin, all had the Alu 1 RFLP marker. Two control cases were also tested and did not have the RLFP marker.

By light microscopy no specific changes were seen in the H&E-stained sections. The green birefringence typical of Congo-red-stained amyloid viewed in polarized light was less obvious than the brown colour of the avidin-biotin complex. The birefringence of collagen sometimes made interpretation of the Congo-red-stained sections difficult. The immunohistochemically stained sections revealed smaller deposits of amyloid than could be detected by Congo red staining. Description of the distribution of amyloid in the skin of HCCA patients is therefore based mainly on the immunohistochemically stained sections.

Amyloid was never found in the epidermal layer. Amyloid deposits were always seen just below the epidermal-dermal junction and in the most severe cases the brown colour of the avidin-biotin complex formed a continuous ribbon below the epidermis (Fig. 1A).

Staining for cystatin C amyloid was frequently found in lymphatic vessels (Fig. 1B). It was also found in the connective tissue surrounding sebaceous glands and sweat glands, the reaction product sometimes completely enveloping these organs (Fig. 1C, D). Positive staining was also seen in the adventitia of arteries and around nerves, and in a few instances staining was found in the arterial wall (Fig. 1E) or within the nerve. In some cases the arrector pili muscles (Fig. 1F) and the connective tissue around hair follicles stained positively for amyloid. In the subcutaneous layer of the skin amyloid staining could be found around individual fat cells, forming so-called amyloid rings, as has been reported by others (Rubinow and Cohen 1978). The amyloid depositions in the skin did not seem to vary from different locations of the body.

Paraffin sections, from the skin of a few patients, were stained immunohistochemically for keratin, as such staining has been reported in amyloid deposits of localized cutaneous amyloidosis (Masu et al. 1981). Positive staining of the amyloid found in our cases was not seen. Sections from 6 patients were stained with anti-SAP and staining was found in various locations in the skin as reported by others (Breathnach et al. 1981). In 5 of the 6 cases, the staining was strong in the dermis immediately below the epidermis, where the anti-cystatin C staining was found to be most consistent.

When normal rabbit serum was substituted for the primary antibodies, no staining was seen. Inflammatory cells were not seen in association with the amyloid deposits.

All biopsy specimens, except 2, were examined with the electron microscope and fibrils typical of amyloid were found in 13 cases, supporting the light microscopic findings. The 5 remaining cases positive in light microscopic examination, 3 autopsy cases and 2 biopsy cases, were not examined with the electron microscope. The amyloid fibrils were always found below the epidermal-dermal junction (Fig. 2A, B) and immunogold labelling showed anti-cystatin C antibodies bound specifically to the amyloid fibrils, indicating that cystatin C is a constituent of the fibrils (Fig. 2C).

Discussion

Cystatin-C-positive amyloid was first found outside the central nervous system in the arteries of a submandibular lymph node from one patient (Löfberg et al. 1987).

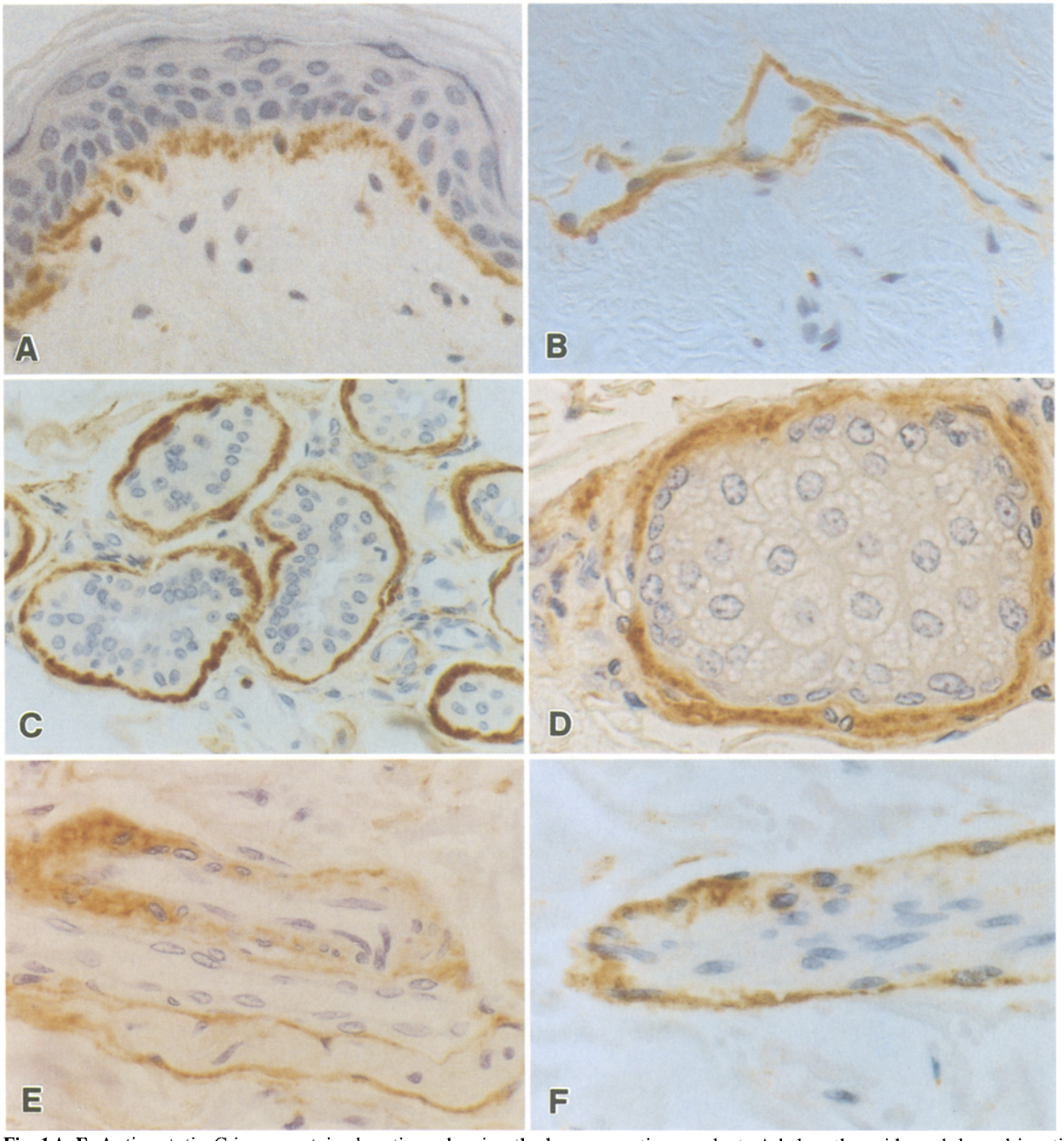

Fig. 1A–F. Anti-cystatin C immunostained sections showing the brown reaction product: **A** below the epidermal-dermal junction ×300; **B** in a lymphatic vessel, ×550; **C** Surrounding sweat glands, ×325; **D** enveloping a sebaceous gland, ×540; **E** in a dermal artery and nerve, ×435; **F** in an arrector pili muscle, ×640

A thorough search for cystatin C amyloid later showed that amyloid was deposited in other lymph nodes and in other organs, including the skin (Blöndal et al. 1989).

This disease has been categorized as a "hereditary localized amyloidosis" (Pepys 1988). In view of new information, the classification of this disease needs to be updated and in the Pepys classification scheme this disease should now be placed with the "hereditary systemic amyloidosis", as a predominantly central nervous system form. Other rare hereditary systemic amyloidoses are predominantly neuropathic, nephropathic or cardiomyopathic forms.

Amyloid deposits in the skin of patients with HCCA could always be demonstrated below the epidermal-dermal junction and often around hair follicles and sebaceous glands. A similar distribution of amyloid has been reported for primary systemic amyloidosis, primary secondary amyloidosis and systemic amyloidosis with myeloma (Rubinow and Cohen 1978) but without special emphasis on those locations. Amyloid deposits in the

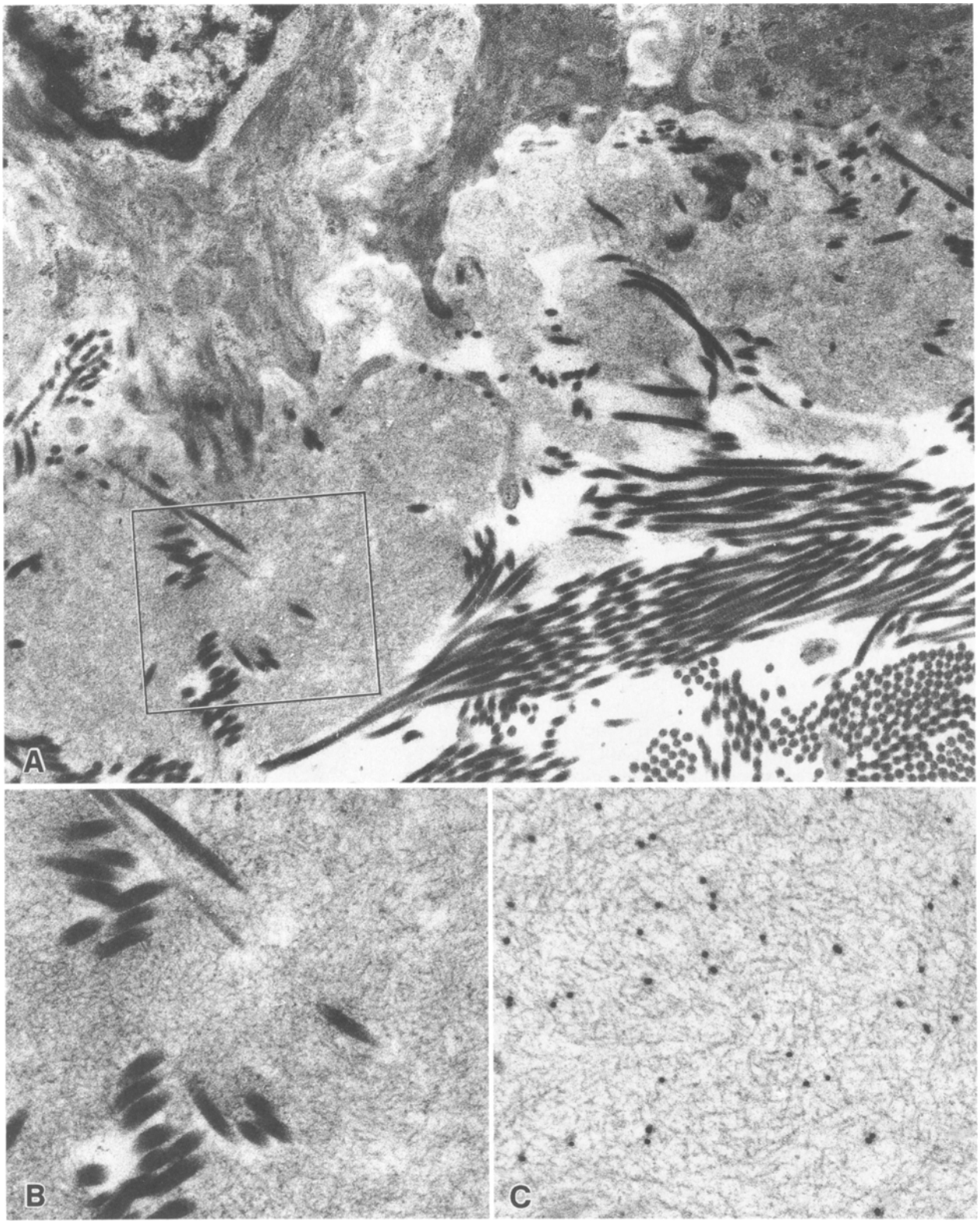

Fig. 2. **A** Electron micrograph showing amyloid fibrils below the epidermal-dermal junction, ×18750. **B** Enlargement from **A**, ×40010. **C** Anti-cystatin C immunogold labelling of amyloid fibrils in the skin, ×56780

skin have been described in patients with familial amyloidotic polyneuropathy. In those patients, amyloid was not found below the epidermal-dermal junction, but small foci were found elsewhere in the dermis. However, amyloid deposits were always seen around sweat glands (Rubinow and Cohen 1981), which was a common finding in our cases. In other primary amyloidoses and in secondary amyloidosis, amyloid around sweat glands

has been reported, though it is apparently not as common (Rubinow and Cohen 1978; Brownstein and Helwig 1970). Amyloid deposits in arterial walls in the skin have been reported in association with all types of amyloidoses, where the skin is involved, and is considered, by some, to be the most common location for amyloid in the skin. In our investigation amyloid deposits were sometimes seen in the walls of dermal blood vessels. Anti-cystatin C staining was frequently seen in lymphatic vessels. To our knowledge, amyloid deposits have not been reported there previously.

It is interesting to note that Rubinow and Cohen (1981) emphasized that amyloid was not found in dermal nerves of their cases of familial amyloidotic polyneuropathy. In this investigation it was not uncommon to find cystatin C amyloid around dermal nerves in the more advanced cases. As in other amyloidoses, amyloid could be found around the arrector pili muscles and individual fat cells.

The distribution of the amyloid deposits found in the skin of HCCA patients is similar to that reported in other amyloid diseases, where the skin is affected but the emphasis is different. This mainly applies to the epidermal-dermal junction and the lymphatic vessels. The fundamental difference between HCCA and other amyloid diseases with skin involvement is that HCCA is the only one known to have cystatin-C-derived amyloid deposits.

Among those we investigated were 6 individuals (12, 14, 17, 21 and two 24 years of age) who had the Alu 1 marker and cystatin C amyloid in the skin but no symptoms or clinical history of the disease. At the present time it is not known how early amyloid deposits can be detected in the skin of affected individuals. It is apparent from this investigation, though attempts at quantification were not made, that the amyloid deposits were most prominent in the skin of those individuals who had the longest history of the disease.

The glycoprotein amyloid P component, which is a normal constituent of most tissues, including skin, is also found in all types of amyloid (Khan and Walker 1984). Amyloid P component in normal skin seems to have a similar distribution to the cystatin C amyloid deposits. It has been suggested that the amyloid P component may be of importance in the pathogenesis of amyloidoses. It is a normal constituent of elastic fibres (Breathnach et al. 1981) and has been shown to have a specific binding affinity for isolated amyloid fibrils. It has been suggested that the P component could act as a framework for the deposition of amyloid fibrils in vivo in association with normal elastic fibre microfibrils (Breathnach 1985). It has also been suggested that amyloid P component may protect amyloid fibrils from digestion and thus lead to the accumulation of amyloid (Breathnach 1985; Coria et al. 1988). These ideas are supported by in vitro investigations.

Three individuals belonging to the HCCA families were without amyloid in the skin but had the Alu 1 RFLP marker. This discrepancy is difficult to interpret. The 3 individuals in question were 17, 27 and 46 years of age (B-2888, B-1589 and B-1789; case B2888 is the daughter of case B-1789). All 3 are asymptomatic, which must be considered unusual for case B-1789 (age 46) as the average age at onset is 25.2 years and the average age at death is 33.1 years. If the Alu 1 RFLP marker is considered a reliable disease marker, this discrepancy could possibly be explained by different progression rates of the disease in affected individuals. Amyloid deposition in the skin could thus be used to diagnose the disease and evaluate its progression. All individuals with clinical symptoms of the disease had amyloid in the skin along with 6 asymptomatic individuals. We suggest that in individuals with the Alu 1 marker and no amyloid depostits in the skin, the disease has not progressed far and we hope that our continuing research will clarify this matter.

At the present time skin biopsy is the only convenient method of demonstrating cystatin C amyloid deposits in the tissues of patients with HCCA directly and we consider that punch biopsy of the skin is an easy, rapid and reliable method for diagosis of HCCA.

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