Sulfhydryl and Disulfide Stainings in Amyloids of Skin-Limited and Systemic Amyloidoses

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Disulfide (S-S) bonds and sulfhydryl (—SH) groups in skin-limited and systemic amyloidoses in frozen and paraffin-embedded sections were examined with a thiol reagent, N-(7-dimethylamino-4-methyl-3-coumarinyl)-maleimide (DACM). In frozen sections, dermal amyloids of skin-limited amyloidoses contained a large number of S-S bonds but no —SH groups [macular amyloidosis (9 cases), lichen amyloidosis (4)], and skin tumor-associated (seborrhic keratosis) amyloidosis (1)]. In contrast, amyloids of systemic amyloidoses contained no S-S bonds or —SH groups [primary and myeloma-associated amyloidosis (1 each)]. The identical results were obtained from paraffin-embedded sections in skin-limited amyloidoses [macular (31), biphasic (4), lichenoid (9) and skin tumor-associated Bowen’s disease (3), seborrhic keratosis (2), solar keratosis (2), porokeratosis Mibelli (1), and basal cell epithelioma (1) amyloidoses]. Systemic amyloidoses [primary (3), myeloma-associated (2), and secondary (2) amyloidoses] and tumefactive amyloidoses of the tongue (2).

Furthermore, amyloid-like deposits confirmed by various histochemical stainings were found in the epidermis in 27/67 cases of skin-limited amyloidoses in both frozen and paraffin sections. These intraepidermal amyloid-like deposits contained S-S bonds in all cases (27/27) and —SH groups in 10 of 27 cases. In contrast, an intraepidermal amyloid-like deposit was not observed in any systemic amyloidoses (0/9) or tumefactive amyloidoses of the tongue (2).

These results showed that skin-limited amyloidoses could be differentiated from systemic amyloidoses by DACM methods (this appears to depend upon the differences of amino acid composition between skin-limited and systemic amyloidoses) and that paraffin-embedded sections were usable for DACM methods. Present study further suggests that amyloids in skin-limited amyloidoses are, at least in part, derived from epidermal keratinocytes.

Ogawa et al. [1] examined sulfhydryl (—SH) groups and disulfide (S-S) bonds in human epidermis, using histochemical techniques with a fluorescent thiol reagent, N-(7-dimethylamino-4-methyl-3-coumarinyl)-maleimide (DACM) and they found that DACM staining methods were very sensitive and highly specific for the detection of —SH groups in various tissues. It has been suggested that amyloids of skin-limited amyloidoses are derived from epidermal keratinocytes by morphologic [2,3], histochemical [4], and immunologic studies [5, 6]. If amyloids of skin-limited amyloidoses are derived from epidermal keratinocytes, they may be rich in S-S bonds in contrast to amyloids of systemic amyloidoses which contain few or no S-S bonds as shown by amino acid analysis or X-ray diffraction [7–10].

We investigated these S-S bonds and —SH groups in various skin-limited and systemic amyloidoses by DACM methods, and report the differences in contents of S-S bonds between skin-limited and systemic amyloidoses.

MATERIALS AND METHODS

Tissue Specimens

Biopsy sections were obtained from typical lesions of various amyloidoses. The tissue samples were divided into 3 pieces. One part was immediately frozen in acetone-dry ice mixture and stored at −80°C until used for DACM stainings, and the second part was observed after staining with hematoxylin and eosin (H&E), alcaine Congo-red, Thioflavin-T, periodic acid-Schiff (PAS), and Dylon [11]. The third part was used for the electron microscope studies. Tissues were fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) and processed thereafter for electron microscopy as described previously [12]. Thin sections were stained with uranyl acetate and lead citrate [13] and observed in a Hitachi HU-12A electron microscope at 75 kV.

Frozen sections: Sixteen patients with amyloidoses were examined: macular amyloidosis (9 cases), lichen amyloidosis (4), skin tumor-associated (seborrhic keratosis) amyloidosis and primary systemic (1), and myeloma-associated (1) amyloidoses.

Paraffin-embedded sections: Sixty-two patients with amyloidoses were examined: macular amyloidosis (31 cases), biphasic macular and lichenoid amyloidosis (4), lichen amyloidosis (9), skin tumor-associated [Bowen’s disease (3), seborrhic keratosis (2), solar keratosis (2), porokeratosis Mibelli (1), and basal cell epithelioma (1)] amyloidoses, primary systemic amyloidosis (3), myeloma-associated amyloidosis (2), secondary systemic amyloidoses due to pulmonary and renal tuberculosis (1) and rheumatoid arthritis (1), and tumefactive amyloidosis of tongue (2). In these patients with tumefactive amyloidoses, systemic workups did not reveal any involvement of other organ systems or clinical or laboratory signs of primary and secondary amyloidoses including Bence-Jones proteins except for polyclonal hyperimmunoglobulinemia (M type). These paraffin-embedded specimens did not include any cases of amyloidoses examined in frozen sections. In addition, 10 patients of various skin diseases without amyloid deposition or dyskeratotic cells were examined; these were prurigo nodularis (4 cases), lichen simplex chronicus (2), seborrhic dermatitis (2), and psoriasis vulgaris (2).

DACM Stainings

Sulfhydryl groups and S-S bonds in frozen sections, which were sliced to a thickness of 2 μm in a cryostat, were stained as described by Ogawa et al. [1] and Taneda et al. [14]. Briefly, for detecting —SH groups, a section was stained with 0.01 mM DACM (Teikoku Seiyaku Co., Ltd., Toyama, Japan) solution for 3 min. For detecting S-S bonds,
-SH groups were first blocked by 0.15 mM N-ethenemaleimide for 3 min, then S-S bonds were reduced to -SH groups using 40 mM dithiothreitol and stained with DACM. Observation was made by a fluorescence microscope with an excitation filter (V-V filter) which excites 400 nm and a barrier filter which eliminates wavelengths below 460 nm. Exposure time was 3 s in our optical system (Nikon fluorescent microscope).

Paraffin sections, sliced to a thickness of 3 μm, were stained with DACM as described in frozen sections above except for a 5-fold longer reaction time. Exposure time was 1 s.

RESULTS

Light and Electron Microscope Findings

Amyloid masses and fibrils in papillary dermis or lamina propria mucosae were observed in all sections of skin-limited and systemic amyloidoses and tumefactive amyloidoses of the tongue by H&E, alkaline Congo-red (Figs 1, 2), PAS, Thioflavin-T, and Dylon stains, and by electron microscopy (Figs 3, 4). Furthermore, amyloid-like deposits (Fig 5) confirmed by various histochemical stainings (Thioflavin-T, PAS, alkaline Congo-red, and Dylon) were observed in the epidermis in skin-limited amyloidoses (27/67 cases) but not in systemic (0/9) or tumefactive (0/2) amyloidoses. Amyloid deposits of tumefactive amyloidoses were observed in and around walls of blood vessels, not beneath the lingual epithelium. This deposit pattern was similar to those found in systemic amyloidoses.


FIG 3. Electron micrograph of macular amyloidosis shows amyloid fibrils. Bar = 0.1 μm.

FIG 4. Electron micrograph of myeloma-associated amyloidosis shows amyloid fibrils, which are identical to those shown in Fig 3. Bar = 0.1 μm.

FIG 5. Macular amyloidosis of skin shows amyloid-like deposits (arrowheads) in the epidermis. Thioflavin-T stain, × 520.
DACM Stainings

Frozen sections: The results of DACM stainings in frozen sections are summarized in Table I. Sulphydryl groups were not detected at all in either skin-limited (0/14 cases) or systemic amyloidoses (0/2) (Figs 6, 7). Disulfide bonds (Fig 8) were shown to be rich in dermal amyloids in all cases of skin-limited amyloidoses (14/14). In contrast, S-S bonds were not detected in amyloids of primary or myeloma-associated amyloidoses (0/2) (Fig 9). Furthermore, intraepidermal amyloid-like deposits were observed in skin-limited amyloidoses (6/14) and these contained S-S bonds in all cases (6/6) and −SH groups in 2 of 6 cases (Figs 6, 8). In contrast, intraepidermal amyloid-like deposit was not observed in systemic amyloidoses (0/2) (Figs 7, 9).

Paraffin-embedded sections: The results of DACM stainings in paraffin-embedded sections are summarized in Table II. These obtained from paraffin sections were identical to those from frozen sections, i.e., −SH groups were not detected in amyloids of skin-limited (0/53), systemic amyloidoses (0/7), and tumeafactive amyloidoses of the tongue (0/2), and S-S bonds were observed in all cases of dermal amyloids of skin-limited amyloidoses (53/53) (Fig 10), but not in systemic amyloidoses (0/7) or tumeafactive amyloidoses (0/2). In addition, intraepidermal amyloid-like deposits were observed in skin-limited amyloidoses (21/53) and these contained S-S bonds in all cases (21/21) (Fig 10) and −SH groups in 8 of 21 cases. In contrast, intraepidermal amyloid-like deposit was not observed in systemic amyloidoses (0/7) or tumeafactive amyloidoses of the tongue (0/2).

Table 1. Results of DACM stainings in frozen sections

<table>
<thead>
<tr>
<th></th>
<th>−SH staining</th>
<th>S-S staining</th>
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<tbody>
<tr>
<td></td>
<td>Intraepi</td>
<td>Dermal</td>
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<tr>
<td>Skin-limited amyloidosis (Primary)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Macular</td>
<td>1/9</td>
<td>0/9</td>
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<tr>
<td>Lichen</td>
<td>0/4</td>
<td>0/4</td>
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<tr>
<td>(Secondary)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Seborrheic keratosis</td>
<td>1/1</td>
<td>0/1</td>
</tr>
<tr>
<td>Systemic amyloidosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary(1) and myeloma-associated(1)</td>
<td>0/2</td>
<td>0/2</td>
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* Intraepidermal amyloid-like deposits.
† Dermal amyloids.
‡ Positive cases.
§ Total cases.

Fig. 7. Sulphydryl staining in myeloma-associated amyloidosis. No fluorescence is detected in amyloid deposits around walls of blood vessels (arrowheads) of lamina propria mucosae. Fluorescence is seen in cytoplasm of mucous epithelium (ME). × 260.

Fig. 8. Disulfide staining in macular amyloidosis. Fluorescence is seen in amyloid masses of papillary dermis and intraepidermal amyloid-like deposits (arrowhead) as well as in the horny cells (H). Fluorescence of S-S positive amyloids was observed to be solid ovoid masses comparable to those shown in Fig 1. × 520.

Fig. 9. Disulfide staining in myeloma-associated amyloidosis. No fluorescence is detected in amyloid deposits around walls of blood vessels (arrowheads) of lamina propria mucosae. × 260.

Fig. 6. Sulphydryl staining in macular amyloidosis. Stronger fluorescence is seen in amyloid-like deposits (arrows) in epidermis (E) as compared to the cytoplasm of keratinocytes. No fluorescence is detected in amyloid masses in papillary dermis. × 520.
**TABLE II. Results of DACM stainings in paraffin sections**

<table>
<thead>
<tr>
<th>Skin-limited amyloidosis</th>
<th>~SH staining</th>
<th>S-S staining</th>
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<tr>
<td>(Primary)</td>
<td></td>
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<tr>
<td>Macular</td>
<td>2/31(d)</td>
<td>0/31</td>
</tr>
<tr>
<td>Biphasic</td>
<td>0/4</td>
<td>0/4</td>
</tr>
<tr>
<td>Lichen</td>
<td>1/9</td>
<td>0/9</td>
</tr>
<tr>
<td>(Secondary)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bowen’s disease(3), seb-</td>
<td>5/9</td>
<td>0/9</td>
</tr>
<tr>
<td>borheic keratosis(2),</td>
<td></td>
<td></td>
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<tr>
<td>solar keratosis(2),</td>
<td></td>
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</tr>
<tr>
<td>basal cell epithelioma(1),</td>
<td>5/9</td>
<td>9/9</td>
</tr>
<tr>
<td>porokeratosis</td>
<td></td>
<td></td>
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<tr>
<td>Mibelli(1)</td>
<td></td>
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<tr>
<td>Tumefactive amyloidosis</td>
<td>0/2</td>
<td>0/2</td>
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<tr>
<td>of tongue</td>
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<tr>
<td>Systemic amyloidosis</td>
<td>0/7</td>
<td>0/7</td>
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<tr>
<td>Primary(3), myeloma-ass</td>
<td>0/7</td>
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<td>ary(2)</td>
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<td>Control groups</td>
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\(a\) Intraepidermal amyloid-like deposits.
\(b\) Dermal amyloid.
\(c\) Positive cases.
\(d\) Total cases.

In the current study, a preliminary result indicated that AA amyloids of secondary amyloidoses are S-S bonds and −SH groups negative in the skin; this seems to make DACM staining more specific for skin-limited amyloidoses.

We have clearly demonstrated S-S bond-positive intraepidermal masses (dyskeratotic cells) together with dermal amyloid deposits in skin-limited amyloids. These intraepidermal dyskeratotic cells probably correspond to cytid bodies undergoing filamentous degeneration [2]. In the epidermis, S-S bonds are mainly present in the terminally differentiated horny cells, whereas −SH groups are present in viable keratinocytes. Damaged or injured keratinocytes such as sunburn cells rapidly undergo premature keratinization and become S-S positive. Positive staining of dermal amyloids in skin-limited amyloids for S-S bonds and negative staining for −SH groups may indicate that the epidermal source of amyloid in these conditions is prematurely keratinized cells and not normal keratinocytes. It is not known what type of damage causes filamentous degeneration of epidermal keratinocytes in the lesion.

**REFERENCES**

2. Kumakiri M, Hashimoto K: Histogenesis of primary localized cutaneous amyloidosis: sequential change of epidermal keratin-