Electron Microscopic, Histochemical and Disc Gel Electrophoretic Studies on the Deoxycholate Soluble Proteins from Human Plantar Horny Layers

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TEZUKA, T. Electron Microscopic, Histochemical and Disc Gel Electrophoretic Studies on the Deoxycholate Soluble Proteins from Human Plantar Horny Layers. Tohoku J. exp. Med., 1976, 118 (3), 209-221 — The matrix proteins of human plantar horny layers were extracted with deoxycholate, purified and examined with the light and electron microscopes, and their histochemical characteristics were investigated. About 44.3% of the total proteins was released into the 270,000 × g supernatant fraction, following incubation with deoxycholate, and the precipitate obtained by dialysing this supernatant fraction consisted of fine granular materials which were found to be electron dense, round material under the electronmicroscopy. Small electron dense dots were seen on the surface of this material. This pellet was positive with Pauly’s reagent, toluidine blue and Harris hematoxylin-eosin. However, the material further purified by the molecular sieve chromatography was negative with Pauly’s reagent. And it had been proved to be of keratohyalin granules origin by the indirect immunofluorescent study. Another protein fraction which was obtained by the molecular sieve chromatography was positive with diazotized sulfanilic acid and showed tinctorial properties as keratohyalin granules show in vivo. —— matrix protein; keratohyalin granule; Pauly’s reaction positive proteins

It would be valuable to examine biochemically keratohyalin granules of abnormal horny cells in order to elucidate the nature of keratohyalin granules in disorders of keratinization such as ichthyosis. Since it is almost impossible to isolate a sufficient amount of keratohyalin granules for the biochemical examination from the skin of such patient, little is known about keratohyalin granules in such skin diseases.

In horny layers, the materials of keratohyalin origin are thought by Mercer (1961) and Brody (1960) to exist in two forms. Mercer interpreted that keratohyalin is added to tonofibrils and actually becomes involved in these morphological changes occurring in tonofibrils which result in the unstained filaments observed in cornified cells and Brody thought that keratohyalin remains as the interfilamentous matrix in cornified cells.

Radioautographic studies have shown that ³H-labeled amino acids are incorporated in keratohyalin granules for a period of one to six hours after injection.
and later they are detected in the cornified layer (Fukuyama et al. 1965; Fukuyama and Epstein 1966, 1967). In addition to these radioautographic observations, with 0.1 N NaOH keratohyalin granules and the electron dense background substances of the horny cells were extracted from glutaraldehyde fixed epidermis (Fukuyama et al. 1968).

When newborn rat epidermis was extracted with sodium deoxycholate, keratohyalin granules were extracted; the electron dense material disappeared and the fibrous structure appeared in the lowermost layers of stratum corneum under electronmicroscopy (Tezuka and Freedberg 1972). These observations suggest that keratohyalin granules, actively synthesized in granular cells, move into the horny layer, and both keratohyalin granules in the granular cell and the electron dense material in the horny cell can be removed with 0.1 N NaOH or sodium deoxycholate.

Since it was proved by indirect immunofluorescent study that the purified, deoxycholate extractable material from human plantar horny layers was of keratohyalin granules origin (Tezuka 1975), the histochemical and electronmicroscopic characteristics of the deoxycholate extractable materials were studied in this paper.

**MATERIALS AND METHODS**

**Materials.** Human plantar skins were obtained from three patients who had been operated on for osteosarcoma. Sodium deoxycholate was purchased from Difco Laboratories, Detroit, Mich.; Sephadex G-200 and Sepharose 6 B from Pharmacia Fine Chemicals Inc., Fair Lawn, N.J.; glass homogenizers were purchased from Kontes Glass Company, Vineland, N.J.; hematoxylin and toluidine blue from Merck Company, Darmstadt, and sulfanilic acid from Daiichi Kagaku Yakuhin Inc. All other chemicals were of reagent or analytic grade obtained from various commercial sources.

**Preparation and extraction of tissue.** Human sole skin was removed immediately after surgery and was cut into thin strips (approx. 7 cm × 1 cm) after dissection of subcutaneous tissue. The strip was placed on a stretching apparatus under moderate tension and was sliced carefully with a razor blade to obtain sheets consisting of only horny layers. Horny layers were minced and homogenized in either 50 mM Tris-HCl buffer, pH 8.6, containing 3% deoxycholate or redistilled water, and stirred for 48 hr at 4°C. The extraction procedure was repeated three times, changing the extracting solution every 48 hr. The homogenate was centrifuged at 15,000 ×g for 10 min (Tominaga refrigerated automatic centrifuge) and the resulting deoxycholate supernatant fraction was recentrifuged at 270,000 ×g for 60 min (Beckman-Spinco ultracentrifuge Model L-2–65). The lipid layer was discarded and the high speed supernatant fraction was dialysed against 10 mM Tris-HCl (pH 8.8) for 24 hr at 4°C, concentrated by lyophilization and stored at -80°C until further use. Protein content was assayed according to the method of Lowry et al. (1951).

**Microscopic studies.** Samples of tissue during the fractionation procedures and the aggregates obtained by dialysis of the supernatant fraction of the second centrifugation were fixed in buffered formalin for light microscopic studies, and in 2.5% buffered glutaraldehyde, and postfixed with osmium tetroxide for electron microscopy. Prior to fixation, deoxycholate was removed from specimens by a wash in 50 mM Tris-HCl buffer (pH 8.6). Tissues for electron microscopy were fixed for 1 hr, dehydrated, embedded in Epon and cut on a Porter Blum MT–2 ultratome (Ivan Sorvall Inc.). Thin sections were stained with 1% uranyl acetate, counterstained with lead and examined in a Hitachi 11 electron microscope. One drop of the dialysate suspension was placed on a grid.
The Matrix Protein

which was previously coated with carbon, dried and stained with 3.5% uranyl acetate.

_Histochemical studies._ 1 ml of the concentrated supernatant fraction of the second centrifugation and the material of the second peak from the Sepharose 6B column in the presence of DOC were dialysed against two liters of redistilled water at 4°C for 72 hr, changing redistilled water every 24 hr. The fractions from 29 to 40 in tube number, which were eluted from the Sephadex G-200 column, were dialysed against redistilled water for 48 hr and centrifuged at 15,000 × g for 10 min. The supernatant fraction was collected and lyophilized. This concentrated fraction was dissolved in 1 ml of redistilled water, then mixed with 9 ml of 95% ethanol and kept in iced-cold water for 48 hr. Precipitates were harvested by the centrifugation at 700 × g for 10 min, and used for the histochemical examination. The pellets and normal human plantar skin were sectioned in a cryostat at 6 nm and fixed in 95% ethanol for 5 min. They were stained with toluidine blue (Kramer and Windrum 1965), diazotized sulfanilic acid (Reaven and Cox 1963) and Harris hematoxylin-eosin. Counter stains were not employed in any of the histochemical techniques.

_Gel filtration studies._ The macroaggregates, which were obtained by dialysing the supernatant fraction of the second centrifugation were harvested by the centrifugation and dissolved in 50 mM Tris-HCl (pH 8.6) so as to make up 3% deoxycholate solution, and were subjected to gel filtration on a column (2.5 cm × 34 cm) of Sephadex G-200. The profile was monitored at 280 nm during elution with 50 mM Tris-HCl (pH 8.6) containing 10 mM 2-mercaptoethanol and the first peak which was eluted immediately after the void volume was further fractionated on a Sepharose 6B column (2.5 cm × 34 cm) using 50 mM Tris-HCl (pH 8.6) containing 0.5% deoxycholate.

_Gel Electrophoresis._ Various fractions were subjected to disc gel electrophoresis in the presence of sodium dodecylsulfate using the technique of Weber and Osborn (1969).

RESULTS AND DISCUSSION

_Tissue source and separation techniques._ The plantar skin consists of very thick stratum corneum, three layers of stratum granulosum, several layers of spinous cells, a single basal cell layer and the dermis (Fig. 1-A). Fig. 1-B represents horny layers which were separated by the stretch method. Fig. 1-C shows that after the procedure, the residual skin has a thick horny layer. This separation technique was used in order to get a pure horny cell preparation without any contamination of granular or spinous cells. Adult human plantar skin is an appropriate material for obtaining the horny layers as its horny layer is usually so thick (around 3 mm thick) that a sufficient amount of pure horny cells can be obtained.

_ Extraction of the deoxycholate soluble materials._ When horny cells separated were homogenized and incubated in deoxycholate as described above, the protein components could be extracted. As is shown in Table 1, about 44.3% of the total protein was released into the supernatant fraction, following incubation with deoxycholate, but only 19.4% of the total protein was extracted with redistilled water. Only several protein bands, which were of small molecular size, were found to be present in this redistilled water extractable fraction, as compared with the electrophoretic profile of the extract with DOC in the disc gel electrophoresis in the presence of SDS (Fig. 2-A).

The electron micrographs of sections from control tissue and specimens from the pellet of the first centrifugation following three successive extraction for 48 hr
Fig. 1. Histologic and electron microscopic pictures of human plantar skin. A-C: Tissue from adult human sole skin was fixed in buffered formalin, embedded in paraffin, cut and stained with hematoxylin eosin. A represents tissue prior to the treatment; B is the horny layer separated from the skin with a razor blade under a moderate tension; and C is the residual skin. A, B and C; × 50, D and G represent the insoluble material which sedimented at 15,000 × g following three times 48 hr extraction in deoxycholate (D) and redistilled water (G). E is the 270,000 × g pellet and F represents control tissue following a separation with a razor blade. Arrow points out a electron dense tonofibril. d: desmosome, × 23,000 (D), × 26,000 (E), × 23,000 (F), × 23,000 (G). Reduced to 6/7.
TABLE 1. Extraction of the matrix protein either with 3% deoxycholate or redistilled water at 4°C. These numbers are the average of the duplicate experiments. About 44.3% of the protein was extracted into the 270,000 × g supernatant fraction with deoxycholate.

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<th>DOC-Tris buffer*</th>
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<tr>
<td></td>
<td>ng of protein</td>
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<tr>
<td>270,000 ×g supernatant</td>
<td>72,580</td>
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<td>fraction</td>
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<td>270,000 ×g pellet</td>
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<td>15,000 ×g pellet</td>
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<td>8 M alkaline</td>
<td>27,340</td>
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<td>Urea soluble</td>
<td>18,750</td>
<td>11.5</td>
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<td>1 N NaOH soluble</td>
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* 50 mM Tris-HCl buffer, pH 8.6, containing 3% DOC.

Fig. 2. Acrylamide gel electrophoretic profiles of the extracts from human plantar horny layers. The 270,000 × g supernatant fractions, which were extracted with redistilled water (A) and with deoxycholate (B), were analysed in the presence of sodium deoxycholate using acrylamide gel of 10% as described in Results and Discussion. A: 160 ng, B: 180 ng.

Fig. 3. Aggregates were yielded by dialysing the 270,000 × g supernatant fraction against redistilled water for 24 hr (C), 48 hr (B) and 72 hr (A).
are shown in Fig. 1-F and D. The electron dense background disappeared and many electron dense fibrous structures became visible and they remained apparently unextracted as shown with arrow in Fig. 1-D. Fig. 2-E demonstrates that the pellet fraction from such high-speed centrifugation contains only debris of fibrous materials and desmosome-like structures. Fig. 1-G shows that the electron dense material is still remained and only partially the fibrous structures were revealed when redistilled water was used instead of Tris-deoxycholate buffer. By these electron microscopic findings, the materials forming the electron dense background of the horny cells (Fig. 1-F) were shown to have been removed into the supernatant fraction.

The precipitate was obtained by dialysing the supernatant fraction of the second centrifugation against redistilled water for 72 hr (Fig. 3-A) and these precipitates were shown to consist of small, round particles in the light micrograph (Fig. 4-A), and electron dense substances in various sizes and amorphous materials under the electron microscope (Fig. 4-B). An electron less dense particle was attached to the electron dense material indicating with arrow in Fig. 4-B. Thin section shows that the pellet consisted of fine granular materials which formed electron dense, round material. Small electron dense dots were present on the surface of this round material (Fig. 4-C and D). The same observation on rat keratohyalin granules, namely the presence of electron dense material, was noted by Matoltsy and Matoltsy (1970, 1972).

In addition to these electron microscopic findings on the dialysed pellet of the deoxycholate soluble material, its histochemical characteristics were examined in order to confirm that this pellet was derived from the matrix proteins of the horny layer. The pellet was stained orange-yellow with Pauly’s reagent, blue with toluidine blue and basophilic with Harris hematoxylin-eosin (Fig. 5-B, D and F). The control sections showed that the horny layer was stained light orange-yellow, blue and light basophilic, and keratohyalin granules were also stained orange-yellow, blue and basophilic with Pauly’s reagent, toluidine blue and hematoxylin-eosin, respectively. The fact that the pellet showed tinctorial properties similar to those shown in horny layer and in keratohyalin granules in vivo, suggests that the materials forming this pellet were of the horny layers origin, since any granular cell was not contained in the original material, and this pellet was the non-fibrous, matrix proteins of the horny cells (Fig. 1-D). Taking both the electron microscopic and histochemical findings on this pellet into consideration, the proteins forming this pellet was the matrix proteins of the horny cells, which was of keratohyalin granules origin.

Molecular sieve Chromatographical studies. Both the supernatant of the second centrifugation or macroaggregate fraction were subjected to column chromatography on Sephadex G-200 in order to purify the material which was positively stained with Pauly’s reagent. The eluate was 50 mM Tris-HCl buffer (pH 8.6), containing 10 mM 2-mercaptoethanol without deoxycholate. The supernatant fraction of the second centrifugation yielded an initial large peak of
Fig. 4. The light and electron microscopic examination of the precipitates, which were yielded by dialysing the 270,000 × g supernatant fraction. A: The pellet in the light microscope (HE stain), × 500. B: A drop of this precipitate was placed on a carbon coated grid and stained with 3.5% uranyl acetate. Many round, electron dense particles and amorphous electron translucent material were observed. An electron less dense particle was attached to the electron dense particle (arrow). × 11,500. C: The pellet obtained from this precipitate was consisting of small electron dense particles and many linear, small, electron dense particles, ×11,500. D: Magnified view of C, ×46,000. The surface of the small electron dense particle is granular. Reduced to 6/7.
Fig. 5. Histochemical examinations.
Cryostat sections (6 microns) of control human plantar skins demonstrating numerous in situ keratohyalin granules and the horny layers (Figs. 5-A, C and E); the pellet of the precipitate obtained by dialysing the 270,000 × g supernatant fraction (Figs. 5-B, D and F). Figs. 5-A and B are stained with diazotized sulfanilic acid; C and D are with toluidine blue at pH 7.0; E and F are with Harris hematoxylin eosin. Reduced to 6/7.
The Matrix Protein

Fig. 6-A. Molecular sieve chromatography (Sephadex G-200) of the deoxycholate-soluble supernatant fraction. Elution from the Sephadex column was monitored at 280 nm and the eluate was 50 mM Tris-HCl buffer (pH 8.6), containing 10 mM 2-mercaptoethanol. o, the concentrated 270,000 × g supernatant fraction; Δ, the aggregates obtained by dialysing the high speed supernatant fraction.

Fig. 6-B. Molecular sieve chromatography (Sepharose 6 B).
The dialysed, lyophilized and resolubilized initial peak fraction from the Sephadex G-200 column was rechromatographed on Sepharose 6 B in the presence of 0.5% sodium deoxycholate. o, optical density at 280 nm; ●, optical density at 630 nm (dextran blue); Δ, optical density at 555 nm (phenol red).

280 nm absorbing material at the void volume followed by a plateau, a small second peak and a final peak which has been identified as deoxycholate (Fig. 6-A). The macroaggregates yielded an initial large peak at the void volume followed by several minor peaks. There were several minor peaks which were eluted very...
slowly, though the optical density of these minor peaks were lower than that of peaks when the supernatant fraction of the second centrifugation was applied to the column. The large initial peak was dialysed, lyophilized and rechromatographed on Sepharose 6 B in the presence of 0.5% sodium deoxycholate, and three peaks were obtained (Fig. 6-B).

**Electrophoretic studies.** When 50 ng of the dialysed pellet of the supernatant fraction of the second centrifugation which was the Pauly's reagent positive fraction, was subjected to disc gel electrophoresis using 10% acrylamide gel, two major and several minor bands migrated (Fig. 7-A). When the material of the second peak from the Sepharose 6 B column in the presence of 0.5% sodium deoxycholate were run, two bands were migrating in a 10% gel (Fig. 7-B), which had been proved to be identical with major bands in a gel A (Tezuka 1975). The material of the C-fraction from the Sephadex G-200 column from 28 to 40 in tube number was run, there were three, rapidly migrating bands (Fig. 7-C, arrow), which were electrophoretically identical with histidine-rich protein bands seen in a gel D. The precipitate obtained by dialysing the supernatant fraction of the second centrifugation from newborn rat granular cell layers was run in a gel D. In order to detect the histidine rich protein bands, the gels to which the supernatant fraction of the second centrifugation, the second peak fraction from the Sepharose 6 B column in the presence of 0.5% DOC and the rat histidine rich fraction (Tezuka and Freedberg 1974) were applied, were directly stained with Pauly's reagent after the
Fig. 8. Histochemical results. Cryostat sections (6 microns) of the precipitates obtained by dialysing the C-fraction from the Sephadex G-200 column (Figs. 8-A, B, and D) and the second peak fraction from the Sepharose 6 B column (Figs. 8-C and E). A was stained with Pauly’s reagent; B and C were with toluidine blue at pH 7.0; D and E were with Harris hematoxylin-eosin. Reduced to 6/7.

electrophoresis was finished and the gels were rinsed in redistilled water, but no positively stained protein bands were observed. The reason why protein bands were negative with Pauly’s reagent may be that SDS binds to histidine residues in protein molecules (Seibles 1969) and once bound, it could not be dissociated so that there
was no free histidine residue left capable of reacting with Pauly's reagent. The cryostat sections of the materials of the second peak from the Sepharose 6 B column in the presence of DOC and of the C-fraction from the Sephadex G–200 column were histochemically examined. The precipitate from the second peak of the Sepharose 6 B column chromatography, which was the purified material of the dialysed pellet, was positively stained with toluidine blue and hematoxylin-eosin, but not with Pauly's reagent (Fig. 8-C and E), though the crude material, which was the pellet obtained by dialysing the supernatant fraction of the second centrifugation, was positively stained with all of three reagents. The material from the second peak fraction is shown to be of keratohyalin granules origin by the indirect immunofluorescent study (Tezuka 1975) and it was not stained with Pauly's reagent, therefore the Pauly's reagent-positive fraction might have been dissociated during the purification procedures. In our previous paper (Tezuka and Freedberg 1974), the histidine rich proteins ‡T,‡U of the newborn rat epidermis were contained in the C-fraction from the Sephadex G–200 column. These proteins were gel-electrophoretically identical with the proteins in the C-fraction extracted from the human plantar horny layers. The precipitate from the human C-fraction was positively stained with Pauly's reagent, toluidine blue and hematoxylin-eosin (Figs. 8-A, B and D). Since the small amount of this fraction was found to be contaminated in the precipitate of the supernatant fraction of the second centrifugation, Pauly's-positive reaction of the pellet obtained by dialysing the supernatant fraction of the second centrifugation might lie in the contamination of the proteins of the C-fraction.

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References