Ultrastructural Studies of Elastic Material and Elastic Fibers in Aged Skin before and after Autoclaving

TAKUO TSUJI, M.D., PH.D.
Department of Dermatology, Osaka City University Medical School, Osaka, Japan

Elastic material and aged elastic fibers of patients with solar elastosis before and after autoclaving was studied using the tannic acid stain as well as the conventional stain. The amorphous matrix of the elastic material and the elastic fibers stained positive with the tannic acid stain. In the fibrous form of elastic material and in the elastic fibers both electron-dense inclusions and the amorphous matrix remained unchanged after autoclaving, while microfibrils were removed. In the amorphous form the fine granular component and round bodies were removed after autoclaving, and the moderately electron-dense amorphous component with a reticular or net-like appearance remained.

It has been suggested that the elastic degenerative changes are (1) expansion of the electron-dense inclusions to form a larger part of the fiber; (2) transformation of the inclusions into moderately electron-dense material, composing mainly of a fine granular component and an amorphous one; and (3) disorganization of the moderately electron-dense material.

Autoclaving has long been used by biochemists to separate collagen from elastin [1-3]. By suitable modification of autoclaving, a new method for use in scanning electron microscopical visualization of dermal elastic fibers has been developed [4]. The method is based on the fact that when the connective tissue is autoclaved or boiled, collagen and ground substances are removed, leaving elastic fibers in their native state. A transmission electron microscopical study confirmed these facts and also disclosed that no ultrastructural changes were induced in the elastic fibers [4]. A scanning electron microscopical study on solar elastosis has been recently carried out on hyaluronidase-treated and autoclaved specimens [5]. In the former, both the amorphous and fibrous structures of elastic material remained, whereas in the latter, only the fibrous structures remained. Thus, autoclaving appears to remove the amorphous structures from the elastic material.

The purpose of this paper is to examine the ultrastructural changes in the elastic material after autoclaving and also to compare these changes with those of normal elastic fibers in some unexposed skin on each patient as a control. In this study, in addition to the conventional method, a tannic acid staining method for elastic fibers [6] was used.

MATERIALS AND METHODS

Specimens were taken from the neck and the buttock of 8 patients (62 to 82 yr old) with cutis rhomboidalis nuchae. Vertical sections of the specimens approximately 1-mm thick were cut and autoclaved in water for 8 hr at 121°C at a pressure of 18 psi. All the specimens were processed for light microscopy (LM) and transmission electron microscopy (TEM).

For the LM, the specimens were fixed in 10% formalin and paraffin sections were stained with hematoxylin-eosin or the Weigert stain for elastic tissue. For the TEM, the specimens were fixed at 4°C for 1 hr in 2% glutaraldehyde buffered with 0.2 M phosphate (pH 7.4). After thorough rinsing they were immersed in 1% osmium tetroxide buffered with 0.2 M phosphate (pH 7.4) for 1 hr and then dehydrated by graded ethyl alcohol concentrations and embedded in epon 812. Ultrathin sections were cut with a Porter-Blum MT-2 ultramicrotome. Some sections were stained with a uranyl-acetate solution for 1 hr and with Reynolds's lead citrate for 10 min. Others were stained with a tannic acid-uranyl acetate solution [6] for 10 min and with Reynolds's lead citrate for 10 min. The tannic acid-uranyl acetate solution was prepared as follows: 0.2 g of tannic acid and 0.5 g of paranitrophenol were dissolved completely in 35 ml of distilled water with the aid of heat. After cooling, 0.2 to 0.4 ml of 5% uranyl acetate was added to this solution. They were examined with a Hitachi HS9 electron microscope.

RESULTS

Light Microscopy

Unexposed skin from the buttock

Appearance before autoclaving. Staining with hematoxylin-eosin revealed no basophilic degeneration of the connective tissue in any of the specimens taken from the patients. With Weigert stain elastic fibers tended to be finer, and vertically oriented in the papillary dermis. In the reticular dermis they were thicker and formed a horizontal network which was scattered among the collagen fibers. There were no big differences in the size, density, and distribution pattern of the elastic fibers depending on the specimens.

Appearance after autoclaving. Staining with hematoxylin-eosin revealed that no collagen fibers and skin appendages remained. All the elastic fibers remained reacted positively with Weigert stain. They revealed a similarity in organization in comparison with those before autoclaving.

Exposed skin from the neck

Appearance before autoclaving. Staining with hematoxylin-eosin revealed in the upper dermis basophilic degeneration of the connective tissue separated from a somewhat atrophic epidermis by narrow band of normal collagen. With Weigert stain large masses of wide, twisted, elastic material (some were fibrous and others were amorphous) was found in the area corresponding to the basophilic degeneration. In areas of severe solar degeneration seen in some specimens most elastic material was amorphous and extended into the lower portions of the dermis (Fig 1a).

Appearance after autoclaving. The elastic fibers and most of the elastic material remained and stained positively with Weigert stain, as did they stain before autoclaving. On the other hand, the other tissues including collagen fibers was completely removed. Irrespective of the specimens the elastic material appeared fibrous and stained basophilic with hematoxylin-eosin (Fig 1b).

Electron microscopy

Unexposed skin from the buttock

Appearance before autoclaving. The elastic fibers consisted of an amorphous matrix and microfibrils. The amorphous ma-
trix was located centrally and the microfibrils were distributed around the periphery with orientation parallel to the long axis of the fiber. There were various amounts of electron-dense areas in the amorphous matrix, some of which contained various sized vesicles (Fig 2a). These electron-dense areas and vesicles appeared to be increased with age, whereas the microfibrils were decreased.

With the tannic acid stain, the amorphous matrix except for the electron-dense areas stained black, but microfibrils as well as the electron-dense areas seen in the conventionally stained section stained lightly because of the counterstains with uranyl acetate and lead citrate.

**Appearance after autoclaving.** No changes were seen except for the microfibrils which were completely removed (Fig 2b). With the tannic acid stain the amorphous matrix still stained black.

**Exposed skin from the neck**

**Appearance before autoclaving.** There were variations in the structure of elastotic material depending not only on the degree of degeneration each specimen had, but also on the portions of the dermis where it was located. In general, however, 2 forms of elastotic material could be distinguished: fibrous and amorphous forms, as described in some previous reports [7-9]. Figure 3 shows low power views of these two forms.

The fibrous form of elastotic material resembled the aged elastic fiber in ultrastructural pattern [10-12]. It was characterized by irregular electron-dense inclusions and an electron-lucent amorphous matrix (Fig 4). In comparison with the aged elastic fiber, it was larger in size and more irregular, and had fewer microfibrils at the periphery. There were no vesicles or holes within the electron-dense inclusions as seen in the aged elastic fiber. This form of elastotic material predominated in the specimens which showed relatively mild degeneration at the light microscopical level, and was mainly located in the lower portion of the degeneration.

The amorphous form of elastotic material was characterized by moderately electron-dense masses consisting mainly a mixture of finely granular and amorphous components (Fig 5, 6). No microfibrils were seen at the periphery. Various sized electron-dense round bodies were seen within the mass. Some of the masses had relatively few bodies of this type (Fig 5), but others had a lot of them, being partially disintegrated into fragments of varying size (Fig 6). In the latter, the fragments were randomly scattered and in places appeared deposited on poorly defined strands of moderate electron density, forming large composite masses. This form of elastotic material predominated in the specimens which showed severe degeneration at the light microscopical level, and was located in the upper and middle portion of the degeneration.

There was a transitional form between the two forms of elastotic material. The transitional form consisted of the electron-dense inclusions and the moderately electron-dense matrix.
consisting of fine granular and amorphous components (Fig 7). This form of elastotic material was located mainly in the upper portion of the degeneration.

With the tannic acid staining, the amorphous matrix in the fibrous form stained black, while little or none in the mass of the amorphous form was stained (Fig 8).

**Appearance after autoclaving.** The changes in elastotic material after autoclaving were different depending on its form.

![Fig 3. A low power view of the 2 forms of elastotic material before autoclaving. (a): a fibrous form, and (b): an amorphous form. (Conventional stain; scale = 1 μm).](image)

![Fig 4. Electron micrograph of the fibrous form of elastotic material from the neck. Numerous, various sized, and irregular electron-dense inclusions (white D) and, an electron-lucent amorphous matrix (AM) can be seen. Microfibrils (MF) are present at the periphery. (Conventional stain; scale = 1 μm).](image)

![Fig 5. Electron micrograph of amorphous form of elastotic material from the neck. A moderately electron-dense material consisting mainly of a mixture of a fine granular component (G) and an amorphous one (A) can be seen. Round bodies (B) of various sizes and electron density are scattered in this material. No microfibrils are present at the periphery. (Conventional stain; scale = 1 μm).](image)

![Fig 6. Electron micrograph of the amorphous form of elastotic material. This shows a mass of fragments consisting mainly of a fine granular component (G) and an amorphous one (A), which are randomly scattered and in places appeared deposited on poorly defined strands of moderate electron density. (Conventional stain; scale = 1 μm).](image)

![Fig 7. Electron micrograph of the transitional form of elastotic material. Irregular electron-dense inclusions (white D) and the moderately electron-dense matrix (M) consisting of a fine granular component and an amorphous one can be seen. (Conventional stain; scale = 1 μm).](image)
Fig 9 shows low power views of the changes in the 2 forms of elastic material after autoclaving.

In most of the fibrous form of elastic material, both the electron-dense inclusions and the electron-lucent amorphous matrix remained unchanged except for the periphery where microfibrils were completely removed (Fig 10a). In some of this form, however, there were areas of reticular or worm-eaten appearance composing of many tiny spaces within and around the electron-dense inclusions (Fig 10b). These findings were not seen in any of the elastic material before autoclaving.

In the amorphous form of elastic material a moderately electron-dense amorphous component forming a reticular or net-like appearance was disclosed (Fig 11a). The size of the space varied from tiny and worm-eaten to large and vacuolar appearance. In some of the elastic material only the strings of the amorphous component remained, that might result from the fusion of such large vacuoles (Fig 11b). In this form a fine granular component and round bodies seen before autoclaving were completely removed.

In the transitional form of elastic material, electron-dense inclusions remained, and a moderately electron-dense amorphous component with a reticular or net-like appearance was disclosed. Sometimes, isolated electron-dense inclusions were seen floating together with the fine granular material among the elastic material.

With the tannic acid staining, the amorphous matrix of the fibrous form and some parts of the transitional form stained black, but none of the amorphous form was stained.

The Table shows the components of the aged elastic fiber and elastic material, and summary of the results of tannic acid staining and autoclaving.

DISCUSSION

The ultrastructural findings of both the aged elastic fibers and the elastic material before autoclaving seen in the present study were identical to those demonstrated by previous reports using the conventional staining technique [7-19] as well as the tannic acid one [11, 12]. After autoclaving it was confirmed that
in aged normal skin only elastic fibers remained, and in solar elastosis, most of the elastic material and the elastic fibers remained. The other dermal components including collagen fibers were completely removed, as revealed in the previous reports [4, 5].

Detailed observation by electron microscopy revealed that autoclaving also altered some components of the elastic fibers and the elastic material. In elastic fibers microfibrils were removed, while amorphous matrix and electron-dense inclusions remained. This may suggest that the microfibrils have a different nature from the amorphous matrix and the electron-dense inclusions. In fact, the microfibrils of elastic fiber are susceptible to digestion by proteolytic enzymes such as trypsin, chymotrypsin, and pepsin, in contrast to the amorphous matrix, and have a significantly different amino acid composition from the latter [20]. On the other hand, the amorphous matrix in the elastic fiber has been identified as elastin on the basis of its amino acid composition, and characterized by the insoluble nature of an extensively cross-linked protein as evidenced by its relatively great resistance to solubilization by most solvents including hot alkali [20]. The present study confirmed its insoluble nature using autoclaving.

In the fibrous form of elastic material, microfibrils were removed, but electron-lucent amorphous material and electron-dense inclusions remained, which was the same results as seen in the elastic fiber. In addition to these, the presence of a common nature on the affinity for tannic acid between the amorphous matrix in the elastic fiber and the electron-lucent amorphous material in the fibrous form of elastic material suggests the strong possibility of the transformation from the former to the latter [12]. In the amorphous form and the transitional form of elastic material, a fine granular component and round bodies were completely removed, while an amorphous component remained forming a worm-eaten, reticular, or net-like appearance. The nature of the fine granular component and the round bodies are not clear, but these may be related to disruption or dissolution of the elastic material since these increase as elastotic changes progress. In addition, the material which makes up the reticular or net-like appearance after autoclaving may be identical to the amorphous component of the amorphous form of elastic material. The fact that both the fine granular and the amorphous component did not stain with tannic acid suggests that the origin of these are not amorphous matrix, but electron-dense inclusions in the fibrous form of elastic material. This would be supported by the findings that electron-dense inclusions were often seen in contact with moderately electron-dense material the fine granular component of which were removed after autoclaving, resulting in worm-eaten appearance.

In the previous report using the tannic acid staining method we suggested that elastotic degeneration might progress in the order of the fibrous, the transitional, and the amorphous form [12]. And it could be supposed from the present study that the elastotic degenerative changes are (1) expansion of the electron-dense inclusions to occupy a larger part of the fiber (fibrous form); (2) transformation of the inclusions into moderately electron-dense material which compose of amorphous and finely granular components (transitional form); and (3) being occupied by the moderately electron-dense material (amorphous form) with increase in the granular component, disruption or dissolution of parts of the elastic material, resulting in its fragmentation. This proposal denies some previous descriptions or hypotheses such as (i) the amorphous matrix might change from the electron-lucent to fine granular appearance (granular matrix) [7, 10, 21], and (ii) the electron-dense inclusions might form by partial condensation processes in the
granular matrix [19]. Thus, autoclaving is a useful technique for defining more clearly the degree and process of degeneration in elastotic material, since it can completely remove the fine granular component and the round bodies which increase as elastotic changes progress.

Finally, there is a question as to the nature of the amorphous masses which were present before autoclaving and removed after autoclaving at the light microscopical and scanning electron microscopical level [5]. Judging from the present study, they might be disorganized elastotic material composed of the fine granular component and the round bodies which are largely disorganized and could be completely removed by autoclaving.

REFERENCES