Scanning Electron Microscopic Studies of Tissue Elastin Components Exposed by a KOH-Collagenase or Simple KOH Digestion Method

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Summary. A KOH-collagenase or simple KOH digestion method was employed for scanning electron microscope (SEM) studies of elastin components in the rat thoracic aorta, mouse urinary bladder, and human ductus deferens. Immersion of the fixed tissues in 30% KOH solution for 8-10 min at 60°C, with or without subsequent collagenase treatment, successfully removed collagen fibrils and basal laminae while leaving cellular and elastin elements unchanged at their original shapes and locations.

The internal elastic lamina of the rat aorta appeared as a solid sheet formed by elastin fibrils 0.1-0.2 μm thick, while the medial elastic laminae were more fibrous because of the presence of numerous fine elastin fibers on their surface. Adventitial elastin fibers were of a cord-like shape complicatedly entangled among the adventitial fibroblasts. These fibers were seen as bundles of fibrils 0.1-0.2 μm thick.

In the mouse urinary bladder, elastin formed a thin lace-like sheet just beneath the serosal covering of the peritoneum. This sheet was composed of small bundles of fine (0.1-0.2 μm thick) fibrils.

The external connective tissue of the human ductus deferens was made up of a three-dimensional loose network of elastin fibers 0.1-1.5 μm thick. These fibers also appeared as bundles of the fine fibrils.

These findings indicate that the present method is useful for SEM studies of elastin as well as cellular components in various tissues and organs. This study also maintains that elastin fibers and laminae are basically composed of unit fibrils of 0.1-0.2 μm thickness. As elastin components are arranged specific to individual organs and tissues, it is reasonable that these components are concerned in the characteristic mechanical properties of these tissues and organs.

Elastin is one of the structural proteins distributed in most connective tissues throughout the body. This protein, in association with microfibrils (Low, 1962), forms fibers and sheets, which are considered to give proper resilience to tissues of the organs (see review by Dempsey and Lansing, 1954; Ross, 1973; Sandberg et al., 1981). It is therefore important to understand precisely the organization of the elastin components in relation to the mechanical properties of the tissues and organs.

Scanning electron microscopy (SEM) is useful for demonstrating the three-dimensional organization of the elastin components in tissues. Here, however, these observations are difficult since elastin components are intermingled with collagen fibrils and cellular elements in tissues. Therefore, SEM investigators have attempted to extract elastin components by autoclaving tissues (Grut et al., 1977; Tsuji et al., 1979), or by utilizing treatments with chemical agents and enzymes; e.g., guanidinium chloride, collagenase, sodium hydroxide, and formic acid (Kuhn, 1974; Kewley et al., 1977; Wasano and Yamamoto, 1983; Crissman and Pakulski, 1984; Song and Roach, 1985; Crissman, 1987). These procedures are indeed useful for observing elastin-rich tissues such as large arteries, but are not practical in tissues containing a small amount of elastin because the latter tissues easily collapse or break during the preparations.

In order to overcome this problem, we have applied a KOH-collagenase or simple KOH digestion method to the SEM observation of elastin components in tissues. This method was originally developed by Miller et al. (1982) for SEM observations of vascular smooth muscle cells, and our modification of his method has proven applicable to SEM studies of the three-dimensional cytoarchitecture of a wide variety of tissues (Usuki and Ide, 1987, 1988; Ushiwata and Usuki, 1990; Usuki, 1990). In the course of these
studies, we have noticed that this method, if properly handled, preserves elastin as well as cellular elements, thus enabling the demonstration of the three-dimensional organization of elastin components in tissues.

The present study, therefore, firstly evidences the applicability of the KOH-collagenase and KOH digestion methods to SEM studies of tissue elastin components, secondly shows the ultrastructure of elastin components treated with this method, and thirdly demonstrates their arrangements specific to the rat thoracic aorta and other certain tissues.

MATERIALS AND METHODS

The materials used were the thoracic aorta of adult rats (WKA strain), urinary bladder of mice (dd strain), and human vas deferens obtained from a 54-year-old patient surgically operated on for bladder cancer. These materials were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) or a fixative containing 2% paraformaldehyde and 2.5% glutaraldehyde in the buffer by either perfusion or immersion, cut into small pieces (about 3x3x5 mm), and further fixed in the same fixative over one day at room temperature. They were rinsed several times in 0.1 M phosphate buffer (pH 7.4), placed in 25-30% KOH for 8-10 min at 60°C and washed in 0.1 M phosphate buffer (pH 7.4) for about 1 h or more. Most of these tissues were immersed in a collagenase solution (Sigma, type II, about 1 mg/ml in 0.1 M phosphate buffer, pH 7.4) for about 3 h at 37°C and washed in the buffer solution, while the others were not treated with collagenase. All tissues were conductive-stained by MURAKAMI's tannin osmium method (1973); they were put in an aqueous solution of 1% tannic acid for 2-3 h, rinsed in distilled water for more than one hour, and stained with 1% OsO₄ solution for several hours at room temperature. The tissues were then dehydrated with ethanol at increasing concentrations, transferred to isoamyl acetate, and dried in a critical point dryer with liquid CO₂. The dried tissues were then attached to aluminum stubs with double-sided adhesive tape, coated with platinum-palladium in an ion coater, and examined in an SEM (S-430 or S-4000, Hitachi). Some tissue pieces at the 100% ethanol step were embedded in Epon and processed for transmission electron microscopy (TEM) to examine how elastin components were preserved after digestion.

RESULTS

Treatment of the tissues with a KOH solution followed by collagenase effectively and consistently removed collagen fibrils and basal laminae, exposing the cellular and elastin elements (Fig. 1). In some cases, digestion with KOH alone also completely removed the collagenous elements from the tissues.

TEM observations confirmed that the non-cellular components exposed by the KOH-collagenase or KOH treatment were actually elastin fibers and laminae; they were well preserved in their natural shapes and locations (Fig. 1b). At a high magnification, they were composed only of amorphous, elastin structures stained black with tannic acid and osmium (Fig. 1c). Elastin-associated microfibrils were completely dissolved in this treatment.

Elastin fibers in the rat aorta

The wall of the thoracic aorta was composed of 7-8 concentric laminae of elastin components sandwiching smooth muscle cells (Fig. 1a). Each laminae appeared as a solid sheet about 2 μm thick. When observed in those places where the endothelium was artificially peeled, the internal elastic lamina had numerous oval fenestrations of varying diameter from 1-10 μm (Fig. 2a). At a high magnification, the luminal surface of the elastic lamina was somewhat fibrous; fibrils of 0.1-0.2 μm thickness appeared to be densely packed in the lamina to form a felt-like sheet (Fig. 2b). Some of these fibrils extended toward the fenestrations and spanned them in various directions to form a meshwork like a wire fence.

The medial elastic laminae were 2-3 μm in thickness and had an irregular profile because of the presence of numerous fine (0.1-1.0 μm in diameter) fibrous structures on their surfaces (Fig. 3a). Tangentially cut surfaces of the media showed that the

Fig. 1. Scanning (a) and transmission (b, c) electron micrographs of a transverse section of the rat aortic wall treated with the KOH digestion method. a, and b. Elastic laminae (E), interlaminar elastin fibers (arrowheads), smooth muscle cells (M) are well preserved in their original configurations, while collagen fibrils have been apparently removed by this method. a: ×3,000, b: ×2,600. c. Higher magnification of a part of the aortic media. Collagen fibrils and basal laminae have been completely removed. Elastin-associated microfibrils have also been dissolved away. E elastic lamina, M smooth muscle cell. ×11,000
Fig. 1. Legend on the opposite page.
thicker fibers were formed by the fusion of smaller ones at random points. These fibers, however, basically ran along the long axis of the smooth muscle cells (Fig. 3b). Some of these fibers left their surface, ran between two adjacent muscle cells and formed interlaminar fibers connecting two adjacent laminae.

Elastin in the aortic adventitia was built into fibers of various sizes from 0.1 to 2 μm (Fig. 4a, b). They branched here and there, ran in different directions and were complicatedly entangled within the meshworks of the adventitial fibroblasts. These fibers were sometimes enfolded by the fibroblasts in their sheet-like processes (Fig. 4b). At a high magnification, the elastin fibers were composed of bundles of fine (0.1-0.2 μm in diameter) fibrils. These bundles were often unraveled into thinner bundles or even into separate fibrils (Fig. 4c), which later united with other bundles, thus producing a branching or anastomosing appearance of the elastin fibers.

**Elastin network in the serosa of the mouse urinary bladder**

In the mouse urinary bladder, elastin formed a thin lace-like sheet just beneath the serosal covering of the peritoneum (Fig. 5a). This sheet consisted of fine fibers ranging from 0.1 to 1.0 μm thick; these fibers ran in various directions and were elaborately interwoven, forming a delicate lacework-pattern (Fig. 5b). Fibroblasts with flat sheet-like processes were scattered just beneath this elastin network, but not present above it.

In higher magnification views, thicker fibers of the elastin network were seen as bundles of thin fibrils about 0.1-0.2 μm in diameter (Fig. 5c). These bundles were often unraveled into thinner ones and even into individual fibrils, which could then rejoin other bundles in turn.
Elastin fibers in the fibrous adventitia of the human ductus deferens

Elastin in the fibrous adventitia of the human ductus deferens formed cord-like fibers 0.1-1.5 μm in diameter. These fibers ran in various directions, branched in places and were complicatedly intertwined to form a three-dimensional loose network in the adventitia (Fig. 6a). Within this elastin network, fibroblasts extended dendritic processes two-dimensionally; some of these processes appeared to be in contact with elastin fibers. Nerve bundles and blood vessels were also present in the elastic network. The nerve bundles consisted mainly of unmyelinated fibers which were identifiable as interconnecting cords with longitudinal mesaxonal furrows on the surface (USHIKI and IDE, 1988). Blood vessels were, on the other hand, characterized by dense networks of elastin fibers surrounding them.

At a higher magnification, the elastin fibers appeared as bundles of thin fibrils 0.1–0.2 μm thick (Fig. 6b). These fibrils often separated from the bundles and united with other bundles, thus producing a branching and anastomosing appearance.

DISCUSSION

Methodology

Following our introduction of KOH-collagenase digestion methods for the SEM observation of peripheral nerves, we have been applying them to the SEM studies of cellular elements in various tissues and organs (USHIKI and IDE, 1987, 1988; USHIWATA and USHIKI, 1990; USHIKI, 1990). The present study, however, clarified that these methods, which removed collagen fibers completely, preserve elastic fibers as well as cellular elements in their original shapes and
Fig. 4. The aortic adventitia treated with the KOH digestion method. a. An overview of the adventitia. Elastin fibers run in various directions and become intricately entangled among adventitial fibroblasts (F). ×1,700. b. A closer view of the adventitia. An elastin fiber (arrowheads) is partially enfolded in the sheet-like processes of a fibroblast (F). ×3,600. c. Higher magnification of an elastin fiber in the adventitia. The elastic fiber is seen as a bundle of fine fibrils. ×11,000
Fig. 5. Elastin observed in the serosa of the mouse urinary bladder. a. An overview of the serosa. The mesothelium (P) is removed on the lower right of the micrograph, where elastin forms a delicate pattern of lacework. Beneath this network are found smooth muscle cells (lower left) and fibroblasts (F). ×2,200. b. High magnification of the elastin network. The network is composed of fine elastin fibers, which run in various directions and are elaborately interwoven. ×4,000. c. Higher magnification of the elastin network. Thicker elastin fibers are found as bundles of thin elastin fibrils. ×11,000
locations. Elastin is known to be highly resistant to alkali degradation or boiling water (Richards and Gies, 1902; see review by Sandberg et al., 1981). Several investigators succeeded in demonstrating elastin components by treating tissues with 0.1 N NaOH at high temperatures (Gotte et al., 1972; Hart et al., 1978; Song and Roach, 1985; Roach and Song, 1988). Therefore, it is reasonable that elastin is preserved after treatment with hot KOH, although its concentration is rather high.

As described in a previous study (Ushiki and Ide, 1988), treatment with collagenase after KOH digestion is not indispensable for removing collagen components, and can be omitted for collagen-sparse tissues. This treatment, however, seems to be effective for cleaning the cellular and elastin surfaces by digesting the collagen residues attached there.

Previous methods for SEM studies of elastin components were applied only to such elastin-rich tissues as blood vessels and ligamentum nuchae (Kewley et al., 1977; Hart et al., 1978; Wasano and Yamamoto, 1983; Crissman and Pakulski, 1984), and not in tissues containing a small amount of elastin, since the latter tissues easily collapse during the specimen preparation. The present method can clearly demonstrate the organization of elastin components even in elastin-sparse tissues. It also has the advantage of visualizing the spatial relationship between the elastin fibers and cellular components in tissues. Our KOH-collagenase and simple KOH digestion methods are, therefore, highly useful for SEM observations of elastin components in various tissues and organs.

Fig. 6. Elastin observed in the fibrous adventitia of the human ductus deferens. a. An overview of the adventitial connective tissue. Elastin fibers run in various directions and are complicatedly intertwined to form a loose three-dimensional network. Arrows indicate unmyelinated nerve fibers, F fibroblasts, M smooth muscle cells. x 900. b. Higher magnification of elastin fibers in the adventitia. The fibers are composed of fine unit fibrils. x 18,000
Presence of unit fibrils within elastin components

The present study has demonstrated that tissue elastin components are basically composed of thinner fibrils 0.1-0.2 \( \mu \)m thick. These fibrils are present individually or in bundles, thus forming elastin fibrils, fibers and/or laminae in individual tissues. These fibrils are apparently different from elastin-associated microfibrils, since the latter structures are removed by the present methods. Similar structures have been demonstrated in studies using other treatments such as guanidine extraction (Kewley et al., 1977) and hot-formic acid extraction (Iyama and Braverman, 1988; Ushiki, 1991). Several TEM investigators have demonstrated that the elastic fibers develop through the coalescence and fusion of similar elastin fibrils (Albert, 1972; Spicer et al., 1975). We therefore consider these fibrils to be a structural unit of elastin which produces sheets or branched fibers of various sizes.

Gotte et al. (1974), however, reported in their TEM studies of the bovine ligamentum nuchae using a negative staining technique that alkali-extracted elastin consists of slender filaments of 3-4 nm arranged roughly parallel to the long axis of the fiber. Since then, similar filaments have been reported by several investigators using such methods as freeze-fracture and freeze deep-etch techniques (Fornieri et al., 1982; Nakamura and Ooyama, 1988). These filamentous structures are apparently much thinner than the fibrils observed in the present study; the latter fibrils may presumably be made up of the former filaments. Further studies using other techniques are needed to clarify this point.

Organization of elastin components

The present study has demonstrated the detailed three-dimensional organization of elastin components in several tissues and organs. This is also the first report which has directly shown the spacial relationship between elastin components and cellular elements by SEM.

Several SEM investigators have demonstrated that the aortic elastic laminae extracted with chemicals were fibrous (Hart et al., 1978; Masuda, 1984; Liu et al., 1988), while others have considered them a smooth sheet (Wasano and Yamamoto, 1983). However, we recently clarified that the surface structure of elastic laminae is changeable, depending on tissue preparations after formic acid extraction, and showed evidence that the aortic laminae are fibrous when the extracted tissues are adequately treated (Ushiki, 1991). Our findings using KOH digestion well correspond to the previous observations.

The presence of the interlaminar elastin fibers connecting two neighboring laminae is reasonable since blood pressure can be distributed uniformly around the whole circumference of the vascular wall (Wolinsky and Glagov, 1964). The arrangement of the interlaminar fibers along the long axis of smooth muscle cells may indicate that these fibers play an important role in effectively distributing blood pressure to the smooth muscle cells.

Another remarkable finding in the present study is the existence of an elastin sheat lining the serosal covering of the peritoneum in the urinary bladder. As far as we know, a similar structure has been demonstrated only by Monson et al. (1988) in their light microscopic studies of the rabbit bladder. Since this elastin sheet is very thin, it may not much contribute to resilience of the bladder. This sheet is conceived to give elasticity to the serous membrane and protect the mesothelium against any distention and contraction of the bladder.

The loose elastic network found in the adventitia of the ductus deferens is obviously a typical organization of elastin fibers in the loose connective tissue. This loose network, in association with collagen bundles, maintains the microenvironment suitable for conducting blood vessels and nerves.

It is thus evident that the elastin component shows morphological feature specific to individual tissues and organs. The organization of the component apparently influences the resilience of tissues suitable for their mechanical properties. Studying pathological alterations in arrangements of elastin components using the present methods will give us further information.

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