Effects of a Triple Enzyme Digestion Method on a Diamine Reaction for Glycosaminoglycans of the Rat Aorta in Electron Microscopy

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ABSTRACT. Effects of a triple enzyme digestion method using glycosaminoglycans-degrading enzymes upon a diamine reaction have been tested for electron microscopic histochemical detection of glycosaminoglycans in extracellular matrix of the rat aorta. The triple enzyme digestion method consists of a sequence of chondroitinase B, testicular hyaluronidase and heparitinase. The results obtained by the present experimental and control studies indicated that dermatan sulfates, chondroitin sulfates (A and/or C) or heparan sulfates were apparently observed in various ultrastructural features of aortic extracellular matrix, such as bundle of collagen fibers and soluble matrix of interstitial space. Particularly, we found that both heparan sulfates and chondroitin sulfates (A and/or C) were detected in association with the basal lamina of smooth muscle cells and the external surface of elastic lamina, and in the latter heparan sulfates were frequently recognized as a mass, whereas chondroitin sulfates (A and/or C) were found intermittently along the external surface of elastic lamina. This suggests that the triple enzyme digestion method which combines the glycosaminoglycans-degrading enzymes with the diamine reaction can be postulated to represent efficient and useful technique for precise electron microscopic histochemical detection of the glycosaminoglycans in the extracellular matrix of the rat aorta. — KEY WORDS: diamine reaction, electron microscopy, glycosaminoglycan, rat aorta, triple enzyme digestion.

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Glycosaminoglycans are negatively charged polysaccharides consisted of repeating disaccharide units and are sulfated at varying degrees in most cases. The sulfated glycosaminoglycans are generally recognized as four types of classes: dermatan sulfate, chondroitin sulfate, heparan sulfate and keratan sulfate. In tissues, they occur as proteoglycans composed of one or more polysaccharide chains attached to a core protein [5]. In the arterial wall, the proteoglycans and their constituent glycosaminoglycans are only minor components, although they play essential physiological roles in a variety of arterial functions. It has been demonstrated that they are of enormous importance in influencing such arterial properties as viscoelasticity, permeability, lipid metabolism, hemostasis and thrombosis [16, 17]. In addition, they have been known to accumulate within intimal lesions in both large and small vessels related to atherosclerotic development [11, 18].

In histochemistry, one of the most reliable methods for detection of glycosaminoglycans is the use of enzymes which selectively degrade or eliminate the carbohydrates from tissues. The enzyme digestion methods have been used by a combination of a kind of glycosaminoglycan-degrading enzymes and several histochemical staining techniques in general [3, 7, 13]. Further extensions of enzyme digestion methods in light microscopic histochemistry of the glycosaminoglycans in extracellular matrix have been made by appropriate combinations of chondroitinase B and testicular hyaluronidase in the buccal mucosa and periodontium [4] and chondroitinase ABC and keratanase in fish skin [14] to establish double enzyme digestion methods for the precise detection of two kinds of molecules or moieties in tissue glycosaminoglycans. In the extracellular matrix of arterial wall, it has been known to contain at least three kinds of the glycosaminoglycans, such as dermatan sulfates, chondroitin sulfates and heparan sulfates [15, 16]. In addition, these glycosaminoglycans have been frequently observed to be coexistent at the same component of the arterial extracellular matrix [1, 13, 15]. In view of this, it is found to be difficult to detect specifically their localization with conventional enzyme digestion techniques in tissues containing three kinds of the glycosaminoglycans in electron microscopy.

In the present study, chondroitinase B, testicular hyaluronidase and heparitinase digestion methods were sequentially combined so as to develop a triple enzyme digestion method, in an attempt for electron microscopic histochemical detection of the glycosaminoglycans such as dermatan sulfates, chondroitin sulfates (A and/or C) and heparan sulfates in the extracellular matrix of the rat aorta. The results obtained in the present study are believed to provide basic data indispensable for investigations into the nature and localization of glycosaminoglycans in the extracellular matrix of the rat aorta and consequently to be significantly useful for research in the alteration of atherosclerosis.

MATERIALS AND METHODS

Reagents: N,N'-dimethyl-m-phenylenediamine (2HCl), N,N'-dimethyl-p-phenylenediamine (HCl), and thiocarbohydrazide were obtained from Sigma (St Louis, U.S.A.), and silver protein from Merck (Darmstadt, Germany). Of the four chemicals involved in a physical developer [6] used, gum arabic, silver nitrate, and citric

acid were from Katayama Chemicals (Osaka, Japan) and bromohydroquinone was from Eastman Kodak (New York, U.S.A.). Chondroitinase B and heparitinase were obtained from Seikagaku Kogyo (Tokyo, Japan) and testicular hyaluronidase was purchased from Sigma (St Louis, U.S.A.).

Preparation of Tissues: A total of 5 Fischer male rats (8 weeks of age, 150–180 g) were anesthetized with Nembutal and perfused through the left ventricle of the heart first with Ringer's solution and then with a solution of 0.5% glutaraldehyde and 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at room temperature for 5–10 min. After perfusion, thoracic aorta was removed, cut into cube of 1 mm and then fixed by immersion in the same fixative at room temperature for 120 min. The tissue blocks were rinsed in the same buffer, dehydrated in an ethanol series and then embedded in LR-White [9]. Ultrathin sections with an approximate thickness of 80–100 nm were prepared on a Reichert ultramicrotome, mounted on nylon grids and subjected to staining as described in detail below.

Staining Protocols: High iron diamine/thiocarbohydrazide/silver protein/physical development (HID/TCH/SP/PD) staining procedure. Ultrathin sections were stained with a solution of a high iron diamine (HID) (N,N'-dimethyl-m-phenylenediamine, 120 mg; N,N'-dimethyl-p-phenylenediamine, 20 mg; Distilled water, 50 m*l*; 40% ferric chloride solution containing 5% hydrochloric acid, 1.4 m*l*) at room temperature for 60 min and then washed in distilled water for 10–15 min. The sections were incubated in 0.5% thiocarbohydrazide (TCH) in 20% acetic acid at room temperature for 30 min and rinsed in distilled water for 10–15 min. The sections were immersed in a 1% aqueous solution of silver protein (SP) for 30 min at room temperature in a dark box and washed in distilled water for 10–15 min. The sections were developed in the physical developer at 20°C for 12–15 min in the dark box, washed in distilled water for 10–15 min, treated with photographic fixer for 5–10 seconds, and then rinsed in distilled water [13]. The sections were stained with 2% methanolic uranyl acetate and lead citrate.

Triple enzyme digestion method: Triple enzyme digestion method consisted of a sequence of chondroitinase B, testicular hyaluronidase and heparitinase was used in combination with the HID/TCH/SP/PD procedure. To degrade dermatan sulfates, chondroitin sulfates (A and/or C) and heparan sulfates in the aorta tissues, ultrathin sections were subjected to first digestion with 0.01 U/m*l* of *Flavobacterium heparinum* chondroitinase B in 0.1 M Tris-HCl buffer (pH 8.0) [8, 10] for 60 min at 30°C, secondly with 0.005 mg/m*l* of bovine testicular hyaluronidase in 0.1 M phosphate buffer (pH 5.5) [7] for 60 min at 37°C, and then finally with 0.01 U/m*l* of *Flavobacterium heparium* heparitinase in 0.1 M Tris-HCl buffer (pH 7.3) [19] for 60 min at 37°C (chondroitinase B/testicular hyaluronidase/heparitinase) before the HID/TCH/SP/PD procedure. For the triple enzyme digestion method, four types of control procedures were performed, as follows. Prior to the HID/TCH/SP/PD procedure, ultrathin sections were incubated in the specified buffer solution without respective enzymes instead of incubation with (a) chondroitinase B (buffer/testicular hyaluronidase/heparitinase) for degradation of both chondroitin sulfates (A and/or C) and heparan sulfates, (b) testicular hyaluronidase (chondroitinase B/buffer/heparitinase) for elimination of both dermatan sulfates and heparan sulfates, (c) heparitinase (chondroitinase B/testicular hyaluronidase/buffer) for exclusion of both dermatan sulfates and chondroitin sulfates (A and/or C), or (d) chondroitinase B, testicular hyaluronidase and heparitinase (buffer/buffer/buffer) under the same conditions of duration and temperature as the triple enzyme digestion method.

RESULTS

HID/TCH/SP/PD procedure: In the extracellular matrix of the rat aorta examined, positive HID/TCH/SP/PD reactions of various intensities were obtained in the bundle of collagen fibers, basal lamina of smooth muscle cells and elastic lamina. Further, HID/TCH/SP/PD reaction products were found in areas appeared to be amorphous and devoid of fibers. They show a major component of the compartment known as the "soluble matrix" of interstitial space. Of these structures, the basal lamina of smooth muscle cells and external surface of the elastic lamina showed strong positive reactions (Figs. 1A, 1B).

Triple enzyme digestion method: Three types of control procedures using specific buffer without chondroitinase B (buffer/testicular hyaluronidase/heparitinase), testicular hyaluronidase (chondroitinase B/buffer/heparitinase) or heparitinase (chondroitinase B/testicular hyaluronidase/buffer) diminished the intensities of the HID/TCH/SP/PD reactions in various ultrastructual features in the extracellular matrix of the rat aorta (Figs. 2A, 2B, 2C). Particularly, in the control procedure with the buffer/testicular hyaluronidase/heparitinase, the stainings of the soluble matrix of interstitial space, the basal lamina of smooth muscle cells and the external surface of elastic lamina were either negative or exceedingly weak, whereas the reactions in the bundle of collagen fibers tended to be diminished slightly in intensity (Fig. 2A). The control procedure using chondroitinase B/buffer/heparitinase either abolished or diminished the intensities of the HID/TCH/SP/PD reactions in the bundle of collagen fibers, and the staining intensities by the HID/TCH/SP/PD procedure in the basal lamina of smooth muscle cells and external surface of elastic lamina showed a moderate decline. Further, positive HID/TCH/SP/PD reactions were found intermittently along the latter ultrastructure. In the soluble matrix of interstitial space the staining reactions remained unaffected, or tended to be slightly diminished in intensity (Fig. 2B). In the control procedure incubating with the chondroitinase B/testicular hyaluronidase/buffer, intensities of the staining reactions of the bundle of collagen fibers and the soluble matrix of interstitial space were markedly diminished. In contrast, the reactions of the basal lamina of smooth muscle cells and the external surface of elastic

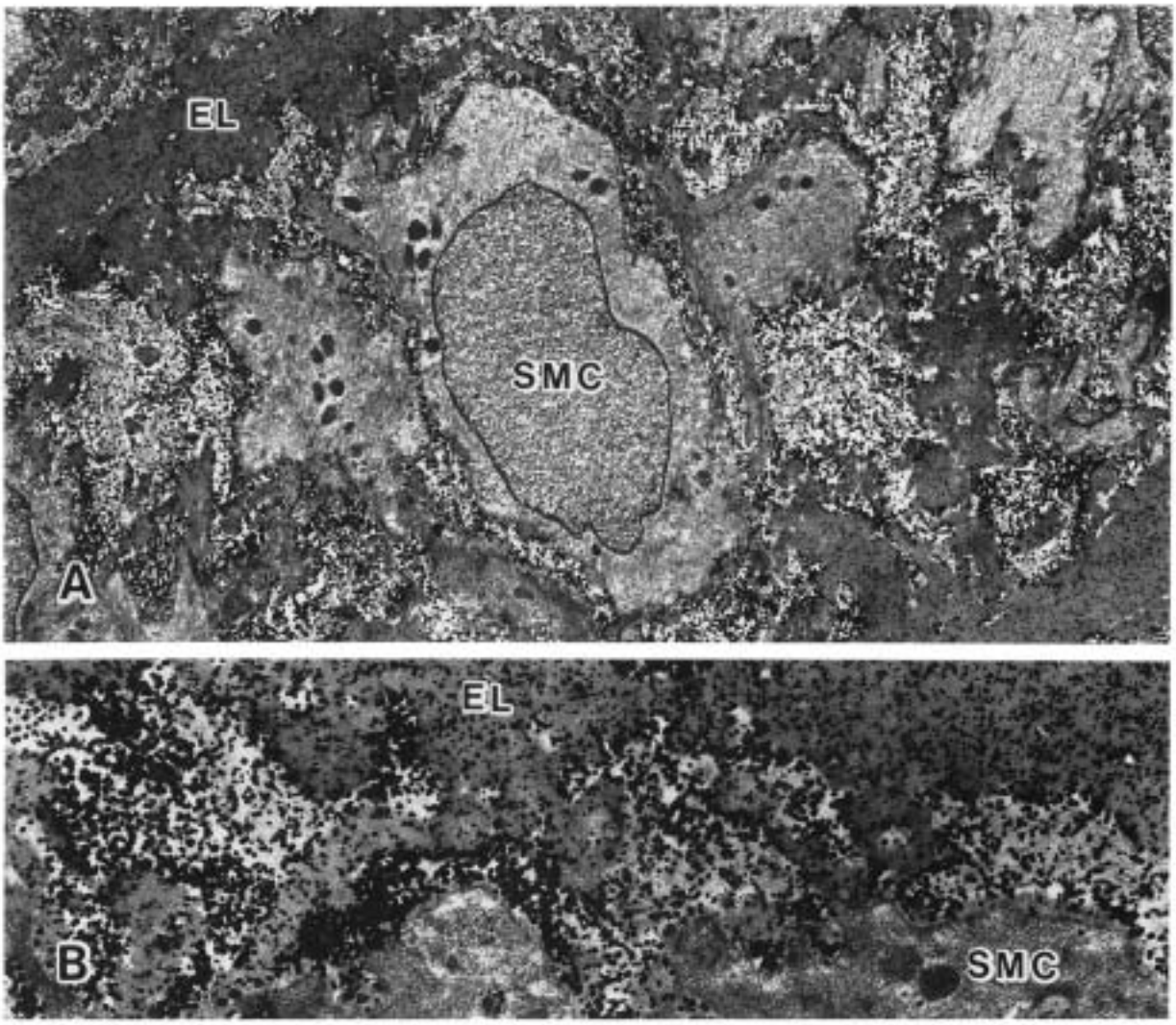

Fig. 1. Rat aortic tissue stained with HID/TCH/SP/PD. EL: elastic lamina, SMC: smooth muscle cell. (A): A lower magnification in a part of the media of the rat aorta. The bundle of collagen fibers (asterisk), the elastic lamina and the basal lamina of smooth muscle cells exhibit positive reactions. × 7,600. (B): The bundle of collagen fibers, the soluble matrix of interstitial space, the elastic lamina and the basal lamina of smooth muscle cells show positive reactions. × 18,000.

lamina showed a slight or moderate decline in intensity (Fig. 2C). In the external surface of elastic lamina, in addition, positive HID/TCH/SP/PD reactions were frequently observed as a mass (Fig. 2C). The control procedure using buffer solutions without three types of enzyme (buffer/buffer/buffer) did not affect the HID/TCH/SP/PD reactions. The triple enzyme digestion method with chondroitinase B/testicular hyaluronidase/heparitinase either abolished or greatly diminished the reactivities of the HID/TCH/SP/PD-stainable all ultrastructural features in the extracellular matrix of the rat aorta. These features included the bundle of collagen fibers, the soluble matrix of interstitial space, the basal lamina of smooth muscle cells and the external surface of elastic lamina (Fig. 2D). However the stainings of the interior of elastic lamina remained unaffected, or tended to be slightly diminished in intensity by means of experimental and control procedures used in the present study (Figs. 2A, 2B, 2C, 2D).

The majority of the results obtained are summarized in Table 1.

DISCUSSION

In the present study, the triple enzyme digestion method consisted of a sequence of chondroitinase B, testicular hyaluronidase and heparitinase was utilized successfully to detect localization of glycosaminoglycans, such as dermatan sulfates, chondroitin sulfates (A and/or C) and heparan sulfates in the extracellular matrix of the rat aorta in electron microscopy.

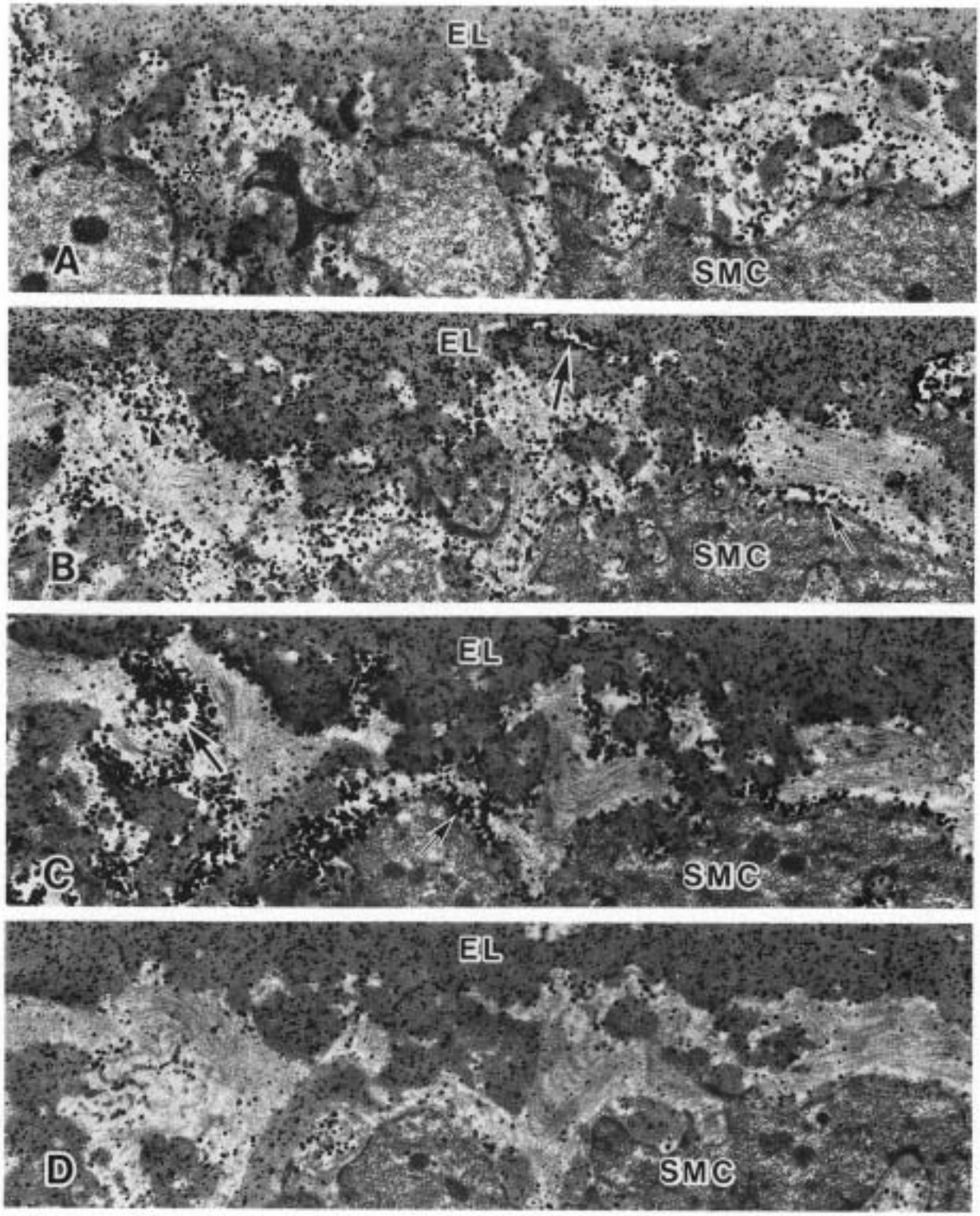

Fig. 2. Rat aortic tissue stained with (A) HID/TCH/SP/PD after control with buffer/testicular hyaluronidase/heparitinase, (B) HID/TCH/SP/PD after control with chondroitinase B/buffer/heparitinase, (C) HID/TCH/SP/PD after control with chondroitinase B/testicular hyaluronidase/buffer and (D) HID/TCH/SP/PD after triple enzyme digestion with chondroitinase B/testicular hyaluronidase/heparitinase. EL: elastic lamina, SMC: smooth muscle cell. × 18,000. (A): The soluble matrix of interstitial space, the external surface of elastic lamina and the basal lamina of smooth muscle cells exhibit either negative or exceedingly weak reactions,

Table 1. Effects of triple enzyme digestion upon the HID/TCH/SP/PD reaction of various ultrastructures in connective tissues of rat aorta

	HID/TCH/SP/PD	Buffer/T-Hylase/Hase HID/TCH/SP/PD	Chase B/Buffer/Hase HID/TCH/SP/PD	Chase B/T-Hylase/Buffer HID/TCH/SP/PD	Chase B/T-Hylase/Hase HID/TCH/SP/PD
Bundle of collagen fibers	++to+++	++	–to±	–to±	±to+
Soluble matrix of interstitial space	++to+++	–to±	++	–to±	–to±
Basal lamina of smooth muscle cells	+++	–to±	+	++	–to±
External surface of elastic lamina	+++	–to±	+to++	+to++	–to±
Interior of elastic lamina	++to+++	++	++to+++	++	++

T-Hylase: testicular hyaluronidase, Hase: heparitinase, Chase B: chondroitinase B, HID: high iron diamine, TCH: thiocarbohydrazide, SP:silver protein, PD: physical development. Staining intensities are scored from – (negative) to +++ (intensely positive). ±: A doubtful or exceedingly weak reaction.

The aim of this paper is to reveal localization of these glycosaminoglycans by means of the triple enzyme digestion method in electron microscopic histochemistry in tissues which include at least three kinds of the glycosaminoglycans, such as the extracellular matrix of the aortic media.

According to the results obtained in this study, the triple enzyme digestion method with chondroitinase B/testicular hyaluronidase/heparitinase resulted in a further diminution of the HID/TCH/SP/PD reactivities of the ultrastructural features in the extracellular matrix of the rat aorta, as compared with those in the extracellular matrix treated with three types of control procedures (buffer/testicular hyaluronidase/heparitinase, chondroitinase B/buffer/heparitinase and chondroitinase B/testicular hyaluronidase/buffer). Particularly, such a diminution was prominent in (1) the bundle of collagen fibers, (2) the soluble matrix of interstitial space, the basal lamina of smooth muscle cells and the external surface of elastic lamina and (3) the basal lamina of smooth muscle cells and the external surface of elastic lamina in comparison with (1) buffer/testicular hyaluronidase/heparitinase, (2) chondroitinase B/buffer/heparitinase and (3) chondroitinase B/testicular hyaluronidase/buffer controls, respectively. In view of staining selectivity of the HID/TCH/SP/PD procedure and substrate specificity of the glycosaminoglycan-degrading enzymes employed in the present study, it seems that dermatan sulfates are closely associated with collagen fibers, whereas chondroitin sulfates (A and/or C) are predominantly found in the soluble matrix of interstitial space. Such results obtained by the present study corresponded with those demonstrated by the previous histochemical studies of the extracellular matrix of the arterial wall [2, 15]. In the present study, in addition, both heparan sulfates and chondroitin sulfates (A and/or C) were observed in association with the basal lamina of smooth muscle cells and the external surface of elastic lamina. In a variety of tissues, the basal lamina has been known to contain not only heparan sulfates described as its major proteoglycan component but also chondroitin sulfates [1, 12, 13]. To the best of our knowledge, however, there is no report which reveals that both heparan sulfates and chondroitin sulfates coexist at the external surface of elastic lamina in the extracellular matrix of the aorta by electron microscopy. Contrary to this, the present method can distinguish ultrastructural localization of two types of glycosaminoglycans at the external surface of elastic lamina, that is, heparan sulfates are recognized as a mass, whereas chondroitin sulfates (A and/or C) are found intermittently along the external surface of elastic lamina. In the early phases of atherosclerosis the proteoglycans and their constituent glycosaminoglycans have been demonstrated to accumulate within intimal lesions in blood vessels. These increases appear to involve mainly dermatan sulfates and chondroitin sulfates, while heparan sulfates are indicated little change or even a decrease [11, 18]. Therefore, the present triple enzyme digestion method can be postulated as a reliable technique for precise detection of the glycosaminoglycans in the extracellular matrix of blood vessels and seems to be applied to visualization of a quantitative and/or qualitative modification of the arterial glycosaminoglycans in the atherosclerosis in electron microscopy.

whereas the bundle of collagen fibers (asterisk) show apparent positive reactions. (B): The bundle of collagen fibers shows either negative or exceedingly weak reactions, whereas the soluble matrix of interstitial space (arrowhead), the external surface of elastic lamina (big arrow) and the basal lamina of smooth muscle cells (small arrow) show distinct positive reactions. (C): The bundle of collagen fibers and the soluble matrix of interstitial space exhibit either negative or exceedingly weak reactions, whereas the external surface of elastic lamina (big arrow) and the basal lamina of smooth muscle cells (small arrow) show distinct positive reactions. (D): The bundle of collagen fibers, the soluble matrix of interstitial space, the external surface of elastic lamina and the basal lamina of smooth muscle cells show either negative or exceedingly weak reactions.

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