

Pathogenesis of Focal Cytoplasmic Necrosis of the Smooth Muscle Cells in Hypertensive Rat Arterial Media

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Hypertensive rat arteries exhibited severe medial smooth muscle cell injury and necrosis. Electron microscopic observations showed the smooth muscle cells of these arteries exhibited characteristics of focal cytoplasmic necrosis forming new cytodemarcating membrane between the healthy cytoplasm and necrotic cytoplasm. When the focal necrotic cytoplasm disappeared from the injured smooth muscle cells, it left it with a moth-eaten leaf-like appearance (moth-eaten necrosis). At an advanced stage of injury, smooth muscle cells changed to islet-like cell bodies with newly formed basement membranes around them, and further islet-like cell bodies and cell debris disappeared leaving lamellar and reticular basement membranes.

In hypertensive rats injected with nitroblue tetrazolium (NBT), formazan deposits were observed in the medial cells and nitrotyrosine, a biomarker of peroxynitrite, were immunohistochemically observed in the arterial media. Nick-end positive extranuclear small granular bodies, which might have derived from focal necrotic cytoplasm and nucleus, were detected in the arterial media using DNA nick-end labeling method. Based on electron microscopical and histochemical findings, we conjectured that the focal cytoplasmic necrosis of the smooth muscle cells in the arterial media depended on injury arising from mitochondria-derived oxidants.

Key words: hypertension, arterial injury, mitochondria-derived oxidant, focal cytoplasmic necrosis, smooth muscle cell necrosis

I. Introduction

Hypertension-induced ultrastructural alterations of smooth muscle cells, with focal fragmentation and cytoplasmic lysis, were observed in the arteries and arterioles [10, 36]. Smooth muscle cell fragmentation resulting from focal cytoplasmic necrosis differs from apoptotic, autophagic, and necrotic cell death. When observed using electron microscopy, the lesions showed focal cytoplasmic necrosis and a characteristic moth-eaten leaf- or islet-like structure

of the smooth muscle cells [34]. The pathogenesis of the smooth muscle cell fragmentation and cytoplasmic lysis has yet to be elucidated.

Oxidative stress, a state of excessive reactive oxidative species (ROS) activity, is associated with vascular disease such as hypertension [6, 33]. Many ROS possess unpaired electrons and thus are free radicals. These ROS include superoxide anion (O_2^-), hydroxyl radical ($HO\cdot$), nitric oxide ($NO\cdot$), and lipid radical. Other ROS, such as hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$) and hypochlorous acid ($HOCl$) are not free radicals, but have oxidizing effects that contribute to oxidant stress. Superoxide production by vascular tissues and its interaction with NO play important

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roles in vascular pathophysiology [6]. Hypertension increases O_2^- production in the blood vessels in spontaneously hypertensive rats or spontaneously hypertensive stroke-prone rats [18, 30]. Mitochondria can contribute to the generation of oxidant species in cell, tissues, and organism [16], as well as vessel wall [20]. The production of these oxidant species are regulated by receptor activation and stimuli such as stretch [37]. Hypertension-induced oscillatory shear has also been linked to an increase in ROS production, resulting in oxidative damage to the vascular wall cells [27]. NO is produced by mitochondrial NO synthase [12, 13] and the reaction of NO with superoxide anion yields the ROS, peroxynitrite [12, 13, 16].

Peroxyntirite has been defined as a potent oxidant [3, 4] and potential mediator of vascular tissue injury [3, 15]. Peroxyntirite anion can undergo protonation to form peroxyntirous acid, and peroxyntirous acid can undergo cleavage to yield nitrate, OH^- , NO_2^- , NO_2^+ , NO_3^- and NO_3^+ [7, 28]. Three of these cleavage products (OH^- , NO_2^- and NO_2^+) are among the most reactive and damaging species in biological system [7]. In the present study, we investigated the pathogenic role of these oxygen species and the morphogenesis of focal cytoplasmic necrosis of the medial smooth muscles in renal hypertensive rat arteries.

II. Materials and Methods

Male Wistar rats weighing under 50 g (3 weeks age) were subjected to bilateral renal artery constriction with silver clamps 0.25 mm in internal diameter. When systolic blood pressure measured indirectly with the tail using the Ueda sphygmomanometer (Ueda Co., Tokyo, Japan) had risen above 200 mmHg (210–260 mmHg), at 3, 4, and 5 weeks after operation, 5 animals each were sacrificed under deep ether anesthesia for the examination of the cerebral, coronary, and mesenteric arteries in the present experiment under the methods indicated.

Light microscopic methods

Six rats were sacrificed under ether anesthesia. At this time, the animal was perfused with heparinized saline, which was injected through a catheter into the left ventricle under a pressure of 120 mmHg and allowed to flow out from the inferior vena cava. Thereafter, 500 ml of 0.1 M cacodylate buffered 2.5% glutaraldehyde (pH 7.4) was perfused under a pressure of 120 mmHg and the cerebral, coronary, and mesenteric arteries were removed and refixed for 24 hr in the same fixative. These specimens were embedded in paraffin and the paraffin sections were stained with hematoxylin eosin for light microscopy.

Transmission electron microscopic methods

For transmission electron microscopy, 12 rats each were sacrificed under ether deep anesthesia at 3, 5, 6, and 9 postoperative weeks. Specimens of the 0.1 M cacodylate buffered 2.5% glutaraldehyde (pH 7.4) perfused arteries

were fixed with 1% osmium tetroxide in cacodylate buffer. They were then routinely dehydrated in a graded ethanol series, embedded in Quetol 812, sectioned by ultramicrotome, and stained with uranyl acetate and lead citrate for transmission electron microscopic observation.

Cytochemical demonstration of ATPase activity

Eight rats each were sacrificed under ether deep anesthesia at 3, 5, 6, and 9 postoperative weeks, and after simultaneous fixation of the arteries by perfusion with 1.25% glutaraldehyde and 4% paraformaldehyde solution (0.1 M cacodylate buffer, pH 7.4) for 5 min from the left ventricle, the cerebral, coronary, and mesenteric arteries were removed, and immersed in the same fixative for 30 min. The arteries were then cut into pieces and rinsed in 5% saccharose-supplemented 0.1 M cacodylate buffer for one hour. For electron microscopy 50 μ m frozen sections were immersed in Wachstein-Meisel medium according to Marchesi and Palade [23] at 37°C for one hour, then fixed with 1% OsO_4 for 30 min, stained with 2% uranyl acetate and after dehydration, embedded in Epon 812 and ultrathin section were prepared and stained with uranyl acetate and lead citrate for transmission electron microscopic observation.

Nitroblue tetrazolium chloride method

Four rats each at 3, 4, 5, 6, 7, 8, and 9 weeks after operation were perfused with Krebs-Henseleit buffered nitroblue tetrazolium chloride (NBT) (Funakoshi Co., Tokyo) (0.5 mg/ml) from left ventricle and followed by 100 ml of 4% paraformaldehyde under deep ether anesthesia which was allowed to flow out from inferior vena cava. The cerebral, coronary, and mesenteric arteries were removed after sacrifice and fixed with 10% formalin solution overnight. Paraffin sections were stained with nuclear fast red for light microscopic observation.

Immunohistochemistry and DNA nick-end labeling methods

Four rats each at 3, 4, 5, 6, 7, 8, and 9 weeks after operation were sacrificed under deep ether anesthesia and the blood was flushed out by heparinized saline perfused from left heart to inferior vena cava. Brains were removed and fixed for 4 hr with 10% formalin solution. The cerebral, coronary, and mesenteric arteries were removed and washed in 0.1 M phosphate buffered sucrose solution (10% sucrose in phosphate buffer, pH 7.4) for one hour, and after embedding in O.C.T. compound were frozen with liquid nitrogen. Six μ m thick tissue sections were prepared on a cryostat, dried at room temperature, and used for immunohistochemical localization of nitrotyrosine. Nitrotyrosine was detected by anti-nitrotyrosine (Upstate Biotechnology, Chicago, IL, USA) using a Histofine Streptavidin-Biotin Peroxidase kit (Nichirei Co., Japan). A negative control section, to which normal rat serum was applied, was included in immunostaining.

DNA fragmentation in situ associated with apoptosis

was detected by DNA nick-end labeling (transfer of biotinylated deoxyuridine to the 3'-OH ends of DNA) known as TUNEL method, according to the method of Gavrieli *et al.* [11]. Briefly, the formalin-fixed paraffin-embedded sections were deparaffinized, washed with distilled water (DW), treated with proteinase K (20 ng/ml) in 10 mM Tris-HCl buffer (pH 7.4), incubated at room temperature for 20 min, washed with DW, and incubated with 2% aqueous H₂O₂ solution to eliminate endogenous peroxidase. Each section was then washed with DW and treated with TdT buffer solution (potassium cacodylate 100 mM, CoCl₂ 2 mM, dithiothreitol 0.2 mM, pH 7.2) (Gibco BRL Life Technologies Inc. Gaithersburg, MD) containing TdT (terminal deoxynucleotidyl transferase, 0.3 equivalent U/ μ l) and biotinylated deoxyuridine triphosphate (dUTP, Boehringer Mannheim/Yamanouchi, Tokyo, Japan, 0.04 nmol/ μ l). Sections (with incubation mixture) were covered by coverslips and incubated at 37°C for 60 min in humidified chamber. Coverslips were then removed and sections washed with buffer solution (sodium citrate 30 mM, NaCl 300 mM) for 15 min, washed with PBS, washed with 10% normal rabbit serum for blocking of non-specific protein adsorption, washed again with PBS, incubated with streptavidin labeled with peroxidase for 30 min and finally stained with DAB/HO solution. For the positive control, a tissue section was treated with DNase I (0.7 μ /ml potassium cacodylate buffer, pH 7.2) (Stratagene Co., La Jolla, CA) for 10 min before treatment with TdT. For negative control, a sample was treated similarly, but with the addition of TdT buffer lacking TdT.

Normotensive rats (90–130 mmHg) were used as controls, and specimens of six rats for light and scanning electron microscopy, four rats for transmission electron microscopy, and two rats for immunohistochemical examination were prepared by the same procedure as described above. This study was carried out in conformity with the ethics criterion of the Geriatrics Research Institute.

III. Results

Normotensive rats

NBT perfused rat cerebral, coronary, and mesenteric arteries were macroscopically normal, and histologically NBT-reduced formazan deposits were not found in the arterial wall. Immunohistochemically, the arteries stained negative for nitrotyrosine. Negative control sections did not show any specific staining, with only slight background staining visible.

Electron microscopically, the medial smooth muscle cells showed a cobblestone-like arrangement with abundant myofilaments in the cytoplasm (Fig. 1a).

Hypertensive rats

Macroscopically, nodular lesions of the mesenteric arteries were found at 3 postoperative week and arterial lesion-induced cerebral softening and nodular lesion of the

coronary arteries were found at 6 and 9 postoperative week. Microscopic arterial lesions of the mesenteric artery, cerebral artery, and coronary artery showed the same findings showing characteristic of fibrinoid degeneration. Arteries without nodular lesions showed the same findings as normotensive rat arteries.

Electron microscopic findings

In transmission electron microscopic studies, early smooth muscle cell lesions exhibited focal electron dense cytoplasmic necrosis containing degenerated mitochondria and other cell organelle in the peripheral area. The focal necrotic cytoplasm changed to medium electron dense coagulation necrosis. Granular, vesicular, and tubular cell debris, and rupture of the mitochondria membrane and destruction of their crista were observed in the necrotic area of the smooth muscle cells (Fig. 1b). Nuclear fragments were not observed in the focal necrotic cytoplasm. Focal necrotic cytoplasm disappeared leaving cytoplasmic invagination of the smooth muscle cell cytoplasm resembling a moth-eaten leaf (moth-eaten necrosis) and with advanced lesions, many moth eaten leaf-like findings of the smooth muscle cells were observed (Fig. 1c). New cytodemarcating membrane was found between the healthy cytoplasm of the smooth muscle cell and cell debris which was derived from focal cytoplasmic necrosis of the smooth muscle cells. Injured smooth muscle cells changed to a small number of islet-like cytoplasm forming basement membrane-like lamellar and reticular substances as a result of repeated focal cytoplasmic necrosis in the peripheral cytoplasm around the islet-like smooth muscle cells (Fig. 1d). Usually the islet-like cytoplasm did not contain a nucleus, but a normal nucleus was occasionally observed in the islet-like cytoplasm (Fig. 1e). Normal nucleus of the injured smooth muscle cell which showed necrotic change of the whole cytoplasm was occasionally observed (Fig. 1f). A small or large amount of cell debris was found between the newly formed lamellar and reticular basement membrane-like substances, and nuclear debris was rarely found among the cell debris. After the islet-like cytoplasm and cell debris had disappeared, the lamellar or reticular basement membrane-like substances remained in the severely injured arterial media. Coagulation necrosis affecting whole cell bodies in the media was observed in rare instances. Electron microscopic findings for smooth muscle cell injury of the arterial media were the same as those for cerebral, coronary, and mesenteric arteries.

Cytochemical demonstration of ATPase activity

ATPase positive cell membrane was visualized as an electron dense reaction product. ATPase positive cell membrane formation was found between the normal cytoplasm and focal necrotic cytoplasm showing electron dense homogenous changes (Fig. 1g). The cell membrane of the islet-like cytoplasm that detached from smooth muscle cells was positive for ATPase.

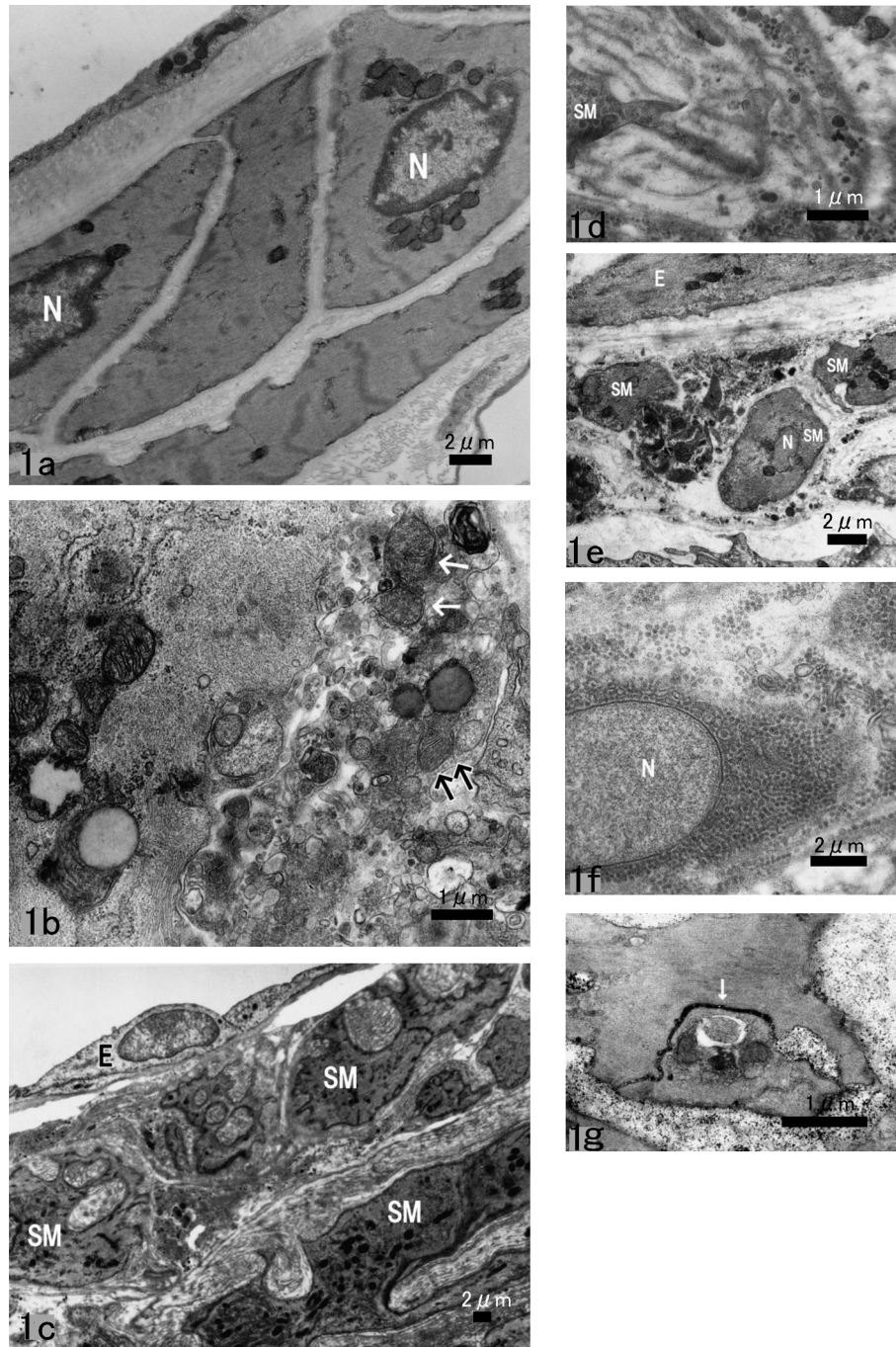


Fig. 1. a. Medial smooth muscle cells in cobblestone arrangement with smooth periphery. N: nucleus. Cerebral artery of normotensive rat. $\times 4,260$. b. Granulovesicular cell debris and rupture of the mitochondria membrane and destruction of their crista (arrow) are observed in the focal necrotic cytoplasm of a smooth muscle cell. Hypertensive rat mesenteric artery at 3 weeks after bilateral constriction of renal arteries. $\times 12,300$. c. Invagination and cribriform changes of the smooth muscle cell (SM) cytoplasm resulted of focal cytoplasmic necrosis is resembles a moth-eaten leaf (moth-eaten necrosis). E: endothelial cell. Hypertensive rat coronary artery at 6 weeks after bilateral constriction of renal arteries. $\times 1,870$. d. Newly formed basement membrane-like lamellar and reticular substances and granular cell debris in them are seen around the smooth muscle cell (SM). Hypertensive rat mesenteric artery at 6 weeks after bilateral constriction of renal arteries. $\times 12,200$. e. Smooth muscle cell (SM) cytoplasm shows islet-like separation. Some of them show changes to granulovesicular and electron dense cell debris. Nucleus (N) of an islet-like smooth muscle cell exhibits normal findings. E: endothelial cell. Hypertensive rat coronary artery at 5 weeks after bilateral constriction of renal arteries. $\times 3,900$. f. Nucleus (N) of smooth muscle cell shows normal findings and cytoplasm of smooth muscle cell shows granular changes. Hypertensive rat cerebral artery at 6 weeks after bilateral constriction of renal arteries. $\times 5,600$. g. Cell membrane (arrow) formed between normal cytoplasm and cytoplasm showing vacuolar and degenerative changes of cell organelles tests positive for ATPase. Hypertensive rat mesenteric artery at 3 weeks after bilateral constriction of renal arteries. $\times 16,500$.

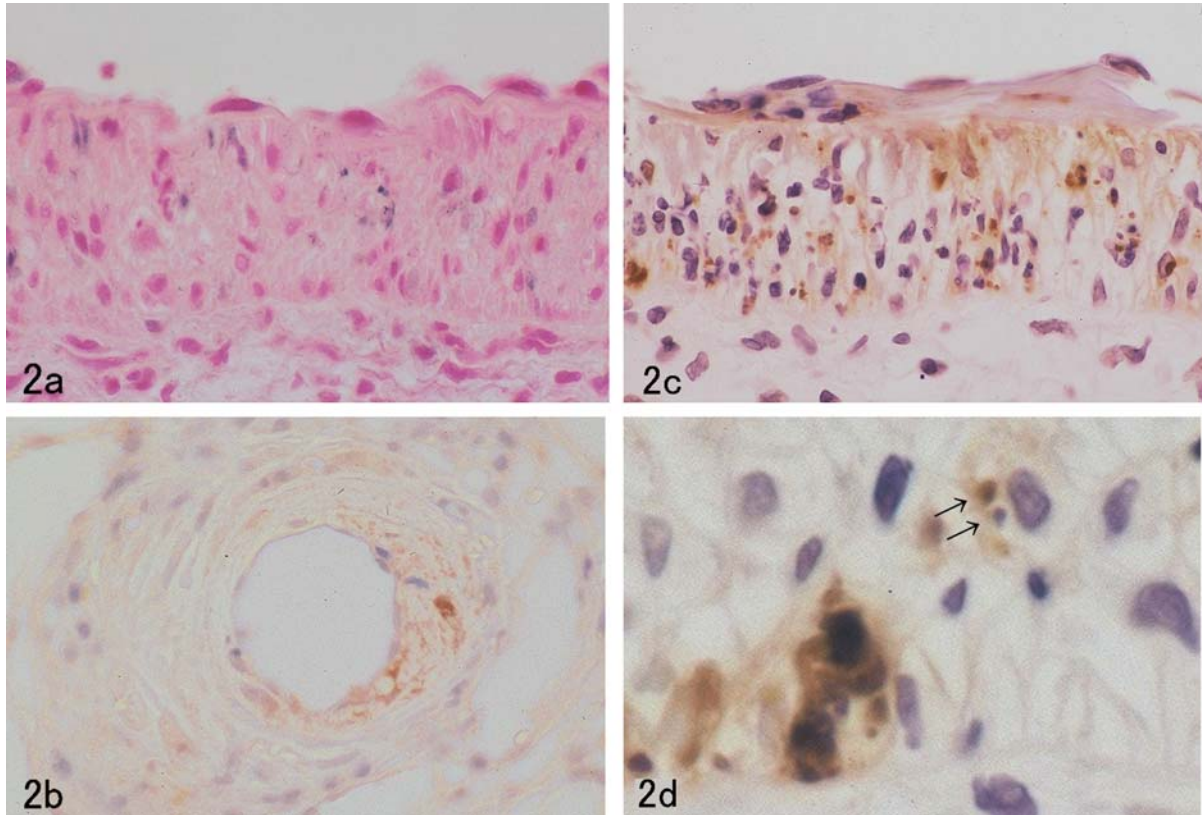


Fig. 2. a. Formazan deposits in NBT-injected rat in the medial smooth muscle cells. Hypertensive rat mesenteric artery at 5 weeks after bilateral constriction of renal arteries. Nuclear fast red stain. b. Nitrotyrosine tested positive for medial smooth muscle cells. Hypertensive rat cerebral artery at 5 weeks after bilateral constriction of renal arteries. Streptavidin-biotin peroxidase method. c. Whole and fragmented nuclei of smooth muscle cells were DNA nick-end positive. Hypertensive rat mesenteric artery at 3 weeks after bilateral constriction of renal arteries. DNA nick-end labeling methods. d. Nick-end positive granular bodies (arrows) were observed around normal nuclei which were positive with hematoxylin staining. Hypertensive rat mesenteric artery at 3 weeks after bilateral constriction of renal arteries. DNA nick-end labeling.

Macroscopic and microscopic findings of NBT

Macroscopically, formazan deposits were not observed in the arteries. Microscopically, however, NBT-reduced formazan deposits were sporadically observed in the endothelial cells and medial smooth muscle cells of the hypertensive rat arteries (Fig. 2a).

Immunohistochemical findings

Immunohistochemistry showed that the smooth muscle cells and interstitial tissue of hypertensive rat arterial media were positive for nitrotyrosine (Fig. 2b).

Nick-end labeling method findings

The whole and fragmented nuclei of smooth muscle cells tested positive when examined using nick-end labeling methods and nick-end positive small granular bodies were found close to the nuclei of smooth muscle cells (Fig. 2c). Nick-end positive granular bodies were observed around normal nuclei that stained positive with hematoxylin staining (Fig. 2d).

IV. Discussion

Electron microscopic findings showed that hypertensive medial lesions were characterized by focal cytoplasmic necrosis of the medial smooth muscle cells (moth-eaten necrosis). We observed that degenerated mitochondria and other cell organelles were present in the area of focal cytoplasmic necrosis, but no nuclear fragments. As a result of the focal cytoplasmic necrosis of the smooth muscle cells, dense granular, vesicular, and tubular cell debris were observed around the remaining non-injured cell bodies. The tubulovesicular cell debris may have originated from extracellularly discharged pinocytotic vesicles and Golgi vesicles. The granular cell debris may have been derived from myofilaments, since electron dense and filamentous structures were observed in the cell debris. In advanced lesions of smooth muscle cells, multiple areas of focal cytoplasmic necrosis were observed, creating a moth-eaten leaf-like structural appearance. In more advanced lesions, the smooth muscle cells changed to solitary islet-like cell bodies. These solitary islet-like cell bodies might have retained the function of smooth muscle cells because they

possessed native myofilaments, an ATPase-positive cell membrane, and very rarely contained normal-appearing nucleus.

The apoptosis of smooth muscle cells has been reported by many authors [8, 22] and the DNA fragmentation of smooth muscle cells has been observed [5]. Apoptosis is characterized by the budding and formation of apoptotic bodies [17]. Electron microscopically we did not observe any budding or apoptotic bodies in the injured smooth muscle cells of hypertensive rat arteries. Focal cytoplasmic necrosis does not belong to any of the three types of necrosis: type I (apoptotic cell death), type II (autophagic cell death), and type III (necrotic cell death) [19]. Thus, it appears to be a specific fourth type of cell death occurring in vascular smooth muscle cells. These findings without the apoptotic nuclear changes of arteriolar smooth muscle cell were observed in arterioles in swine infected with toxin-producing *Escherichia coli* [24]. In the present experiment, electron microscopy observations revealed whole body necrosis of smooth muscle cells, but nuclear fragmentation and budding were not observed. Using nick-end labeling method we observed positive smooth muscle cell nuclei and extranuclear small granular bodies in the vicinity of normal-appearing nuclei. Budding and apoptotic bodies [17] of smooth muscle cells were not observed in this experiment using electron microscopy. The nucleus in the islet-like cytoplasm of the arterial media was normal and did not show signs of apoptotic nuclear condensation [17]. Both DNA fragments derived from nuclear DNA and mitochondria DNA of the smooth muscle cells would be expected to stain positive using DNA nick-end labeling method. However, nick-end positive granular bodies were extranuclearly observed not only around nick-end positive nuclei but also normal staining nuclei. These nick-end positive granular bodies surely contain DNA fragments and must have derived from mitochondrial DNA.

NBT test revealed that hypertensive rat medial smooth muscle cells definitely produced superoxides. NBT test is widely used to measure superoxide anions released by neutrophils and macrophages during oxidative burst [1, 35], and reduced NBT is clearly visible as an insoluble blue deposit of formazan. Formazan deposits observed in the medial smooth muscle cells may reflect the reduction of NBT by superoxides produced by smooth cell mitochondria.

Mitochondria contain NO synthase [12, 13] and can contribute to the generation ROS [12, 13, 25, 26]. Hypertension induces several forms of stress on medial smooth muscle cells and may result in the production of large amounts of oxidant species by the mitochondria of medial smooth muscle cells [6]. Smooth muscle cells have numerous thin and thick myofilaments that are inserted into the dense bodies which are located in subsarcolemmal lesions [10]. Many mitochondria are found in the central sarcoplasmic core, but many appear in the sarcolemmal area [10]. Those myofilaments and mitochondria in the

subsarcolemmal area are thought to be easily subjected to hemodynamic stress and their destruction is associated with hypertension. The production of oxidant species is subject to regulation by receptor activation and stimuli such as stretch [37], and shear stress or hypertension induces ROS production [27]. Such mechanical stress in hypertension is linked to increased ROS production with consequent oxidative cell damage [27]. The mechanical stress to smooth muscle cells contributes to the production of ROS by the mitochondria [21]. Hypertension-induced mechanical stress is thought to influence mitochondria in the subsarcolemmal area and increase reactive oxygen species production by injury of the mitochondria with consequent focal cytoplasmic necrosis of the smooth muscle cells.

Estradiol reduces superoxides and in effect acts as an antioxidant [9, 29]. We examined whether an antioxidant substance would inhibit the smooth muscle cell lesions of the coronary artery and aorta with aging using the antioxidant 17- β -estradiol [31, 32]. 17 β -estradiol administration inhibited aging changes which showed focal cytoplasmic necrosis of medial smooth muscle cells such as hypertensive rat arterial medial cells [31, 32].

NO reacted superoxide anion forms a ROS, peroxynitrite [6, 12, 13, 33]. Peroxynitrite has been defined as a potent oxidant and potential mediator of vascular tissue injury [3, 15]. Mitochondria-derived NO may react with superoxide and form peroxynitrite which plays a large role in focal cytoplasmic necrosis.

Nitrotyrosine, a specific marker of peroxynitrite generation, is specially derived from the reaction of peroxynitrite with protein [2, 14]. In this study, nitrotyrosine was detected in the medial smooth muscle cells using immunohistochemistry. This result suggests that, in hypertension, the ROS derived from the mitochondria product peroxynitrite are capable of injuring the medial smooth muscle cells, resulting in focal cytoplasmic necrosis (moth-eaten necrosis) and severe medial cell loss.

In conclusion, mitochondria-derived ROS in the medial smooth muscle cells of hypertensive rats may have contributed to resultant fragmentation of mitochondria DNA, formazan deposition, nitrotyrosine deposition in arterial media, and medial cell injuries which are characteristic of focal cytoplasmic necrosis (moth-eaten necrosis).

Based on the observations made in this experiment, we hypothesized that focal cytoplasmic necrosis in the smooth muscle cells of hypertensive rat arteries depended on a mitochondria-derived oxidant and that the smooth muscle cell death is a fourth type of cell death which is different from the other three types of cell death: apoptotic, autophagic, and necrotic cell death.

V. References

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