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Intimal Thickening and the Distribution of Vasomotor Nerves in the Mechanically Injured Dog Coronary Artery

TOMOAKI TAGUCHI, YUTAKA ISHII, FUJIO MATSUBARA, AND KENZO TANAKA

Department of Pathology, Faculty of Medicine 60, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812, Japan

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Intimal injury and atherosclerotic change seem to be causative factors linked to spasm of the coronary artery. Intimal thickening was produced by mechanical injury to the endothelium of the canine coronary artery and we investigated the distribution of adrenergic, cholinergic, and peptidergic nerves in the coronary arteries. Although adrenergic and cholinergic nerves were not altered in density, neuron specific enolase positive nerve fibers were increased in number in dogs killed 1 and 3 months after injury. Substance P-containing fibers were also increased at 3 months after the induced injury.

INTRODUCTION

Spasm is an abnormal, enhanced contraction of the smooth muscle of the epicardial coronary artery. It is now recognized to be one cause of acute myocardial infarction, as well as unstable, variant, and typical effort angina pectoris. Coronary spasm occurs most commonly in atherosclerotic portions of the vessels (Maseri *et al.*, 1978). It has also been shown that severely atherosclerotic coronary segments are hypersensitive to histamine, *in vitro* (Ginsburg *et al.*, 1984) and *in vivo* (Shimokawa *et al.*, 1983). Thus, coronary atherosclerosis may be the primary factor in the pathogenesis of coronary artery spasm.

Although the effect of vasomotor nerves is an important factor in coronary spasm, there is little histological evidence concerning the relationship between the distribution of vasomotor neurons and atherosclerosis of the coronary artery. We induced an intimal thickening by mechanical injury in canine coronary arteries and investigated the distribution of adrenergic, cholinergic, and peptidergic nerves, using immunohistochemical and histochemical techniques.

MATERIALS AND METHODS

Intimal injury of coronary artery. Six adult mongrel dogs of either sex, weighing 7 to 15 kg, were anesthetized with pentobarbital sodium. The hearts were exposed under open chest surgery with mechanical ventilation. Intimal injury was produces with a balloon catheter (Fogarty, Model 12-060-2F) inserted into the left anterior descending coronary artery (LAD) via the anterior left ventricular branch (Baumgartner, 1963). The intima was then mechanically injured without disruption of the elastic lamina. Groups of three dogs were killed at 1 and 3 months (1 month—Group 1, 3 month—Group 2) after the operation. Six intact dogs served as the controls (Group 0).

LAD with surrounding connective tissue and cardiac muscles were dissected from the hearts. Samples of about 1 cm were taken from both proximal and distal sides of treated portions of the LAD. Each sample was cut into 3-mm ring segments. One was fixed with Zamboni's solution (1967) for 24 hr and embedded in paraffin. Two were snap frozen in *n*-hexane cooled by acetone dry ice and kept −70°C.

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Catecholamine fluorescence. The frozen specimens were freeze-dried in an vacuum evaporator and left to react for 1.5 hr at 80°C with paraformaldehyde that had been preequilibrated in a chamber with 60% humidity according to the method of Falck *et al.* (1962). The tissue blocks were embedded in paraffin and sectioned at 6 μm. Sections were viewed under a Zeiss fluorescence microscope.

Acetylcholinesterase (AchE) staining. The frozen specimens were cut transversely on a −20°C cryotome at 6 μm, then dried and incubated in acetylthiocholine iodide (Sigma) in the presence of iso-OMPA (Sigma) for 4 to 6 hr at room temperature (Karnovsky and Roots, 1964). The sections were then washed and observed under a light microscope.

Immunohistochemical study and hematoxylin and eosin staining. The specimens embedded in paraffin were cut into 6-μm-thick sections for immunohistochemistry and routine hematoxylin and eosin staining. For the immunohistochemical study, an enzyme-labeled antibody technique, the avidin–biotin peroxidase complex method was used (Guesdon *et al.*, 1979; Hsu *et al.*, 1981).

Primary antisera used were anti-native bovine brain neuron specific enolase (NSE) rabbit serum (Kuramitsu), anti-synthetic porcine vasoactive intestinal polypeptide (VIP) rabbit serum (R502, Yanaihara 1977), anti-Substance P (S-P) rabbit serum (IBL), and anti-Leu-Enkephalin (Enk) rabbit serum (IBL).

The deparaffinized sections were immersed in 0.03% (v/v) hydrogen peroxide in 0.01 *M* phosphate-buffered saline (PBS, pH 7.4) for 30 min at room temperature. Primary antisera were applied to the sections at 1:500 or 1:1000 dilution and the preparations were incubated at room temperature in a moist chamber for about 20 hr. The diluted biotinylated anti-rabbit IgG goat sera and the diluted avidin–biotinylated peroxidase were each incubated for one hr at room temperature. Visualization of the peroxidase was achieved by the diaminobenzidine method. The sections were then stained with methyl green and examined under a transmitted light microscope. Nonimmune rabbit sera were used instead of the primary antisera for the negative controls.

Quantitation. The thickness of intima was measured at three points equally divided, as shown in Fig. 1. The number of nerve fibers was estimated in every 200 μm of the medioadventitial (M-A) border of arterial wall.

RESULTS

Histology

Intimal thickening occurred in all the treated samples and the degree of thick-

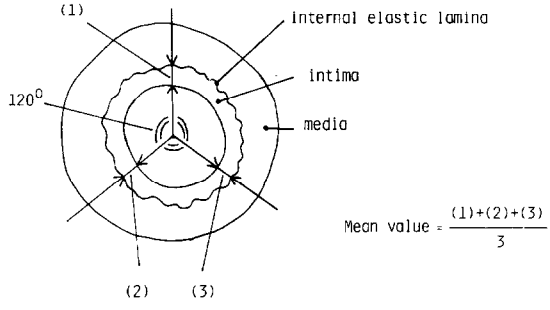

FIG. 1. The measurement of thickness of intima was done at three points equally divided and the mean values were used for the estimation.

ening was proportional to duration of time after the operation (Fig. 2). There were a few variations in the severity of intimal thickening between the dogs in the same group. Arteries in Group 0 (controls) showed no intimal thickening. The thickness measured was Group 0, 6.3 ± 0.6; Group 1, 31.4 ± 5.9; Group 2, 48.5 ± 6.2 μm. All treated arteries had reendothelialized.

Histochemical Analysis of Nerve Fibers

We counted the number of nerve fibers in every 200 μm of M-A border and the findings were summarized in Table 1.

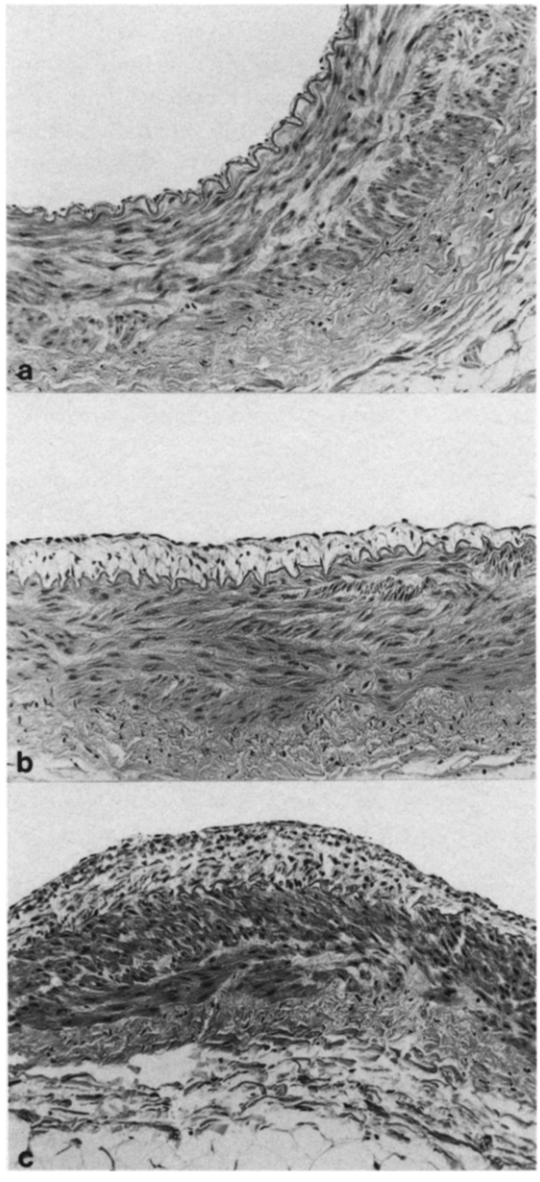

FIG. 2. Intimal thickening of dog coronary arteries after mechanical injury (H&E, ×128). (a) Control, (b) 1 month after injury, (c) 3 months after injury. The degree of thickening is proportional to the duration after injury.

TABLE I
Distribution of Vasomotor Nerves in Canine Coronary Artery

	Group 0 (n = 6)	Group 1 (n = 3)	Group 2 (n = 3)
NA	12.7 ± 3.1	14.3 ± 2.5	13.0 ± 2.6
AchE	13.5 ± 2.7	14.3 ± 2.1	15.3 ± 3.2
NSE	14.3 ± 2.3	24.3 ± 3.5*	25.3 ± 2.5*
S-P	5.7 ± 1.6	5.3 ± 1.5	16.0 ± 2.6*
VIP	0	0	0
ENK	0	0	0

Note. The number of nerve fibers was counted in every 200 μm of medioadventitial border. NA: noradrenergic fluorescent nerve. AchE: acetylcholinesterase reactive nerve. NSE: neuron specific enolase immunoreactive nerve. S-P: substance P immunoreactive nerve. VIP: vasoactive intestinal polypeptide immunoreactive nerve. ENK: Leu-Enkephalin immunoreactive nerve. NSE immunoreactive fibers of Group 1 and 2, and S-P fibers of Group 2 were significantly increased in number, compared with those of Group 0. Mean ± SD.

* $P < 0.01$.

(*A*) *Catecholamine fluorescence.* The blue-green noradrenergic (NA) fluorescent fibers were densely distributed in M-A border of the arterial wall in Group 0. Nerve trunks in surrounding fat tissue also contained NA fibers. The density of NA fibers in all treated groups was similar to that of Group 0 (Fig. 3). The number of NA fibers was Group 0, 12.7 ± 3.1; Group 1, 14.3 ± 2.5; Group 2, 13.0 ± 2.6 / 200 μm. There was no significant change between three Groups.

(*B*) *Acetylcholinesterase (AchE) staining.* AchE-containing fibers were evident in the M-A border of the arteries. There was no difference between treated groups and Group 0 (Fig. 4). The number of AchE fibers was Group 0, 13.5 ± 2.7; Group 1, 14.3 ± 2.1; Group 2, 15.3 ± 3.2 / 200 μm.

(*C*) *Neuron specific enolase (NSE) immunostaining.* NSE immunoreactive nerve fibers, representing whole compartments of the nerve system, were noted

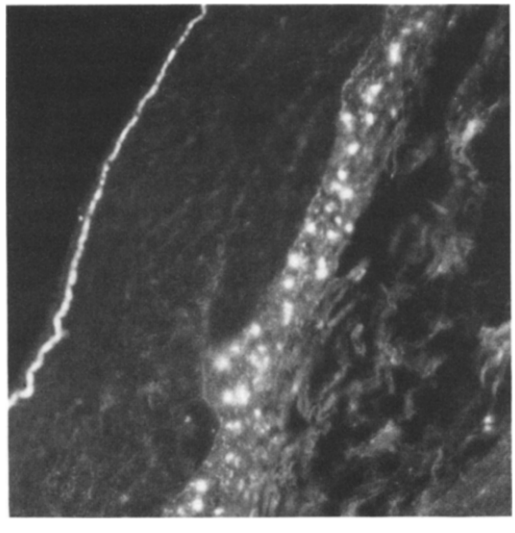

FIG. 3. Noradrenergic fluorescence (×150) in the coronary artery of control dog. Noraurenergic fluorescent fibers are seen in the M-A border of the arterial wall. The densities of fluorescent fibers in all treated groups are similar to that of controls. The fluorescent regular wavy line represents the internal elastic lamina.

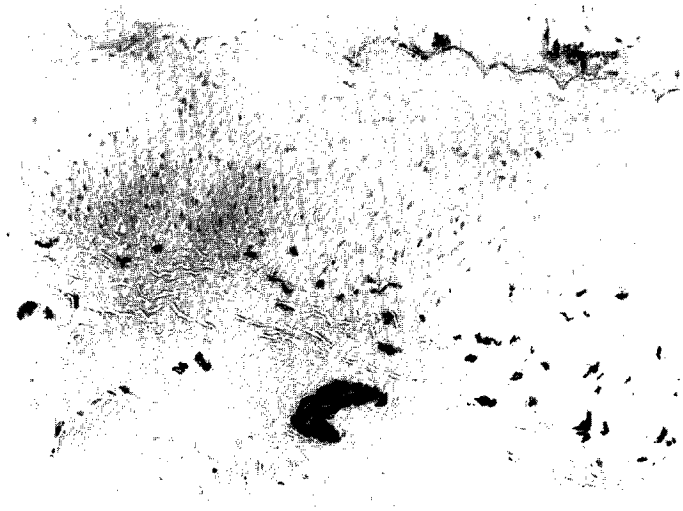

FIG. 4. Acetylcholinesterase staining in the coronary artery of control dog (×200). AchE-positive fibers are seen in the M-A border. All treated groups show similar densities to controls.

in the M-A border of arteries. The number of NSE-fibers was Group 0, 14.3 ± 2.3; Group 1, 24.3 ± 3.5; Group 2, 25.3 ± 2.5 / 200 μm. They were significantly thicker in Groups 1 and 2 than in Group 0 (Fig. 5). All nerve trunks showed NSE immunoreactivity in the surrounding adipose tissue and the cardiac muscle layer.

(D) Peptidergic nerve immunostaining. (i) Substance P (S-P). A few S-P-containing fine nerve fibers were present in the M-A border of the arteries in all groups and were significantly thicker in Group 2 than in the other Groups (Fig. 6). The number of them was Group 0, 5.7 ± 1.6; Group 1, 5.3 ± 1.5; Group 2, 16.0 ± 2.6 / 200 μm.

(ii) Vasoactive intestinal polypeptide (VIP). VIP-containing fibers were absent in almost all cases in each group. A slight VIP immunoreactivity was seen in only a few cases; however, as the numbers were few, a valid comparison could not be made.

(iii) Enkephalin (Enk). Immunoreactivity of Enk noted in the wall of the arteries was nil, in all groups.

DISCUSSION

Furchgott and Zawadzki (1980) observed that removal of the endothelium from isolated blood vessels abolishes the relaxation induced by acetylcholine. Thus, the functional integrity of the endothelium may be essential for maintenance of normal vascular reactivity.

Coronary spasm occurs most commonly in atherosclerotic portions of the vessels (Maseri *et. al.* 1978). Endothelial injury is thought to be one of the most important factors of spasm and several reports concerning the relationship between intimal injury and vascular contractility have appeared (Cocks and Angus, 1983; Kawachi *et al.*, 1984). The vasomotor nerves, localized in the adventitia (Denn and Stone, 1976), directly contract or relax vascular smooth muscle, and alteration of the distribution might relate to the occurrence of coronary vasospasm.

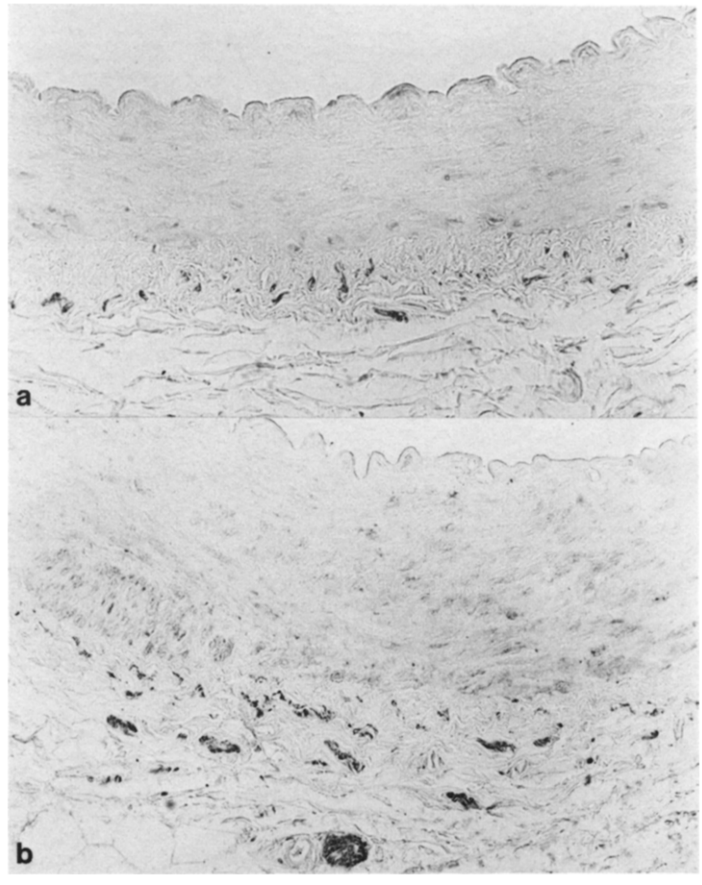

FIG. 5. Neuron-specific enolase immunostaining (×200). (a) Control, (b) 1 month after injury (Group 1). NSE immunoreactive nerve fibers are increased in number in Group 1, compared with controls.

We investigated the long-term effect of deendothelialization on nerve distribution in the coronary arterial wall. As intimal thickening was evident in all treated arteries, the results may also show a relationship between atherosclerotic change in the intima and the distribution of vasomotor nerves.

Both adrenergic and cholinergic fibers are present between the adventitia and the outer smooth muscle cells of the media in the canine coronary arteries (Denn and Stone, 1976; Muntz *et al.*, 1984). Our findings on these two nerve systems are similar. Distribution of these two types of fibers was not altered by the intimal thickening induced by the mechanical injury.

NSE is a superior marker of whole neurons (Bishop *et al.*, 1982). Our study suggested that some nerve fibers increased in number in the M-A border of coronary artery of dogs a few months after the injury (Groups 1,2).

S-P containing nerve fibers were reported to be localized mainly at the larger branches of the coronary vasculature (Weihe *et al.*,1984). The S-P system is considered to be one of the sensory neurons (Weihe *et al.*, 1984; Sharkey *et al.*, 1984). The increase of S-P nerves in Group 2 suggested the possibility of a hypersensitive state. One of the increased NSE-positive nerve components was S-P system.

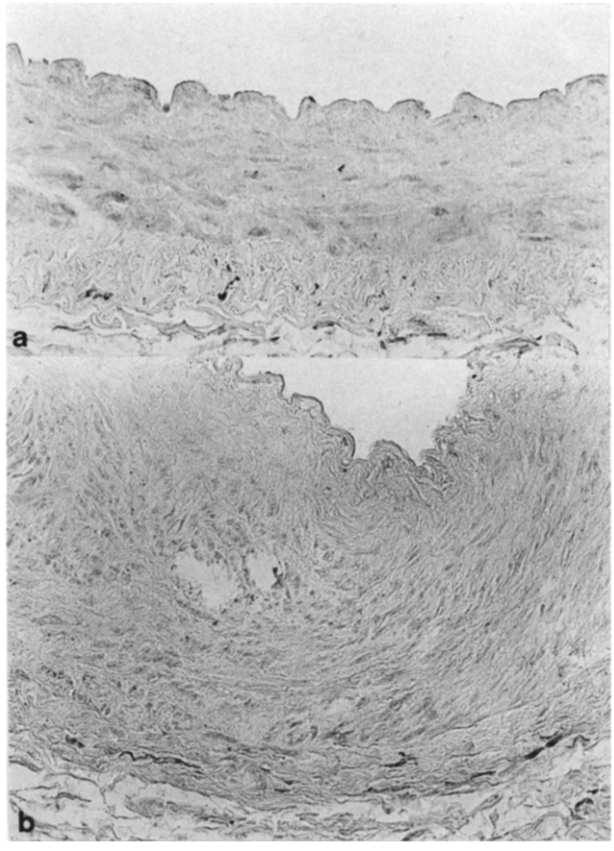

FIG. 6. Substance P immunostaining (× 220). (a) Control, (b) 3 months after injury (Group 2). A few S-P-containing nerve fibers are present in the M-A border. They are thicker in Group 2 than in other Groups.

The relationship between the severity of intimal thickening and the density of nerve fibers was shown in Fig. 7. The numbers of NSE and S-P fibers were not necessarily proportional to the thickness of intima.

VIP-containing nerve fibers are numerous around arteries in whole organs except for splenic, renal, and coronary arteries, in cats (Uddman *et al.*, 1981). Weihe *et al.* (1984) reported that VIP fibers were present around the coronary artery of several mammals, including the dog, but the number was few in the large and small branches, while it was high in smaller vessels and the terminal vasculature. We found no or very few VIP fibers around the main branch of LAD, in any Group. Pharmacologically VIP relaxes the smooth muscle of coronary artery and reduces the vascular tone (Smitherman *et al.*, 1982), and VIP is thought to be a neurotransmitter of nonadrenergic noncholinergic inhibitory nerve (Hunter *et al.*, 1984). The deficiency of VIP nerves around the main branch of coronary artery might be one of the factors that coronary artery would tend to be spastic, compared with other vessels.

There is radioimmunoassay evidence for the presence of enkephalins in the heart was shown by Lang *et al.* (1983). Enkephalins are peptidergic neurotransmitters related to the adrenergic system (Hökfelt *et al.*, 1980). However, we found no immunoreactivity around the coronary artery, in the present report.

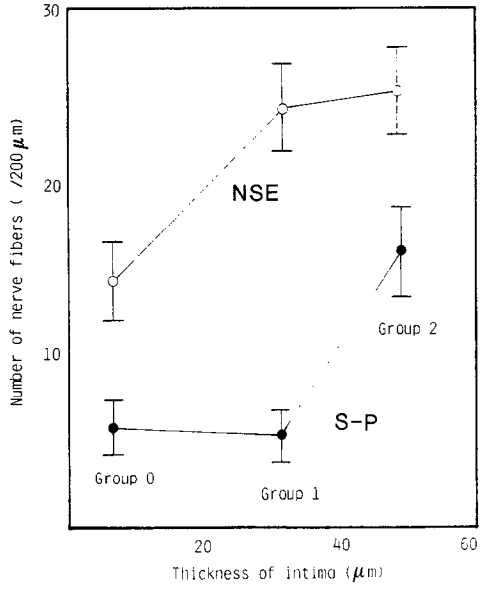

FIG. 7. Relationship between numbers of nerve fibers and intimal thickness. The numbers of NSE and S-P fibers were not necessarily proportional to the severity of intimal thickening.

Permeability to plasma proteins is increased in focal areas in the regenerated endothelium (Masuda and Tanaka, 1984). Regenerated endothelial cells had already covered the denuded surface in all our treated Groups. It has also been reported that permeated plasma proteins include the growth factor of smooth muscle cells. It may be that they also contained the growth factor of nerve fibers.

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