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Ultrastructural Studies of Experimental Arteriosclerosis

Arterial Lesions Produced by Repeated Contraction

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Organic changes in the gastric arteries of rabbits induced by experimentally repeated electric stimulation with A.C. 3 volt were examined electron microscopically in the order of chronology, and the following results were obtained.

As the changes at an early stage of experiments or within 3—5 days after the commencement of electric stimulation, crush-up effect and detachment of a part of cytoplasms in medial smooth muscle cells particularly in those in the vicinity of internal elastic lamina were observed. These fragments showed a tendency to increase electron density, to become granules and to be deposited in the intercellular space of the medial smooth muscle cells. At the same time, focal cytoplasmic necrosis and coagulation necrosis of smooth muscle cells were also observed at various localities. Another change at an early stage was marked increase of rough endoplasmic reticula and Golgi apparatus in fairly numerous smooth muscle cells. These organelles induced subsequently the increase of collagen fibers in the intercellular space of smooth muscle cells. As the changes at the latter stage of the experiments, the increase of collagen fibers and granular materials in the media, and the invasion and proliferation of smooth muscle cells in the subendothelial space (intimal thickening) were noted. It was discussed that repeated vascular contraction results in the lesions resembling the findings of arteriosclerosis of the muscular arteries in man, and that vascular contraction is considered as one of the pathogenic factors of arteriosclerosis in man.

INTRODUCTION

Since arteriosclerosis was reported by Scarpa (1804) and Virchow (1856), a number of outstanding pathological studies have been
presented and the pathogenesis has been attributed to vascular functional disturbance, lipid metabolic disturbance, hormonal disturbance, dietary factor, psychical or neurological factor, hereditary factor, disposition and other various factors\(^{1}\)\(^{18}\). However, all these factors are no other than speculated ones. \textit{Hueper},\(^{13}\), \textit{Byrom}\(^{3}\), \textit{Barnard}\(^{1}\), \textit{Hass}\(^{19}\) and others\(^{17}\)\(^{25}\) attempted to explain some sort of arteriosclerotic change by vascular contraction. Clinically theories attempting to explain various diseases by vascular contraction are not few. Raynaud's disease, anfall of angina, early stage of essential hypertension, angiospasmus theory of gastric ulcer and intracerebral angioneurosis may be enumerated as the examples.

Concerning physiological contraction of vessels, \textit{Semba}\(^{26}\) has given detailed description of the arterial site and vasomotor nerve. Light microscopically, \textit{Takebayashi}\(^{29}\) and \textit{Harano}\(^{9}\) have already reported that repeated vascular contraction by means of electric stimulation results in arteriosclerotic changes of the peripheral vessels such as intimal thickening, lipid deposits on the vascular wall and occasionally angioneurosis (fibrinoid necrosis).

In order to study as to what organic change is caused to the arterial wall concerned when vascular contraction which is a functional change is repeated, the author conducted experiments and observed the specimens light microscopically and electron microscopically. Further, vascular contraction was examined as one of the pathogenic factors of arteriosclerosis in man.

**MATERIALS AND METHODS**

*Experimental Animals:*

Adult white rabbits weighting approximately 3 kg were each placed in a cage irrespective of sex and were fed with Oriental RC 5* solid food. Their physical condition was observed for about 1 week prior to experiments and only those proving to be healthy were used for the experiments.

*Experimental Methods:*

There have long been used many reagents to effect vascular contraction, namely, bichloride of mercury, lead, calcium phosphate, phosphatic acid, barium chloride, alkaloid, ergotine, nicotine, strophanthin, adrenalin, etc. There have been many experiments after Josue (1903) to induce so-called adrenalin type arteriosclerosis using these reagents. However, the author used the method of low voltage stimulation following \textit{Takebayashi} and \textit{Harano} to obtain vascular contraction as natural as possible, since the use of any of the above

* The Oriental Yeast Co., Ltd., Japan.
reagents was suspected not to be able to eliminate such changes as may be caused by the reagent itself.

Seventy rabbits were anesthetized with ether and upper abdominal median incision was performed to expose the gastric anterior wall. At a certain location of the gastric wall with a relatively large artery at the center, silver electrodes measuring 0.3 mm in diameter were embedded into the serosa by approximately 0.2 cm with an interval of approximately 0.5 cm without hurting or oppressing the artery, and were fixed with suture. The small, vinyl-covered lead wires were first fixed with suture at the lesser curvature and the xiphoid process, then lead out through the dorsal subcutaneous region from the abdominal wall to the neck, and fixed with suture at the locations of lead out so as to prevent detachment of the electrodes as well as to have them endure lengthy electric stimulation. The electrodes and the lead wires were soldered and adequately covered with vinyl tube. The experiments were conducted aseptically to a possible extent but 150,000 unit penicillin was injected to each incised region. The stimulation devices used were a transformer with A.C. 100 volt at the primary side and 1-10 volt at the secondary side, and a specially devised automatic current interrupter. As to the procedure for stimulation, such procedure that would unfailingly effect sufficient contraction with a minimum voltage was sought in advance and, as the result, A.C. 3 volt 30 seconds stimulation with 2 minutes pause was found to be required and yet sufficient. This procedure was repeatedly continued for 6 hours daily in the fixing box. Accordingly, the actual stimulation time was one fifth of the period of experiments.

Macroscopic Findings against Stimulation:

Upon stimulation, the area of about 2.5 cm in diameter in the gastric anterior wall where electrodes were fitted showed marked contraction and the serosa became pale and anemic (Figs. 1, 2). Arteries were not at all observed macroscopically but veins became tortured indicating sporadical mass-like expansion. The stimulation being discontinued, these phenomena were gradually abated and finally restored to normal. The change of the gastric anterior wall to become pale and anemic seems to indicate both temporary contraction of the blood vessels and their secondary contraction due to the contraction of muscle layer of the stomach. If electric stimulation was made continuously, the reaction would be decreased gradually after 30-40 seconds and finally brought to naught. In the procedure of 30 seconds stimulation and 2 minutes pause, sufficient reaction was noted against each intermittent stimulation. This procedure provided a minimum and yet sufficient requirement to effect contraction.

Stimulation was commenced from the second day after surgery in consideration of the recovery from surgery. The region where
electrodes were embedded formed granulation after 5–7 days, and sufficient contraction was observed macroscopically though reaction to stimulation decreased in intensity. After the 20th day, reaction became so weak that contraction could not be observed macroscopically. The voltage was measured daily since sudden voltage drop was occasionally encountered despite the fact that the electrodes were completely fitted and no short circuit was noted. Those animals with accident during the experimental period such as death, detachment of electrodes, voltage drop despite the absence of electrode detachment or short circuit, and inadequate fixation, were excluded from the experiments. The control group was also provided with electrodes in the same manner but electric stimulation was not given. Blood flow was not measured since it is constantly variable and measurement method and measured values are least reliable.

The relation between the groups of experimental animals and the days of electric stimulation was as shown in Table 1. A total of 16 rabbits were used and the period of electric stimulation ranged from the minimum of 1 day to the maximum of 60 days.

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<th>1</th>
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<th>5</th>
<th>7</th>
<th>10</th>
<th>15</th>
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<th>40</th>
<th>50</th>
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<td>3</td>
<td>1</td>
<td>1</td>
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</tr>
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<td>1</td>
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<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
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**Fixation Method:**

Under anesthesia with ether, median incision was performed at the anterior neck and the lower abdominal region, and the bilateral jugular veins and the abdominal aorta were exposed. Upon injecting from the jugular veins 10 ml of 5% glucose solution containing 10,000 unit heparin, perfusion was made for approximately 20 minutes by 1% isotonic glutaraldehyde (pH 7.4) from the abdominal aorta with blood transfusion needle. The perfusion pressure at the commencement was 150 mmHg, which was then gradually decreased and was maintained at 110–130 mmHg after the cardiac arrest.

**Specimen Sampling Region:**

Specimens were obtained from the region 0.5 cm distant on the peripheral side from the electrodes in consideration of the effects of granulation and electrodes themselves. Most of the specimens were prepared for electron microscopy. These specimens were subjected to post-fixation with 1% osmic acid, dehydration with
RESULTS

Light Microscopic observations:

Some specimens which were obtained at an early stage of experiments (3–5 days) and showed little change at the light microscopic level, revealed the appearance of sudanophilic granules beneath the internal elastic lamina and the media in case of fat stain. However, these granules were negative in the specimens obtained on or after the tenth day. The Epon-embedded specimens obtained on or after the 30th day (36 or more hours of actual stimulation time) and stained with toluidine blue showed intimal thickening, wherein the proliferating cells were mostly of single layer. No intensive intimal thickening was encountered. Various medial changes seen electron microscopically (described below) could not be readily detected in Epon-embedded toluidine blue stain specimens.

Electron Microscopic Observations:

Clear electron microscopic changes were noted in the specimens obtained on or after the third day of stimulation. One of the changes was the crush-up effect of a part of medial smooth muscle cell cytoplasmas. In these cytoplasmas, a little part was detached from the main part appearing as if it was torn off. As a result, various sizes of fragments were formed (Figs. 4, 7, 8). The appearance of the fragmented cytoplasm was relatively frequent in the smooth muscle cells beneath the internal elastic lamina, partly in the form of protoplasmic process but mostly being detached from the cytoplasm. Such cell debris gradually showed vacuolation and finally became a small electron denser particle (Fig. 9). This change seemed to be more intensified when the artery in question was at the contracted stage (Fig. 7) but a similar change remained to be present even at the dilated stage (Fig. 8).

In the specimens obtained on the third to fifth day of experiments, findings suggesting partial or total coagulation necrosis of the medial smooth muscle cell cytoplasms were encountered in addition to the above change (Figs. 4, 5, 6). In other words, moderate electron dense amorphous materials were noted in the vicinity of irregularly atrophic smooth muscle cells, in most cases, in the basement mem-
brane. No hematogenous finding such as fibrin was noted. In such necrotic lesions or at the periphery, high electron dense granules and vesicles were sometimes observed but most of these materials were thought to be the residues of the crush-up effect\textsuperscript{33}) and necrosis of the above smooth muscle cells.

On the other hand, marked increase of smooth muscle cell organelles particularly of rough endoplasmic reticula and Golgi apparatus was seen where degeneration or necrosis was noted and even where such change was scarce (Figs. 4, 5, 11). In such locations, however, myofibriles contrarily decreased or even disappeared. The electron microscopic changes at the early stage of experiments may be summarized as the crush-up effect\textsuperscript{33}) of smooth muscle cell cytoplasms, partial necrosis of the cytoplasms as well as accelerated protein synthesis of smooth muscle cells in reaction to the stimulation. This seemed to be related to the increase of collagen fibers in the intercellular space of smooth muscle cells, which will be described later\textsuperscript{37}).

At the latter stage of the experiments from the 30th day to the 60th day, the above-stated necrotic lesions of smooth muscle cells decreased but there was a tendency that high electron dense particles and granules increased in the intercellular spaces (Fig. 9). At the same time, increase of collagen fibers in the dilated intercellular spaces became marked (Fig. 11) being accompanied with appearance of a small quantity of elastic fibers (part of amorphous components) in some localities (Fig. 9), and irregularly atrophic smooth muscle cells increased. At this stage, invasion and proliferation of smooth muscle cells in the subendothelial space began to appear (Fig. 11), and many of the cell organelles in the proliferated cells were well developed. In summary, the changes at the latter stage of the experiments were as follows. In the media, increase of collagen fibers in the intercellular spaces as chronic degeneration were generally observed. In the intima, intimal thickening due to the proliferation of smooth muscle cells began to appear.

The endothelial cells showed little or no difference from the control even in electron microscopic examination throughout the entire period of the experiments.

DISCUSSION

Light microscopic observations on the lesions induced by repeated contraction by means of electric stimulation to the blood vessels have already been described by TAKEBAYASHI\textsuperscript{31}) and HARANO\textsuperscript{99}). They mentioned angionecrosis (fibrinoid necrosis) and edematous thickening as the changes at the early stage. These changes seem to be identical
with the electron microscopic findings by the author such as fragmentation and and coagulation necrosis of cytoplasms and dilatation of the intercellular space of the media due to the deposition of many fragments and so on.

Previously, the deposition of high electron dense particles and granules in the intercellular space of smooth muscle cells of small arteries and arterioles in man was observed electron microscopically by many investigators\(^4\)\(^5\)\(^6\)\(^7\)\(^8\)\(^9\)\(^20\)\(^23\)\(^33\). The deposition is frequent particularly beneath the internal elastic lamina and it is considered to be the deposition of lipid particles transintimally transported from the blood. However, the fact that, in the present experiments, partial crush-up effect\(^9\) of smooth muscle cells was noted in the vicinity of the internal elastic lamina and these particles turned out to be high electron dense granules and vesicles, suggests that the above lesions in man are not totally from the blood but partially from the medial smooth muscle cells. The tendency that the crush-up effect of smooth muscle cells appears particularly in the vicinity of the internal elastic lamina may suggest, in consideration of the pictures in Figs. 7 and 8, that a sort of physical pressure of the internal elastic lamina induced by the contraction is applied to these smooth muscle cells.

The immediate causes of focal necrosis of smooth muscle cell cytoplasms and coagulation necrosis of the cells themselves could not be clarified by the present experiments. However, TAKEBAYASHI et al.\(^37\) observed various stages of necrosis ranging from evident focal necrosis of smooth muscle cytoplasms to fibrinoid necrosis in the mesenteric arteries of the rats with bilateral nephrectomy. Accordingly, while sudden contraction may be one of the factors, the presence of chemical material (angiotoxin) should also be considered.

Fibrous thickening and hyalinous thickening which were frequent after the middle stage of the experiments were considered electron microscopically to be caused mostly by the proliferation of collagen fibers in the intercellular spaces of medial smooth muscle cells and partly by the deposition of debris produced by fragmentation of cytoplasms and focal necrosis.

Previously collagen fibers were considered to derive from fibroblast. However, it has been clarified by the recent advancement of electron microscopic studies that smooth muscle cells of the blood vessel also have the function of mesenchymal cells and that collagen fibers are produced by these smooth muscle cells\(^36\)\(^37\)\(^39\). In this case, increase of cell organellas, particularly of rough endoplasmic reticula and Golgi apparatus, first enhances the synthesis of protein and acid mucopolysaccharide, and then the production of tropocollagen and component of collagen, which in turn may deposit on the vascular wall.

In addition to the increase of collagen fibers, increase of elastic
fibers, other amorphous materials and microfibrils is also noted and these materials have been considered to be produced by these modified smooth muscle cells themselves\(^5\)\(^6\)\(^9\)\(^10\)\(^11\)\(^12\)\(^13\)\(^14\)\(^15\)\(^16\)\(^17\)\(^18\)\(^20\)\(^21\)\(^22\)\(^23\)\(^24\)\(^25\)\(^26\)\(^27\)\(^28\)\(^29\)\(^30\)\(^31\)\(^32\)\(^33\)\(^34\)\(^35\)\(^36\)\(^37\)\(^38\)\(^39\). That the increase of cell organellas which is considered as hyperfunction appears at an early stage of vascular contraction suggests that this is the first defensive ecological reaction of the arterial wall concerned against contraction which is an abnormal physiological condition, and then secondarily expedites the formation of fibers for the reinforcement of the vascular wall.

The histogenesis of intimal thickening has been described by SPIRO\(^27\), FISHER\(^5\), ESTERLY\(^4\)\(^5\), TAKEBAYASHI\(^34\)\(^36\) and recent reports \(^14\)\(^19\)\(^24\)\(^30\). In the present experiments also, intimal thickening consisted in the proliferation of smooth muscle cells. These smooth muscle cells are likely to have penetrated through the fenestra of internal elastic lamina.

Intimal thickening is readily induced by anoxia of vascular wall \(^17\)\(^38\) experimental obstruction of vessels\(^7\)\(^12\) or endoarteritis\(^35\), and therefore it cannot necessarily be understood as an defensive or reparatory reaction of the vessel which is an ecological reaction against stimulation. One way or the other, however, it is evident that arterioscleroses in man, particularly intimal thickening which is the main lesion of muscular arteries, is induced even by repeated vascular contraction. It is believed that this fact gives a suggestive solution to the problem\(^18\) that arteriosclerosis in man varies greatly by individual and, even within the same individual, by the tissue or organ where the arteaies in question are distributed.

REFERENCES


ACKNOWLEDGMENT

This thesis submitted to School of Medicine, Nagasaki University, Nagasaki, Japan, in fulfilment of the requirements for the Ph. D. degree in Pathology.
Figs. 1 and 2. The stomach wall of the rabbits inclosing the vessels were subjected to prolonged intermittent electric stimulation. The silver electrodes are embedded in the serosa with interval distance ca. 0.5 cm each containing a large artery, so that the gastric artery together with the smooth muscle may repeat strong contraction. Fig. 2 shows a state which the gastric wall are contracted by electric stimulation, compared with the control (Fig. 1).
Fig. 3. Note a normal gastric artery of a rabbit (control). Endothelial cells (E) form continuously a single thin layer with tight junctions (↑). Internal elastic lamina (EL) shows uniform in thickness. Smooth muscle cells (M) arrange in normal in the media. ×7800
Fig. 4. Experimental group, after 3 days.
The cytoplasm of the some smooth muscle cells (M) beneath the internal elastic
lamina (EL) are fragmented and increased its density (↑). Some of the others
are vacuolated (↑↑). Note an amorphous dense material in the intercellular
matrix (↑↑↑). F- Fenestrae. ×5500
Fig. 5. Experimental group, after 3 days.
The similar alteration as Fig. 4 observe in the intercellular spaces. Some smooth muscle cells (M) increase RER and ribosomes (modified smooth muscle cell). x5000
Fig. 6. Experimental group, after 3 days.
Note the focal cytoplasmic necrosis (N) with dense granules. Some other smooth
muscle cells (M) turn to the modified smooth muscle cell. L- Lumen. ×5500
Fig. 7. Experimental group, after 5 days.
Note the contracted artery which was compelled by electric stimulation. Some of the smooth muscle cell cytoplasm beneath the internal elastic lamina (EL) are crushed-up and minced irregularly (↑). Some other smooth muscle cells show increased densito (big arrow). ×3400
Fig. 8. Experimental group, after 5 days.
Dilated artery of the same experimental rabbit as Fig. 7. The crush-up effect is demonstrated in the smooth muscle cell cytoplasm beneath the internal elastic lamina (EL) as in Fig. 7 (†). ×2600
Fig. 9. Experimental group, after 30 days.
Note the fragmented cytoplasmas and increase of the dense granules (††) in the intercellular space. Some of them are vacuolated (V). ×7800
Fig. 10. Experimental group, after 30 days.
Increase the collagen fibers (C) in intercellular space of the media. The smooth muscle cells are fragmented or irregularly atrophic. ×5300
Fig. 11. Experimental group, after 50 days.
Note the immigration of modified smooth muscle cells (M) increasing RER, into the subendothelial space. Proliferation of collagen fibers (C) and the modified smooth muscle cells are seen in the media. ×6000