Vessel Healing in Dogs after Endarterectomy with and without Aspirin Administration

—Observations by Light and Scanning Electron Microscopy—

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Abstract

Healing of the carotid artery after endarterectomy in adult mongrel dogs was investigated at intervals from 15 minutes to two weeks after surgery by light and scanning electron microscopy. In the control group, a fibrin-platelet carpet was found on the surface of the endarterectomized wall of the vessel soon after surgery. A large, typical, red thrombus was noted on the endarterectomized surface in three of six control animals 15 minutes after the operation, and in another one hour after the operation. On Day 1 after surgery, no new thrombus formation was detectable. On Day 3, most of the mural thrombi had disappeared, and formation of new-endothelium had started and was completed about one week after the operation. In the group treated with aspirin, thrombus formation in the wall was much less just after operation, and no large thrombi were seen in any animals. Almost no newly formed endothelium was detectable on Day 3. After one week, new endothelium covered only about 60% of the inner surface of the artery, but after two weeks the formation of new-endothelium was almost complete in all dogs. Thus, with aspirin treatment, formation of new endothelium seemed to be delayed and the new endothelial cells seemed to grow somewhat differently from those in the control group.

Key words: carotid endarterectomy, aspirin, scanning electron microscopy, new-endothelium

Introduction

Cerebral ischemic diseases in Japan are thought to differ slightly in etiology from those in Europe: Intracranial vascular lesions seem to be frequent causes of cerebral ischemic diseases in Japanese, whereas extracranial lesions are frequent causes of this condition in Europeans. However, this difference in etiology seems to be decreasing, because in increasing numbers, Japanese extracranial vascular lesions have been reported to cause strokes and transient ischemic attacks. Therefore, carotid endarterectomy of the neck has become a rather common operation in Japan.

Aspirin is often used in treatment of cerebral ischemic diseases due to extracranial lesions.^{1,8,20,31,33,38,39} There are many reports on healing of the carotid wall after endarterectomy,^{7,12,23,26,42,43} but there are few descriptions of the effects of aspirin on healing of the intima after surgery.^{13,26}

This paper reports studies by light and scanning electron microscopy on the effect of aspirin on healing of the endarterectomized intima of dogs.

Methods

Adult mongrel dogs weighing 10–15 kg were used. The experimental group was given an oral dose of aspirin of 50 mg/kg/day from 7 days before carotid endarterectomy until the time of sacrifice.

The dogs were anesthetized by intravenous injection of 20 mg/kg of pentobarbital and the common carotid artery was exposed. The exposed vessels were occluded at both ends with low pressure Heifetz aneurysm clips and a longitudinal incision of 6 to 7 mm length was made in the anterior wall of the common carotid artery under a model K-380FW surgical microscope (Asahi Electron Industry Co.). A sheet of tissue from the internal surface of the vessels of 2–3 mm length, including the endothelium, internal elastic laminae, and part of the muscular layer of the media, was cut out *en bloc* circumferentially (Fig. 1). Then, the cavity was washed with heparin solution (200 units/dl) and the opened vessel was sutured with nylon thread (9–0

Fig. 1 A longitudinal incision of 6 to 7 mm length was made in the anterior wall of the common carotid artery under a surgical microscope. A sheet of tissue of the internal surface of the vessels of 2-3 mm length, including the endothelium, internal elastic laminae and part of the muscular layer of the media, was cut out *en bloc*.

monofilament).

The operation was carried out aseptically, and penicillin was administered intramuscularly for three days after the operation. The control group was subjected to carotid endarterectomy only. Platelet aggregation activity was measured preoperatively in both groups. Six dogs from each group were sacrificed at each interval of 15 minutes, 30 minutes, one hour, three hours, one day, three days, one week and two weeks after surgery. At the time of sacrifice, a catheter was introduced into the proximal part to the endarterectomized area of the common carotid artery and the distal side of the endarterectomized area was tied. The operated region was washed with 100 ml of normal saline under a pressure of 100 mm H₂O to remove blood from the lumen. Then, a portion including the endarterectomized region was removed. The longitudinal suture line was opened under a surgical microscope. The specimen was divided into two pieces by a longitudinal incision along the midline. For scanning electron microscopy (SEM), one part was placed in 2.5% glutaraldehyde for two hours, washed with phosphate buffer, treated with 1% osmic acid for two hours at room temperature, and then dehydrated with 50% to 100% acetone. Then, the specimen was mounted on an aluminum grid, coated with gold, and examined with a Hitachi model SSM-2 scanning electron microscope. For light microscope (LM), the other half of the tissue was placed in formalin, embedded in paraffin, and sectioned at right angles to the inner wall of the vessel. Sections were stained with hematoxylin-eosin (HE), Masson's trichrome stain for connective tissues, Lawson's elastin van Gieson stain for collagen fibers, muscle fibers and elastic tissues, and phosphotungstic acid-hematoxylin (PTAH) stain for tissue fibrins.

Results

Platelet aggregation activity (for collagen) was found to be $45.4 \pm 13.1\%$ before carotid endarterectomy in the control group. In the aspirin treated group, the value was $45.7 \pm 18.4\%$ before aspirin administration and $22.7 \pm 12.4\%$ before carotid endarterectomy (Table 1). Thus, aspirin clearly inhibited platelet aggregation.

In the control group, the endarterectomized portion was covered with a relatively thick thrombus 15 minutes after surgery, and formation of large thrombi was noted in three of six dogs (Fig. 2). These thrombi were typical: platelets and red blood cells were closely enmeshed in strands of fibrin. In the other three dogs, the endarterectomized portion was filled with blood cell components. It was easily distinguishable from the surrounding region because a fibrin-platelet carpet, consisting of fibrin, platelets, and some red cells, covered the endarterectomized surface (Fig. 3). White blood cells were seen on the surface of the thrombus in all 6 dogs. At 30 minutes after surgery, no large thrombus was seen, but one of 6 dogs killed one hour after surgery had a large thrombus similar to that seen in the 3 dogs 15 minutes after surgery. The volume and appearance of the fibrin-platelet carpet in the control group were similar 15 minutes, 30 minutes and one hour after surgery. No adhesion of the thrombus to

Table 1 Platelet aggregation activity (for collagen)

Control group before CEA	$45.4 \pm 13.1\%$
Aspirin group before aspirin treatment	$45.7 \pm 18.4\%$
after aspirin treatment for 7 days (before CEA)	$22.7 \pm 12.4\%$

Fig. 2 A large thrombus adhering to the endarterectomized surface 15 minutes after endarterectomy in a control dog. Bar=100 μ

Fig. 3 Scanning electron micrograph showing an acute mural thrombus formed on the endarterectomized surface 15 minutes after surgery in a control dog. The thrombus formed on top of the initial fibrin-platelet layer consisting of fibrin, platelet and red blood cells. Bar=10 μ

the normal endothelium adjacent to the endarterectomized area was observed. In the aspirin treated group, a similar fibrin-platelet carpet was observed in the endarterectomized portion 15 and 30 minutes and one hour after surgery, but thrombus formation was not marked. Thus aspirin inhibited formation of a fibrin-platelet carpet and formation of large thrombi.

Three hours and one day after surgery the endarterectomized portions were covered by a fibrin-platelet carpet in both groups and there were no large thrombi. One day after surgery, a decrease in blood cell components was seen in the carpet in the control group and the surface became smooth (Fig. 4). The aspirin treated group still had a carpet at this time which appeared similar to that of the control group 3 hours after operation.

In the control groups, a swelling, which seemed to be due to neo-intima formation, appeared along the

Fig. 4 Scanning electron micrograph showing the amorphous surface of the platelet carpet, which seemed to be formed by flattened platelets 24 hours after endarterectomy in a control dog. Bar=10 μ

Fig. 5 New endothelial cells on the endarterectomized edge 3 days after surgery in a control dog. Bar=10 μ

Fig. 6 Photomicrograph showing few new endothelial cells 3 days after surgery in an aspirin treated dog. Bar=10 μ

Fig. 7 Endarterectomized area of a control dog on Day 7 almost entirely covered with neo-intima. Bar=10 μ

margin of the endarterectomized portion on Day 3. No adhesion of blood cell components was seen in this area (Fig. 5), but no initial fold, such as that usually seen in normal endothelial cells, was recognized on the neo-intima. In the aspirin treated group, no neo-

Table 2 Morphological changes at the site of carotid endarterectomy

Group	Time	Aspirin group			Control group		
		T.T.	F-PC	RE	T.T.	F-PC	RE
1	15 min	0 case	(6++) cases	0 case	3(++) cases	6(+++) cases	0 case
2	30 min	1(+)	6(+++)	0	0	6(+++)	0
3	1 hour	0	6(+++)	0	1(+)	6(+++)	0
4	3 hours	0	6(+++)	0	0	6(+++)	0
5	Day 1	0	6(+)	0	0	6(+++)	0
6	Day 3	0	6(+)	3(±~+)	0	6(+)	6(+)
7	Day 7	0	6(+)	6(+)	0	5(±~+)	6(+++)
8	Day 14	0	2(±)	6(+++)	0	0	6(+++)

T.T.: Typical thrombi, F-PC: Fibrin-platelet carpet, RE: Reendothelialization. Each group consisted of 6 dogs. Numbers represent those dogs in which the findings were positive.

intima was detectable at this time (Fig. 6). On Day 7, the endarterectomized area was almost entirely covered with neo-intima in the control group (Fig. 7), but in the aspirin treated group only about 60% was covered with neo-intima. Thus, formation of new endothelium was apparently delayed in this group; in the aspirin-treated group the endarterectomized area was not covered with neo-intima until Day 14. These results are summarized in Table 2.

Discussion

In this work, large thrombi consisting of platelets and red blood cells enmeshed in fibrin were found in three of six control dogs on Day 1 after surgery. In the aspirin treated group, thrombus formation was scarcely detectable at these times. In the control group, formation of new endothelium was completed with neo-intima by Day 7, whereas in the aspirin treated group, this was not observed until Day 14.

Three origins of new endothelial cells have been proposed: 1) remaining normal endothelial cells; 2) smooth muscle cells of the media; and 3) blood cells. In scanning electron microscopic studies on endarterectomized areas of the dog carotid artery, Dirrenberger *et al.*⁷⁾ detected a fibrin-platelet carpet from 30 minutes to four hours after surgery, and observed thrombi covered with smooth, flat, amorphous layers, from 48 to 72 hours after surgery without formation of new thrombi. They concluded that these amorphous and homogeneous layers were composed

of fibrin and deformed platelets. They also reported that the new endothelium originated from the remaining normal endothelium that gradually covered the carpet. Many reports support this theory.^{4-6,9-11,23)}

Schwartz *et al.*³⁴⁾ reported studies on the ultrastructure of the aortic lining during repair of mechanically denuded aortic intima in the rat. They used transmission electron microscopy (TEM) to examine the cells appearing during the healing process after denudation of the endothelium and classified them morphologically into 3 types; A, B and C. Type A cells were definite endothelial cells. Type B cells had the following characteristics: 1) they were enmeshed in the sub-endothelial connective tissue in many, but not all cases; 2) they had few vesicles or vacuoli; 3) they contained filaments at the cell periphery; 4) they showed no membrane contact or fusion with other Type B cells or with Type A cells; 5) irregular-shaped deposits of material resembling basement membrane were present on their luminal cell membrane; and 6) they were cuboidal or highly squamous in shape. Type C cells were seen near the central portion of the lesion. The cytoplasm of Type C cells was clearly divided into two regions: 1) the perikaryon, which was embedded in the intimal connective tissue and was not morphologically distinguishable from that of other cells of the intimal thickening, and 2) a portion of peripheral cytoplasm that projected from the luminal surface forming part of the aortic lining. They suggested that Type B and C cells were smooth muscle cells of an undifferentiated type and that these cells disappeared within a month, leaving only Type A cells (definite endothelial cells). They concluded that the Type B and C cells originated from smooth muscle cells of the media.⁴¹⁾ Spaet *et al.*³⁵⁾ also observed the random appearance of new endothelial cells in the early stage of healing of the damaged area. They observed a clear border-line between the remaining normal endothelial cells and the new endothelial cells, and found that smooth muscle cells of the media migrated into the intima in the early stage through the internal elastic laminae. Accordingly, they supported a two-origin theory, namely, that in the early stage new endothelial cells were formed from smooth muscle cells of the media, and in the later stage from remaining normal endothelial cells. Recent work by Yokoyama *et al.*^{42,43)} also supports their two-origin theory. Ghani *et al.*¹⁴⁾ and Stump *et al.*³⁷⁾ reported that monocytes were colonized and modified to form new endothelial cells, and concluded that they originated from blood cells. But there is little other support for this theory at present.^{23,35)} Although theories on the origin varies, there is general acceptance of the changes in the intima over time during the healing proc-

Table 3 Process of reendothelialization

ess.^{15,17,21,24,28,30,32,36}) That is, healing consists first of adhesion of platelets to the endarterectomized surface, followed by aggregation of platelets, and then by precipitation of fibrin which is influenced by intrinsic coagulation. After formation of what Dirrenberger *et al.*⁷⁾ named the fibrin-platelet carpet, the endothelial cells are regenerated (Table 3). In the present work, carpets of fibrin with enmeshed platelets and red blood cells were formed in the endarterectomized area, flattening and fusion of platelets occurred, and then amorphous and smooth substances covered the endarterectomized area. In the control group, the neo-intima originating from existing adjacent endothelium of the ridge of the lesion was formed later.^{4-7,9-11,21})

The absence of isolated islands of endothelium on the endarterectomized surface suggests that the endothelium did not develop from the underlying medial cells^{31,38}) or from blood elements^{14,21,32}) in the control group. In the present experiment, cells named ovoid cells by Yokoyama *et al.*⁴³⁾ appeared during formation of the new endothelium. Ovoid-type cells were more numerous in the aspirin treated group than in the control group. Bhawan *et al.*²⁾ reported that smooth muscle cells had disappeared from the carotid artery of Wistar rats, and that undifferentiated cells appeared in the media three days later, and differentiated cells on Day 7. They considered that these undifferentiated cells might be smooth muscle cells that had lost the morphological features of smooth muscle cells. Fishman *et al.*⁹⁾ reported that in the early stage, new endothelial cells gradually became flat and changed to normal endothelial cells within a month. Reviewing their reports, Yokoyama *et al.*^{42,43)} concluded that the ovoid-type cells might be formed from smooth muscle cells of the media.

It is very interesting that there were many new endothelial cells which might be regarded as originating from smooth muscle cells of the media in the aspirin treated group in our study. A uniform eosinophilic layer was noted with light microscopy and concluded to be a layer of platelets, because it was detected beneath the new endothelium by hematoxylin-eosin stain, and was stained blue with phosphotungstic acid-hematoxylin (Fig. 8A, B). The thickness of this

A

B

Fig. 8 A: Eosinophilic layer, thought to be a platelet carpet, below the new endothelium. (HE stain $\times 400$)
B: The carpet beneath the new endothelium stained blue, suggesting the presence of fibrin. (PTAH stain $\times 400$)

Fig. 9 Scanning electron micrograph of an endarterectomized vessel 3 days after surgery in a control dog showing a fibrin-platelet carpet beneath the new endothelium. Bar=10 μ

layer that stained blue was similar in both the control and the aspirin treated groups. Scanning electron micrograph showed a fibrin-platelet carpet beneath the neo-intima (Fig. 9).

Poole *et al.*²⁷⁾ observed with transmission electron

Fig. 10 Scanning electron micrograph showing ovoid-type cells on the endarterectomized surface 7 days after surgery in an aspirin treated dog. Bar=10 μ

microscopy that platelets aggregated on the surface of the thrombi in the early stage and that the surface then became covered with closely packed platelets. Yokoyama *et al.*⁴³⁾ reported little infiltration of plasma components into the amorphous material layer, which was considered to be a stage before formation of the neo-intima. They suggested that this layer, or the platelets themselves, might have a direct relation with abnormal transmission of vessel walls, and that this layer substituted for endothelial cells until the reendothelialization was completed. Our results suggest that platelets and fibrin of a regular volume on the endarterectomized surface may be necessary for initiating the reendothelialization.

Platelets contain arachidonic acid, which is a precursor of prostaglandin (PG). The arachidonic acid in the platelets is easily released, and is transformed into prostaglandin cyclic endoperoxide in the presence of cyclooxygenase. Then two enzymes transform the endoperoxide PGH_2 into one of two unstable products having strong, yet mutually opposing, biological activities; thromboxane A_2 (TXA_2) and prostacyclin (PGI_2). TXA_2 is produced in the presence of thromboxane synthetase found in platelets, whereas PGI_2 is produced in the presence of prostacyclin synthetase, which is present in abundance in endothelial cells in the arteries and veins but which is present at successively lower concentrations from the intima to the adventitia. Aspirin inhibits production of prostaglandin cyclic endoperoxides by acetylating the cyclooxygenase and irreversibly inhibiting the action of this enzyme. Thus, aspirin prevents production of not only the powerful platelet aggregating substance TXA_2 , producing vasoconstriction, but also of the potent antiaggregating substance PGI_2 , producing vasodilatation.

A small dose of aspirin strongly inhibits the produc-

tion of TXA_2 in the platelets,^{3,8,40)} but a large dose of aspirin inhibits the production of PGI_2 in the wall of the vessels as well. According to Kelton *et al.*,¹⁸⁾ thrombus formation was increased by a large dose of aspirin. Hence, in dogs, a dose of 50 mg/kg/day, by inhibiting cyclooxygenase, prevented the production of TXA_2 in the platelets more strongly than production of PGI_2 in the vessel walls, and so inhibited platelet aggregation. At the same time, the aspirin reduced the activity of the intrinsic coagulation system, especially the activity of the platelet third factor. Subsequently, it reduced fibrin production and the volume of thrombi. When the process of adhesion of platelets is reduced, reendothelialization may be delayed. This was considered to be why reendothelialization was delayed in the aspirin treated group.

Current clinical evidence suggests that many transient ischemic attacks (TIA) in both Europe and America are due to embolization of platelets, fibrin, or atheromatous debris from intravascular atheromatous lesions of extracranial cerebral arteries, including those of the heart. However, in many cases in Japan, it is questionable whether TIA is due to formation of microthrombi. Clinical findings suggest that hemodynamic factors, such as cardiac arrhythmia²⁹⁾ and orthostatic hypotension¹⁹⁾ are as important as stenotic lesions of extracranial cerebral vessels. But, recently in Japan, TIA due to microthrombi has been increasing. Recently the Canadian Cooperative Study Group reported a 48% risk reduction ($P < 0.005$) in stroke or death in male patients with aspirin administration, but there was no apparent reduction in females with this treatment. There is evidence of reduction of venous thrombosis in male, but not in female patients with aspirin treatment after hip-replacement surgery.¹⁷⁾ In another study, thrombosis was significantly reduced only in male rabbits by a small dose of aspirin, and in both sexes by a larger dose. PGI_2 may account for thrombosis in normal vascular endothelium. The greater sensitivity of vascular cyclooxygenase to aspirin in female patients could account for the sex difference. The author is uncertain whether reduction of thrombosis in the acute stage after surgery and delay of reendothelialization in the aspirin treated group indicate that aspirin acts in the postoperative stage of carotid endarterectomy and in the prevention of TIA. Further studies are required on the effects of other doses of aspirin and on the healing process of vessels after endarterectomy.

Conclusions

1. Endarterectomy of the common carotid artery

was performed on adult mongrel dogs treated with aspirin at a dose of 50 mg/kg/day. The effect of aspirin on the healing process of the intima after surgery was studied morphologically.

2. In the aspirin treated group, thrombus formation was retarded in the endarterectomized region in the acute stage after surgery, and reendothelialization was delayed.
3. The number of ovoid-type cells tended to be greater during reendothelialization in the aspirin treated group. These findings suggest that the healing process was somewhat different in the aspirin treated group from that in the control group.

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