Cerebrovascular Injuries Induced by Activation of Platelets

in Vivo

TSUKASA FUJIMOTO, M.D., HIDENORI SUZUKI, PH.D.,
KENJIRO TANOUE, M.D., YOSHIHARU FUKUSHIMA, M.D., AND
HIROH YAMAZAKI, M.D.

SUMMARY Intravascular platelet aggregation induced by ADP injection into the carotid artery of rabbits caused ipsilateral cerebrovascular injuries. We have observed the details of these in vivo vascular changes under the electron microscope. Intracytoplasmic vacuole (1.0-2.0 μm in diameter) formation and partial deendothelialization followed by platelet thrombus formation were characteristic changes in the middle cerebral artery. These vacuoles did not contain horseradish peroxidase (HRP) which was used as a marker of vascular permeability change. Compared with these phenomenon, increased vesicular (0.05-0.2 μm in diameter) transport was prominent, and vacuole formation was rarely seen in small vessels, namely, capillaries and arterioles in the cortex. Endothelial cell damage seemed to be more prominent in large arteries, but only the smaller vessels show marked extravasation of HRP-reaction product and perivascular edema. Blood levels of TXB2 and 6-keto PGF1α were significantly increased 3 min after the ADP injection and returned to pre-injection levels at 60 min after. These results suggest that vasoactive substances resulting from platelet activation may play an important role in producing cerebrovascular injuries caused by platelet aggregation induced with ADP.

Materials and Methods

Experiment 1

Forty-four white male rabbits, weighing 2.9-4.0 kg, were used. Under general anesthesia with sodium pentobarbital (50 mg/kg, intraperitoneally), a polyethylene catheter was inserted into a lingual artery, and its tip was advanced to the carotid bifurcation. Using this method a platelet aggregating substance was injected into the cerebral arteries without disturbing the blood flow of the right internal carotid artery. Five min after an intravenous injection of 100 unit/kg of heparin, 0.7 mg/kg of sodium arachidonate (AA) (Sigma) (concentration: 2 mg/ml) or 20-40 mg/kg of ADP (Sigma) (concentration: 40-60 mg/ml) was injected through the catheter into the right carotid artery of each of 26 rabbits. AA was injected within 5 sec, and ADP was injected within one min. In another 18 animals, after clamping at the proximal part of the right common carotid artery, warm (37°C) physiological saline was injected through the catheter under a pressure of 120 cm H2O for 10 sec to wash out the blood in the right carotid and cerebral arteries. Then the same dose of AA or ADP was injected into the catheter. During the injection and until one min later, the vessels were continuously irrigated with saline and the clamp was released. Immediately after, and 5 and 60 min after the injection, the animals were decapitated and the brains were removed. Several pieces of the middle cerebral artery and its branches in the subarachnoid space were dissected and fixed with 1% glutaraldehyde-2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 90 min at 4°C. The procedure for taking the artery samples was performed within 15 sec after sacrifice of the animals. The samples were rinsed five times in the same buffer and postfixed with 1% osmium tetroxide in the buffer for 90 min at 4°C. After dehydration with
graded ethanol, the specimens were embedded in Epon 812. Ultrathin sections were doubly stained with uranyl acetate and lead citrate and observed under an electron microscope (JEM-100S, JEOL, Japan) at 80 KV.

Experiment 2
To observe the process of vascular changes, horseradish peroxidase (HRP, Type VI, Sigma, USA) was injected as a tracer into 12 rabbits. For injections, a polyethylene catheter was inserted in the same manner as in Experiment 1. Five, 15, or 60 min after ADP injection, the animals were sacrificed. The dose and concentration of ADP were the same as in Experiment 1. Fifteen min before the sacrifice, HRP (25 mg/kg) was injected into the ear vein. One ml/kg of 2% Evans blue was injected 15 min before these animals were sacrificed. Immediately after sacrifice, the brain was removed. Small pieces of the middle cerebral artery with the cerebral cortex were removed and fixed with 1% glutaraldehyde-2% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4) for 90 min at 4°C. After rinsing with the same buffer, 40 μm-thick sections of the samples were cut using a vibratome (Model G, Oxford Laboratory, USA). These sections were incubated for 60 min at room temperature in modified Graham-Karnovsky medium7 (10 ml of 0.05 M Tris-bated for 60 min at room temperature in modified Graham-Karnovsky medium7 (10 ml of 0.05 M Tris-HCL buffer, pH 7.6, containing 0.002% H2O2 and 5 mg of 3-3'diaminobenzidine tetrahydrochloride DBA, grade II, Sigma, USA). As a control, some of the sections were incubated in the same medium without H2O2. They were rinsed three times with cacodylate buffer and postfixed with 1% osmium tetroxide in the same buffer for 30 min at 4°C. After embedding in Epon 812, ultrathin sections stained with uranyl acetate and lead citrate or unstained sections were examined under a JEM-100S electron microscope.

Experiment 3
Blood levels of thromboxane B2 (TXB2) and 6-keto PGF1α were measured before and after ADP injection in 9 rabbits. A polyethylene catheter for injection of ADP was prepared as in Experiment 1, and another polyethylene catheter was inserted into the right internal jugular vein to collect blood samples periodically. Five min after the intravenous injection of 100 units of heparin, 40 mg/kg of ADP was injected into the right internal carotid artery. Thirty min after the ADP injection, 1 ml/kg of 2% Evans blue was injected into the right ear vein. Blood (5 ml) was taken from the internal jugular vein before, 3 and 60 min after the ADP injection. Sixty min after the injection, the rabbits were decapitated and the brains were removed to observe vascular changes. Assay of TXB2 and 6-keto PGF1α were performed as previously described.4 The blood (5 ml) was collected into a heparinized syringe and indomethacin (final concentration of 10^-5 M) was added to it immediately. The plasma collected by centrifugation at 2000 x g for 10 min was stored at -80°C for no longer than 30 days before the measurement of TXB2 and 6-keto PGF1α. Crude lipid extracts of samples obtained by the procedure of Folch et al9 were separated from nonlipid contaminants on Sephadex G-25 columns following the modified method of Wuthier.10 After evaporation of the solvent from the eluate, the residue was dissolved in carbon tetrachloride; the prostaglandins were extracted with 10% methanol/phosphate buffer (pH 6.8). The aqueous phase was acidified with hydrochloric acid to pH 3.0. After extraction of the prostaglandins with ethylacetate, the ethylacetate layer was neutralised with ammonium hydroxide. After evaporation of the solvent, the TXB2 and 6-keto PGF1α fractions obtained were separated by thin layer chromatography on silica gel. The amount of each of the purified prostaglandins was measured by radioimmunoassay using the antisera for TXB2 and 6-keto PGF1α (Ono Pharmaceutical Co., Osaka, Japan).

Results
Experiment 1
In the group of animals in which AA was injected when blood was present in the vessel, remarkable changes were seen in the vessel wall of the injection side, even immediately after the AA injection (fig. 1). Deendothelialization was a characteristic change. Platelets adhered to the luminal surface and the platelets showed shape change. Such a finding was observed in 3 out of 5 animals. In part of the vessel, migration of red blood cells into the internal elastic lamina was observed. Edematous changes in the muscular layer were also observed. Sixty min after the AA, platelet thrombi with fibrin formation on the deendothelialized surface were found abundantly. Marked edematous changes were seen in the subendothelial and muscular layers. These changes were seen in 4 out of 5 animals. When the blood was replaced with saline, similar deendothelialization and edematous

FIGURE 1. Electron microscopic findings of the middle cerebral artery immediately after an injection of arachidonic acid. Characteristic findings were deendothelialization and platelet adhesion on the surface. In some parts, penetration of red blood cells into the internal elastic lamina (arrow) was observed. Edematous changes in the muscular layer (*) was also seen. Abbreviations in figures 1-11: EC: endothelial cell, IEL: internal elastic lamina, RBC: red blood cell, SM: smooth muscle cell, PL: platelet.
FIGURE 2. Middle cerebral artery immediately after an injection of ADP. A small aggregate of platelets with vacuoles (arrow) is seen in the vascular lumen. No injury is recognizable in the vascular wall including endothelial cells, internal elastic lamina and smooth muscle cells.

changes were recognized even immediately after the AA injection.

When blood was present in the vessel, immediately after the ADP injection, small aggregates of platelets were seen in the vascular lumen of the injection side in all 5 animals. Platelets showed slight shape change. However, the vascular wall showed a normal appearance (fig. 2). After five min, intracytoplasmic vacuole formation (1.0–2.0 μm in diameter) in the endothelial cells was seen (fig. 3). Tortuosity of internal elastic lamina which might suggest vasospasm was observed. There was vacuole formation in the muscular and subendothelial layers. After 60 min, abundant intracytoplasmic vacuole formation and tortuosity of internal elastic lamina were seen in 3 out of 5 animals. Deendothelialization was partially observed, and platelets adhered to such regions. Edematous changes in the subendothelial layer and smooth muscle cells were observed (fig. 4). The endothelial junctions were preserved. In some areas, a large number of platelet thrombi were formed on the deendothelialized area

FIGURE 3. Middle cerebral artery 5 min after an injection of ADP. Tortuous internal elastic lamina, vacuole formation in the endothelial cells (arrow), the subendothelial layer and smooth muscle cells (*) are revealed.

FIGURE 4. Middle cerebral artery one hour after an injection of ADP. Vacuolation in the endothelial cells is remarkable. Note the adhesion of platelets (arrow) at the area of deendothelialization. Tortuosity of internal elastic lamina and vacuole formation in subendothelial and muscular layers are remarkable.

(fig. 5). These vascular changes were observed only on the side injected with ADP. On the other hand, when the blood was replaced with saline before the ADP injection, the vascular wall was intact not only immediately after but also at 60 min after the injection (fig. 6).

Experiment 2

Five min after the injection of ADP, a number of small vesicles (0.05–0.2 μm in diameter) which contained black HRP-reaction products were seen beneath the endothelial surface of the middle cerebral artery on the injection side (fig. 7-A). The numbers of vesicles were more than 4 to 6 times compares to those on the noninjection side (fig. 7-B). Vacuoles (1.0–2.0 μm in diameter) were also seen sporadically in the cytoplasm of the endothelial cells on the injection side. The vacuoles did not contain HRP-reaction products. Fifteen min after the injection, intracytoplasmic vacuoles were frequently observed. HRP-positive vesicles
FIGURE 6. Middle cerebral artery one hour after an injection of ADP. Intravascular blood was temporarily washed out with saline throughout from 10 sec before and to one min after the injection. The vascular wall shows no pathological changes.

were seen beneath the endothelial surface, but the increase in their numbers or size was not remarkable compared to that observed after 5 min. Sixty min after the injection, many intracytoplasmic vacuoles were observed in the endothelial cells on only the injection side in 3 out of 5 animals. In these endothelial cells, the cytoplasm was filled with black HRP-reaction products. Tortuosity of the internal elastic lamina was prominent (fig. 8). Even in these arteries, extravasation of HRP-reaction products into the subendothelial and muscular layers was not clear in the non-staining section (fig. 9).

On the other hand, in smaller vessels, namely, arterioles and capillaries in the cortex, endothelial cell damage seemed to be different from that observed in the middle cerebral artery. Fifteen min after the injection, the number and size of HRP-positive vesicles in endothelial cells were increased, but vacuoles were rarely observed. After sixty min various sizes and numbers of HRP-positive vesicles were increased, while vacuoles were rarely seen. Prominent extravasation of black HRP-reaction products into the perivascular area with edematous changes were seen only on the injection side in 3 out of 5 animals (fig. 10 and 11) and these changes were never seen on the noninjection side.

Experiment 3

Before the ADP injection, blood levels of TXB2 ranged from 0.124 to 0.560 ng/ml, with a mean and standard deviation of 0.346 ± 0.143 ng/ml. After three min it increased significantly (p < 0.02) to 1.266 ± 0.741 (0.289-2.410) ng/ml. After sixty min it returned to 0.460 ± 0.294 (0.100-0.999) ng/ml, similar

FIGURE 7. Middle cerebral artery 5 min after an injection of ADP. Increase of the number of small vesicles (arrow), about 5 times of noninjection side which contain black HRP-reaction products are seen beneath the endothelial surface ipsilateral to injection (A). Contralateral to the side of injection (B), only few such vesicles were seen. The vascular lumen is filled with HRP-reaction products.

FIGURE 8. Middle cerebral artery one hour after an injection of ADP. Remarkable HRP-negative vacuoles in the endothelial cells (arrow) are seen. The tortuosity of the internal elastic lamina is also seen.

FIGURE 9. The same case as shown in figure 8 without staining. Many HRP-negative vacuoles are observed in the endothelial cells. The cytoplasm was filled with black HRP-reaction product. In the cytoplasm of an endothelial cell without vacuole formation, HRP-reaction products are not present. Extravasation is not seen.
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FIGURE 10. Capillary in the cortex of the injection side sixty min after an injection. Various sizes and numbers of HRP-positive vesicles (arrow) are increased in endothelial cells, but HRP-negative vacuoles are rarely seen. Prominent perivascular edematous changes (*) are revealed. To the pre-injection level. The level of 6-keto PGF_{1α} was 0.833 ± 0.403 (0.488-1.770) ng/ml before the injection. It increased significantly to 5.427 ± 1.521 (3.750-8.200) ng/ml after 3 min (p < 0.001). After sixty min, it returned to 1.342 ± 0.707 (0.803-2.440) ng/ml.

Discussion

In addition to our reports,1-2 there have been several other reports that induced platelet aggregation may initiate damage of vascular walls.13 However, the morphological changes and mechanism have not been clarified. In the present report, we have observed that vascular injuries occurred after one of the platelet aggregating agents such as ADP was intraarterially injected into cerebral arteries. These injuries were observed when ADP was injected into the cerebral arteries which were filled with circulating blood. When the blood was washed out with physiological saline, the injuries were not observed during the experimental time. When arachidonic acid was injected into the cerebral arteries instead of ADP, the marked injuries were observed even without the presence of circulating blood. Since arachidonic acid is known to have a detergent effect,14 the vascular injuries induced by arachidonic acid may be caused at least partially by this effect. Because of this, we could not determine whether the platelet-induced vascular injuries were present or not in the case of the experiments using arachidonic acid.

The pathological changes in the middle cerebral artery induced by intraarterial injection of ADP were observed only on the ipsilateral side of the brain and these characteristic changes are as follows: 1) Intracytoplasmic vacuole formation and increased vesicular transport in endothelial cells. 2) Sporadic deendothelialization and adhesion of platelets which advanced to thrombus formation with fibrin deposition on this part. 3) Edematous changes in the subendothelial and muscular layers. 4) Tortuous internal elastic lamina, suggesting the presence of vasospasm. 5) The tight junctions were well preserved.

In the middle cerebral artery, increased vesicular transport was recognized beneath the endothelial surface at 5 min after the ADP injection. These vesicles (0.05-0.2 μm in diameter) contained HRP-reaction product and showed little increase as time elapsed. On the other hand, vacuole formation (1.0-2.0 μm in diameter) in the endothelial cytoplasm was enhanced as time elapsed; HRP-reaction products were not found in the vacuoles. In a large number of endothelial cells, the vacuoles were found abundantly and there was a diffuse invasion of HRP-reaction products in the cytoplasm, suggesting that there was cell damage. The tight junctions seemed to be preserved. Even in these injured vessels, extravasation of HRP-reaction products were not seen. This indicates that the enhancement of vascular permeability, which was recognized by the invasion of HRP-reaction products, was not related to the genesis of these types of injuries. Details of the vacuole formation are not clear, but swelling of mitochondria or endoplasmic reticulum would be related to their genesis.

In comparison with the phenomenon in the middle cerebral artery, in the small vessels within the brain tissue, vacuoles were scarce, and there were numerous HRP-positive vesicles in the cytoplasm of endothelial cells. Remarkable extravasation which was associated with the enhanced vesicular transport was seen in these small vessels. Endothelial cell damage was more dominant in large arteries, but extravasation and perivascular edema were mainly seen in smaller vessels. Although the reason why such a difference would occur between large and small vessels is not clear, the vasospasm which was observed in the large arteries may play some role in the changes of large vessels.

In speculating upon the cause of these vascular injuries, it may be necessary to consider the role of ischemia induced by platelet aggregation and/or thrombosis. However, the platelet aggregates were
quickly disaggregated, and no obstruction in blood vessels with platelet aggregates could be found even at 5 min after the injection. Moreover, it is known that vessels themselves are relatively resistant to such a degree of ischemia. As one of the important causes of these vascular injuries, substances released from platelets must be considered because of their strong vasodilating effect.\textsuperscript{15,18} It has been observed that platelets release various vasoactive substances such as serotonin, prostaglandin endoperoxides (PGG\textsubscript{2}, PGH\textsubscript{2})\textsuperscript{19} and thromboxane A\textsubscript{2} (TXA\textsubscript{2})\textsuperscript{20,21} when they aggregate. Although evidence exists that the released substances may have an effect on vessel walls \textit{in vitro}, there are few reports which document its effect on vessel walls \textit{in vivo}.\textsuperscript{11,12} We have measured blood levels of TXA\textsubscript{2} and found an increase in TXB\textsubscript{2}, which is the terminal product of TXA\textsubscript{2}, to about 4 times the pre-injection level at 3 min after the ADP injection. Simultaneously, 6-keto PGF\textsubscript{1\alpha}, which is the terminal product of PGI\textsubscript{2}, was also found to be markedly increased, PGI\textsubscript{2} aggregates platelet aggregates and induces vascular dilatation.\textsuperscript{22} The increase in PGI\textsubscript{2} associated with the increase in TXA\textsubscript{2} was also observed after injecting arachidonic acid into the coronary artery of rabbits.\textsuperscript{8} The phenomenon may provide a protective mechanism against thrombus formation. As a preliminary experiment, we examined whether OKY-046,\textsuperscript{23,24} which is a specific inhibitor of TXA\textsubscript{2} synthetase, protects against these vascular injuries. Only one out of 5 animals pretreated with OKY-046 showed a slight extravasation of Evans blue. On the other hand, 4 out of 5 animals without pretreatment showed remarkable extravasation.

Vascular injury was observed following intravascular platelet aggregation and different responses were found depending on vascular size. Our results suggest that vasoactive substances from activation of platelets in vivo may play an important role in the production of these vascular injuries. Platelet aggregation may occur commonly not only in cerebral infarction, but also in various pathological conditions. In seeking to understand the pathophysiological mechanisms and treatment of various vascular diseases, attention should be paid to this phenomenon.

Acknowledgment

The authors would like to thank Dr. S.M. Jung for revising the language of the text.

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Cerebrovascular injuries induced by activation of platelets in vivo.
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Stroke. 1985;16:245-250
doi: 10.1161/01.STR.16.2.245

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