Circulating Endothelial Cells Fail to Induce Cerebral Infarction in Rabbits

YOSHIO IWATA, M.D., FUMIO KUZUYA, M.D., MICHIHKO HAYAKAWA, M.D.,
MICHITAKA NAITO, M.D., KAZUAKI SHIBATA, M.D., AND HIDETOSHI ENDO, M.D.

SUMMARY Exfoliated cells that appear to be circulating endothelial cells were detected in human blood. Because the size of endothelial cells is larger than that of other circulating blood cells, we investigated whether the increased number of circulating endothelial cells might be responsible for cerebral embolism. We compared rabbits into which suspensions of endothelial cells were injected through the common carotid arteries with control rabbits into which blood clots or arachidonic acid were administered in the same manner. Injected endothelial cells failed to cause cerebral embolism. Because the majority of exfoliated circulating endothelial cells were degenerated and deformed, the suspensions of endothelial cells obtained from rabbit aortas were treated by ultrasonic waves. These suspensions also failed to cause cerebral embolism. We conclude that cerebral embolism cannot be induced by an increase of circulating endothelial cells alone.

ENDOTHELIAL CELLS on vessel walls are thought to be mechanically, chemically, and immunologically injured, and these injuries can be an important factor in atherogenesis, thrombosis, inflammation or degenerative angiopathy. Degeneration of vascular endothelial cell is perhaps the most easily recognizable type of injury to the vessel walls. These deformed cells may trigger vascular injury when they are exfoliated.

Gaynor et al.1 found unusual cells or cell fragments other than ordinary blood cells in rabbit's blood. On comparing them with endothelial cells on vessel walls, they reported that these were apparently detached endothelial cells.

Hladovec et al.2,3 measured the number of apparent endothelial cells in the circulating blood of rats and reported the differences in the cell numbers under various conditions. They also reported that the number of the circulating endothelial cells increases at the time of myocardial infarction and severe angina pectoris,4 but they did not confirm by immunofluorescent methods that these cells were identical with endothelial cells. We counted the number of the endothelial cells in the circulating blood of 72 human samples using the method used by Hladovec et al.2 To confirm the vascular endothelial nature of these cells, we observed them by Giemsa-staining, Ruthenium Red-staining5 and direct immunofluorescent staining using anti-human factor VIII serum6,7 or anti-human α2-macroglobulin serum.8 To prevent the acquisition of endothelial cells while drawing blood, blood samples were also obtained from the arterial side of extracorporeal circulation tubes in hemodialysis-patients suffering from renal insufficiency. The size of the circulating endothelial cells was larger than that of other circulating blood cells. We aimed to determine whether the endothelial cells can cause brain microembolism to the cerebral circulation.

Materials and Methods

Seventy-two specimens from healthy persons and 32 specimens from chronic hemodialysis patients were obtained. Figure 1 shows the method of collecting circulating endothelial cells.2 Silicone coated tools were used for taking blood. Blood samples (5 ml) were obtained from the median cubital vein in the cases of healthy humans and from the artery side of the extracorporeal circulation tube in the cases of chronic hemodialysis patients, and treated with citrate. After centrifuging the blood samples at 4°C for 20 min, at 395 g, the supernatant (herein-after abbreviated PRP) was collected. Then 0.2 ml of ADP (1 mg/ml solution) was added to 1 ml of PRP, and agitated for 10 min to mix completely. This material was again centrifuged at 4°C for 20 min at 395 g to remove platelet aggregates. The supernatant was centrifuged at 4°C for 20 min at 2100 g, and precipitates which appeared to be vascular endothelial cells were obtained.

Then 0.1 ml of 0.9% NaCl was added to the sediment and left for 1 min. A fraction of the resulting suspension was placed in a Bürker-Türk hemocytometer to count the number of cells. Each sample was counted several times to obtain the mean value. A fraction of the remaining suspension was smeared thinly on 2 slides and was dried. One was fixed in methyl-alcohol, and stained with Giemsa-staining by microscopy. The other was fixed in 10% formalin, stained with 500 ppm Ruthenium Red solution for 30–60 min, dried and observed microscopically. The size of these cells was measured by a micrometer. A portion of the remaining suspension was smeared thinly on a glass-slide, dried, fixed in methyl-alcohol, and washed with PBS solution for 5 min. Anti-human factor VIII and anti-human α2-macroglobulin fluorescence-labeled serum diluted to 10-fold volume were placed on this specimen, which was stored in a dark place at 4°C for 30 min. The fluorescence-labeled serum was washed away by the PBS, cleaned and agitated for 10 min. It was treated with glycera buffer (glycerin 9: PBS 1) and observed by a fluorescence microscope. Human erythrocytes were stained by the same method as the control.
In the animal experiments, the intima of the thoracic aortas of male Japanese white rabbits (3–4 kg in body weight) were exfoliated and treated with collagenase to separate each endothelial cell. The sediment of vascular endothelial cells obtained by centrifugation were suspended in saline solution. When counted by a Bürker-Türk hemocytometer, cell-numbers were approximately $2 \times 10^7$ cells/cm$^3$. These cell suspensions (3.3 x 10$^6$ cells/kg B.W.) were injected into 5 rabbits through a catheter inserted into the left common carotid artery which had been exposed in advance under general anesthesia. At the same time, the endothelial cell suspensions which were treated for 10 min by ultrasonic waves to produce lesions in the cells were also injected into 5 other rabbits. For the controls, blood samples which were obtained from the auricular artery of rabbits were left 48 hr at room temperature to clot. These blood clots were dried, crushed and suspended in a saline solution to be injected into 7 rabbits. In addition, 2 mg/kg of arachidonic acid (made by Sigma Chemical Co.) dissolved in saline were injected into 2 rabbits through the common carotid artery as mentioned above. The catheter was pulled out after injection, and the skin was closed by suturing. Each group was observed for 1 week. The brains of the rabbits were removed for pathohistological observation under general anesthesia. Hematoxylin and Eosin (H.E.) staining were performed for histological observation.

**Results**

Figure 2 shows the number of circulating endothelial cells counted in the human samples. The mean value for healthy persons was 1.59 cells/9 µl PRP, while that of the patients treated with hemodialysis was 1.29 cells/9 µl PRP, showing no significant difference between the 2 groups. In Giemsa staining, the majority of the cells were devoid of nuclei, polygonal in shape including occasional crumpled cells (fig. 3 a). Their diameters ranged between 20 and 50 µm. The cell membrane stained by Ruthenium Red (fig. 3 b). Examination by the direct immunofluorescent staining using anti-human factor VIII serum indicated specific fluorescent cells with the same size and shape as seen in Giemsa and Ruthenium Red stainings (fig. 3 c). Human erythrocytes had no fluorescence when stained by the same method. Examination by direct immunofluorescent staining using anti-human $\alpha_2$ macroglobulin serum showed similar specific fluorescent cells as mentioned above (fig. 3 d). $\alpha_2$ macroglobulin, in the same manner as in the human erythrocytes, had no fluorescence.

None of the rabbits injected with endothelial cell suspensions showed any signs of abnormality, particularly no symptoms suggesting cerebral embolism. The brains showed no gross or histological abnormalities. The brains of rabbits which had been injected with endothelial cells disrupted by ultrasonic waves, also, showed no abnormalities. Three control rabbits out of 7 which had been injected with blood clots died immediately after the injection. Transient paresis in the right lower limb was observed in another, pupillary hemorrhage of the left eye in 1 of these 7 rabbits. Multiple emboli by blood clots were observed in the cerebral blood vessels. Many infarctions were observed in the
brain tissue of rabbits that survived for a week after the injection. The control rabbits which were treated with arachidonic acid died immediately after the injection. Histological examination showed widespread platelet thrombi.

Discussion

In the present study, we observed unusual cells in the circulating blood cell components other than ordinary blood cells.

The possibility that these cells originated from vascular endothelium was confirmed with the positive results of direct immunofluorescent staining using anti-human factor VIII serum6,7 and anti-human α2 macroglobulin serum.8 To eliminate possible contamination by vascular endothelial cells scraped by needles while drawing blood, samples were taken from the arterial side of the extracorporeal circulation tubes in hemodialysis patients. The existence of vascular endothelial cells in these samples suggested that such cells were circulating in the blood vessels. The size of these cells was much larger than that of other circulating blood cells and conceivably these circulating endothelial cells could form a microembolus and thereby could become the source of a brain infarct. Endothelial cells in numbers greater than those of circulating endothelial cells were injected into the common carotid artery of normal rabbits. This method could not produce cerebral embolism in rabbits.

The circulating endothelial cells are thought to be exfoliated from the intima because they appeared injured. Taking this into consideration, the normal separated endothelial cells from rabbit aorta were subjected to ultrasonic waves before injection. Factor VIII and α2 macroglobulin were found in these cells too, by direct immunofluorescent staining. Cerebral embolism could not be induced by injections of these endothelial cell suspensions. The injections of homologous blood clots did produce typical cerebral embolism in control rabbits.

The injection of arachidonic acid reportedly can cause platelet thrombi.9,10 We injected arachidonic acid into the carotid artery of rabbits and observed similar results suggesting that platelet thrombi are responsible for the brain infarctions. In our study, an
overwhelming number of endothelial cells (compared with the number of the normal circulating endothelial cells) were injected into the experimental rabbits. We had ascertained that the concentration of circulating endothelial cells in normal rabbits is comparable to that existing in healthy humans. Cerebral embolisms in rabbits was not induced by the injections of endothelial cell-suspensions. Circulating endothelial cells in humans exist both on the arterial and the venous sides.

Increased numbers of circulating endothelial cells in humans are not a factor causing vascular embolism. It is not known why circulating endothelial cells do not cause vascular embolism.

References

Studies of the Influence of Biogenic Amines on Central Nervous System Ischemia
JUSTIN A. ZIVIN, M.D., PH.D., AND UMBERTO DEGIROLAMI, M.D.

SUMMARY Several serotonin inhibitors have been shown to reduce neurologic deficits in experimental CNS ischemia. Using biochemical and histological methods we tested the effects of the serotonin inhibitors cyproheptadine and brom-LSD in a highly reproducible rabbit spinal cord ischemia model. Detailed mapping of regional spinal cord blood flow was used to guide sampling for the biochemical studies. We found that it is possible to study biochemical and morphological aspects of spinal cord ischemia in great detail using a combination of quite precise techniques. However, at this level of resolution there were no substantial changes in biogenic amine concentrations in severely ischemic or marginally perfused tissue after the durations of ischemia that cause the onset of irreversible tissue damage. Treatment with doses of serotonin inhibitors that produce preservation of neurological function did not cause significant alterations of tissue concentrations of biogenic amines or tissue morphology in treated versus untreated animals.

Stroke Vol 17, No 3, 1986

Materials and Methods
Surgical Procedure
New Zealand albino male rabbits weighing 2 to 3 kg were fed rabbit Chow and water ad lib until the time of surgery. As described in detail previously, the animals were anesthetized with ketamine, 15 mg/kg, and open drop ether. Through a midline abdominal incision, the aorta was exposed at the level of the renal arteries. A small diameter plastic line (8 lb test monofilament fishing line) was threaded through two small plastic buttons surrounding the aorta and then a larger diameter plastic tube to form a snare ligature. The incision was closed around the tubing so the free ends were accessible externally. The rabbits were allowed to recover from anesthesia for at least 3 hr. At that time, each animal's motor and sensory functions were apparently normal. The aorta of each animal was occluded by pulling and clamping the fishing line which compressed the aorta between the two buttons. In experiments in which restoration of blood flow was
Circulating endothelial cells fail to induce cerebral infarction in rabbits.
Y Iwata, F Kuzuya, M Hayakawa, M Naito, K Shibata and H Endo

Stroke. 1986;17:506-509
doi: 10.1161/01.STR.17.3.506

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/17/3/506

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Stroke can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Stroke is online at:
http://stroke.ahajournals.org/subscriptions/