Original Article

Attempt to Establish an Experimental Animal Model of Moyamoya Disease Using Immuno-embolic Material—Histological Changes of the Arterial Wall Resulting from Immunological Reaction in Cats—

Ichiro Kamata*, Yoshinori Terai, and Takashi Ohmoto

Department of Neurological Surgery, Okayama University Medical School, Okayama 700–8558, Japan

In this study, we investigated the relationship between intimal thickening of the internal carotid artery (ICA) and immunological reaction, and between occlusion of the ICA and development of basal collateral vessels in moyamoya disease. Rod-shaped lactic acid-glycolic acid copolymer (LGA-50) and N-acetylmuramyl-L-alanyl-D-isoglutamine (muramyl dipeptide: MDP), an immuno-embolic material, were injected into cats unilaterally via the common carotid artery. Histological changes of duplication of the internal elastic lamina could be seen mainly in the terminal portion of the ICA in the animals injected with rod-shaped LGA-50 containing MDP. No angiographic changes were seen in any of the animals. These findings suggest that the immunological reaction induced by MDP caused histological changes in the intima of the ICA similar to those observed in moyamoya disease. This experimental study, however, could not clarify the development of the basal collateral vessels.

Key words: moyamoya disease, etiology, histology, immunological reaction, embolization

Moyamoya disease (Spontaneous Occlusion of the Circle of Willis) is characterized by non-inflammatoriy, bilateral intimal thickening of the siphon of the internal carotid arteries and development of many collateral vessels in the base of the brain [1–3]. However, its pathogenesis still remains unclear. One theory based on the histopathological investigation of autopsy cases posits that the pathogenesis involves abnormal thrombogenesis [4]. Previous authors have reported chronic inflammation above the neck, leptominal infection, and tuberculous meningitis in their patients with moyamoya disease [5]. Recently, Hosoda et al. reported a case in which immune complexes were deposited in the thickened intima [6]. This suggests that an immunological reaction is involved in the development of moyamoya disease. On the other hand, most investigators consider so-called moyamoya vessels as collateral vessels that develop as a result of an occluded internal carotid artery [1]. Based on these data, we tried to establish an experimental animal model using an immunological reaction combined with an arterial occlusion technique. This article describes the relationship between intimal thickening of the internal carotid artery and immunological reaction, and between occlusion of the ICA and the development of basal collateral vessels.

Materials and Methods

Thirteen cats ranging in age from 2 months to 1 year and weighing 1.5 kg to 2.5 kg were used in this study.
The experiments were carried out in accordance with the Okayama University Policies and Guidelines for the Care and Use of Laboratory Animals.

The animals were injected intramuscularly with ketamine hydrochloride (30 mg/kg) immediately before the procedures to facilitate intubation and cannulation of an intravenous catheter in the femoral vein. Lactate Ringer’s solution was administrated intravenously. The animals were mechanically ventilated throughout the procedures after endotracheal intubation. General anesthesia was induced by intravenous administration of sodium pentobarbital (10 mg/kg). The animals were paralyzed by intravenous administration of pancuronium bromide (0.08 mg/kg). After induction of general anesthesia, the right common carotid artery was dissected in sterile fashion, and a 21G elaster needle was cannulated for the following procedure. The contrast material was injected manually. A single photo of the arterial-phase in the axial view was obtained during each procedure (Fig. 1).

**Preparation of immuno-embolic material.** Lactic acid-glycolic acid 50:50 copolymer (3 mg), molecular weight 10,000 (LGA-50) (Shionogi & CO., Ltd., Osaka, Japan) mixed with 1 mg of N-acetylmuramyl-L-alanyl-D-isoglutamine (muramyl dipeptide: MDP) (Shionogi & CO., Ltd.), was used as an immuno-embolic material in each procedure. The material was shaped in a sterile fashion into rod-shaped pieces measuring 1 mm in diameter and 5 mm in length.

**Experimental study with immuno-embolic material.** The 13 cats were divided into 3 groups. In Group 1 (n = 5 cats: No. 1–5), sensitization was performed by intravenous injection of MDP (500 μg/kg). The rod-shaped immuno-embolic material was injected via the elaster needle, which was cannulated into the right common carotid artery, 3 or 4 times at 10-day intervals beginning 2 weeks after the initial sensitization. At this procedure, the temporary clipping of the right external carotid artery was performed just distal to the common carotid artery bifurcation for selective occlusion of the right internal carotid artery with the rod-shaped material. Angiography was performed before and after the embolization procedures. In Group 2 (n = 4 cats: No. 6–9),

![Diagram](Image)

**Fig. 1** Diagram showing the route of the embolus injection via the elaster needle, which was cannulated into the right common carotid artery. CCA, common carotid artery; ECA, external carotid artery; ICA, internal carotid artery; Int. maxillary A, internal maxillary artery.

![Diagram](Image)

**Fig. 2** Diagram showing the experimental procedure for each group. E, surgical exposure of the right common carotid artery; LG, intra-arterial injection of the rod-shaped LGA-50; LM, intra-arterial injection of the rod-shaped LGA-50 with MDP.
rod-shaped LGA-50 that did not contain MDP was injected 3 or 4 times every 10 days without sensitization. In Group 3 (n = 4 cats: No. 10–13), only cannulation of the elaster needle into the right common carotid artery was performed, 3 or 4 times at 10-day intervals, as a control group (Fig. 2).

**Histological examination.** After the final angiography, performed on Days 45 to 63 post-sensitization (Group 1), and on Days 43 to 57 after initial angiography (Groups 2 and 3), the animals were sacrificed and infused intra-arterially with 10% formalin. Both common carotid arteries were cannulated, and both jugular veins were cut in the cervical region. Then, 10% formalin was infused via the cannula for 5 min at a pressure of 100 mm Hg. After this procedure, the brain, including the internal carotid arteries, vertebral and basilar arteries, and external carotid arteries, was removed and preserved in formalin at room temperature for 1 month. The terminal portion of the internal carotid arteries, the proximal portion of the anterior and middle cerebral arteries, the upper one-third of the basilar artery, and the external carotid arteries of the neck were carefully dissected and removed. Specimens of these arteries were cut into small pieces. The samples were dehydrated through a graded ethyl alcohol and xylene series. Following paraffin embedding, 8 μm sections were cut on a steel knife using a rotary microtome, floated on an albuminized slide, and allowed to dry overnight at room temperature. These samples were stained with hematoxylin-eosin and Elastica van Gieson and examined by light microscopy.

**Results**

**Angiographic study.** The initial common carotid angiogram demonstrated the terminal portion of the internal carotid artery, its branches, and its external carotid rete, which were different from those of the human carotid system (Fig. 3A). The angiogram taken immediately after the injection of the rod-shaped LGA-50 with MDP revealed the right internal carotid artery occlusion (not shown). However, because of the right external carotid rete, the right terminal portion of the internal carotid artery at the base of the skull and its intracranial branch were well demonstrated by the contrast material (Fig. 1). The angiogram before second injection of the rod-shaped material demonstrated the re-canalized right internal carotid artery. No general changes or neurological deficits were seen in the animals before and after the injection of the rod-shaped material in each procedure. Comparison of the initial angiogram and the

---

**Fig. 3**  A, Axial projection of the right common carotid angiogram at the initial angiography, arterial phase, showing the internal carotid artery at the base of the skull (arrow), its branches and the right external carotid rete. B, Axial projection of the right common carotid angiogram at the final angiography, arterial phase, showing no stenotic changes in the terminal portion of the right internal carotid artery or development of collateral vessels in the base of the brain (arrow).
Fig. 4  A. Photomicrograph of the terminal portion of the right carotid artery from the No.3 cat of Group 1 showing the duplication of the internal elastic lamina (arrow). Elastica van Gieson stain.  B. Higher magnification photomicrograph of the terminal portion of the right carotid artery from the No.2 cat of Group 1 showing the duplication of the internal elastic lamina (arrow). Elastica van Gieson stain. Each bar indicates 100 μm.

<table>
<thead>
<tr>
<th>Group</th>
<th>Cat No.</th>
<th>GS</th>
<th>LM × 4</th>
<th>Sensitization → Sacrifice</th>
<th>Arterial changes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>IC</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>+</td>
<td>LM × 4</td>
<td>63 days</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>+</td>
<td>LM × 4</td>
<td>61 days</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>+</td>
<td>LM × 4</td>
<td>58 days</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>+</td>
<td>LM × 4</td>
<td>52 days</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>+</td>
<td>LM × 4</td>
<td>45 days</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>-</td>
<td>LG × 4</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>-</td>
<td>LG × 4</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>-</td>
<td>LG × 4</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>-</td>
<td>LG × 4</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>-</td>
<td></td>
<td></td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>-</td>
<td></td>
<td></td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>-</td>
<td></td>
<td></td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>-</td>
<td></td>
<td></td>
<td>-</td>
</tr>
</tbody>
</table>

Histological findings were assessed as (+) when intimal thickening and/or duplication of the internal elastic lamina were seen, and as (−) when no histological change was seen.

A1, proximal portion of the anterior cerebral artery; BA, basilar artery; ECA, external carotid artery; GS, generalized sensitization; IA, intra-arterial injection; IC, terminal portion of the internal carotid artery; L, left; LG, LGA-50; LM, LGA-50 with MDP; M1, proximal portion of the middle cerebral artery; R, right.
final one performed immediately before sacrifice of the animals (Fig. 3B) showed no stenotic changes in the terminal portion of the internal carotid arteries (near the Circle of Willis) or any development of collateral vessels in the basal ganglia in any of the groups.

**Histological examination.** Histological changes of the internal carotid arteries, anterior cerebral arteries, middle cerebral arteries, basilar arteries, and external carotid arteries were studied. Mild intimal thickening accompanying focal folding and duplication of the internal elastic lamina were observed in Group 1 (Fig. 4A and B). No inflammatory cell infiltration was seen in any of the arteries. Intimal changes were mainly observed bilateral to the terminal portions of the internal carotid arteries. Furthermore, in some anterior cerebral arteries, middle cerebral arteries, and external carotid arteries in Group 1, thickening of the intima and duplication of the internal elastic lamina were observed. In Group 2 and Group 3, no histological changes were seen. The histological changes of arteries in all groups are summarized in the table.

**Discussion**

Moyamoya disease is characterized by bilateral arterial wall thickening in the siphon of the internal carotid arteries and the development of many collateral vessels in the base of the brain [1–3]. A hallmark histological characteristic of the stenosed arteries is fibrous intimal thickening associated with duplication of the internal elastic lamina [7–10]. However, the pathogenesis of moyamoya disease remains unclear. Ikeda *et al.* proposed that the pathogenesis involves abnormal thrombogenesis, based on their histopathological investigation of autopsy cases [4]. They reported that the high incidence of thrombi formation, as well as the distribution of thrombi, which was closely correlated with the progression of the intimal lesions of moyamoya disease, strongly suggested that abnormal thrombogenesis is an important etiologic factor in this disease. Aoyagi *et al.* reported that the down-regulation of PDGF receptors in SMC from patients with moyamoya disease might be interpreted as insufficient recycling or a decreased intracellular pool of PDGF receptors, and their results provided evidence that functional alterations in vascular cells were involved in the development of intimal thickening in moyamoya disease [11]. Hoshimaru *et al.* suggested that basic fibroblast growth factor played an important role [12], and Hojo *et al.* reported that transforming growth factor-beta 1 was associated with the pathogenesis in moyamoya disease [13].

On the other hand, that the incidence of the disease is highest in, but not confined to, Japan and that the condition is frequently familial have been considered to suggest the involvement of a genetic factor in its pathogenesis [14, 15]. In fact, approximately 10% of the total number of cases of moyamoya disease occur as familial cases. Ikeda *et al.* reported a linkage between the disease and markers located at 3p24.2–26 in a familial case of moyamoya disease [16]. Hosoda *et al.* [6] reported IgM and IgA deposits on the thickened intima in an autopsy case. And previous case histories of patients with the disease have reported chronic inflammation above the neck, leptospinal infection and tuberculous meningitis [5]. Therefore, moyamoya disease is thought to be a chronic inflammatory disorder in which immunological reactions play an important role in histological change. On the other hand, moyamoya vessels in the base of the brain are thought to be collateral arteries secondary to brain ischemia [1]. From this standpoint, an “inflammatory and immunological theory” has been proposed, and studies have been conducted in experimental models of moyamoya disease in animals [17]. Kasai *et al.* [18] reported pathological arterial changes in mongrel dogs resulting from experimentally induced immunological arteritis. The changes, which included thickening of the intima and duplication of the internal elastic lamina, were observed mainly in the terminal portion of the internal carotid artery, and were identical to those seen in moyamoya disease. However, the changes were minimal and were not sufficient to cause arterial stenosis. Suzuki *et al.* reported an experimental study in which repeated intravenous and/or intrathecal administration of immunological material was performed in Wistar rats. As a result, histological changes consisting of disruption of the internal elastic lamina, degeneration of media, and intimal thickening were observed mainly in the terminal portion of the internal carotid arteries; however, there was no stenosis in the affected vessels [19]. In their study using Japanese white rabbits, Ezura *et al.* reported that the combination of a single intravenous injection of antigens concomitant with intracisternal administration of antibodies induced cerebral arteritis, likely in the initial process of the histological changes of the internal carotid arteries in moyamoya disease [20].

In this study, we attempted to produce an animal
model of moyamoya disease by the combination of temporary occlusion of the carotid artery and an immunological reaction. We used LGA-50 as the embolic material for temporary occlusion. LGA is a water soluble white powder at room temperature, can be formed in any shape, and can be mixed with other types of powders. Because of differences in molecular weight, LGA has a different nature with regard to melting point and solubility time in phosphate buffer at 37°C. In this study, we used rod-shaped LGA-50—which has a molecular weight of 10,000, dimensions of 1 mm in diameter and 5 mm in length, and a solubility time of 7–10 in phosphate buffer at 37°C—as an embolic material. Yamada et al. reported moyamoya-like changes of the intracranial internal carotid arteries in P. aenes-infected rats [21]. These findings suggested that bacteria and immunological factors might play a role in the pathogenesis of moyamoya disease. We used MDP as a sensitization material. MDP is a major constituent of bacterial cell walls, modulates immune responses, and can induce experimental autoimmune disease [22, 23]. This material was first utilized for experimental induction of moyamoya disease by Suzuki et al. [19].

The brain of cats receives a rich blood supply from the external carotid arteries as a result of the external carotid rete (Fig. 1). Therefore, the experimental animals did not suffer from serious cerebral infarction despite temporary internal carotid artery occlusion by the rod-shaped LGA-50. Reduction of the cerebral blood supply was also expected. However, the cerebral hemodynamics produced by repeated internal carotid artery occlusion with LGA-50 in this experiment might be better preserved than that in actual moyamoya disease. This point will require further investigation using an experimental model that more closely approximates the cerebral vasculature in humans. On the other hand, MDP contained within LGA-50 was released continuously to the terminal portions of the internal carotid artery. In the study of Sato et al., experimental models were sacrificed within 29 days after sensitization and the histological changes of the terminal portion of the internal carotid artery were minimal [24]. MDP was only administrated intravenously in their study. In addition, the duration of MDP administration in their study was shorter than that in our study. MDP was released continuously for 45–63 days in our study. Furthermore, the brains of cats in our study were rendered ischemic by temporary internal carotid artery occlusion with rod-shaped immuno-embolic material. The histological changes, which consisted of mild intimal thickening accompanying focal folding and duplication of the internal elastic lamina, were observed only in Group 1 animals. However, the histological changes in the intima were minimal. These findings suggest that the immunological reaction plays an important role in the pathogenesis of moyamoya disease. On the other hand, in Group 1, histological changes were observed in many parts of the intracranial arteries of the experimental animals who showed a prolonged sensitization to sacrifice. These findings suggest that the extent of the histological changes of the intracranial arteries relates to the duration of MDP administration and the number of internal carotid artery occlusions with rod-shaped immuno-embolic material. Further elucidation will require an increase in the amount and duration of injection of the embolic material.

We did not investigate the presence of immunoglobulin deposits on the thickened intima. Whether or not other biological activities of MDP, in addition to immunological effects, play a role in intimal thickening should be evaluated in future studies.

In this study, the vascular intimal changes were most prominent in the terminal portion of the internal carotid arteries in 4 of 5 animals (7 of 10 sides). These findings seemed related to the injection of an embolic agent containing a sensitizing substance into the internal carotid artery. However, the vascular intimal changes were observed bilaterally in the internal carotid arteries and its branches despite injection of an immuno-embolic material only in the right internal carotid artery. Kasai et al. reported that the injection of foreign serum to the unilateral superior cervical ganglion induced the arterial changes at the carotid fork bilaterally [18]. The site of stenosis or occlusion in moyamoya disease is most prominent at both side of the terminal portion of the internal carotid arteries. The vascular nerve fibers of the human intracranial arteries are abundant in the Circle of Willis and its neighboring arteries, but there are no vascular nerves in the small pial arteries over the convexity. These nerve fibers are provided with a cluster of synaptic vesicles as they approach the smooth muscle cells of the media. Noradrenergic axons are also found in these fibers [24]. Some investigators also believe that the intimal thickening is due to Reilly’s phenomenon and may be strongly associated with sympathetic nerves distributed in the vascular periphery [18]. Our results are in accord with this hypothesis. Autopsy studies have also reported moyamoya-like intimal thickening in the coronary arteries.
[25-27], renal arteries [27-29], pancreatic arteries, and pulmonary arteries [30], but the occlusion in these cases was not nearly as pronounced as that in the terminal portion of the internal carotid arteries. This indicates that moyamoya disease has a predilection for vessels in the region of the Circle of Willis. In our study, the vascular intimal changes were also more prominent in the internal carotid arteries than in the external carotid arteries, with particular involvement of the terminal portion of the internal carotid arteries. This suggests that the intimal changes are due to some mechanism other than direct stimulation. Comprehensive investigation of this issue will require further studies that also examine changes in the renal arteries, aortic arch, and coronary arteries.

Vascular occlusion is usually associated with the development of collateral vessels, but in our study, no changes peripheral to the Circle of Willis were noted before or after embolization. Whether this was due to absence of a significant decrease in blood flow in our experimental model or due to a difference in the mechanism of development of moyamoya vessels is unclear. Further elucidation will require measurement of cerebral blood flow before and after embolization, use of an experimental model that more closely approximates the cerebral vascularulture in humans, and changes in the amount and duration of injection of the embolic material.

Acknowledgments. The authors thank Drs. Akira Nishimoto and Kazushi Kinugasa for their encouragement and support of this study.

References