

# Examination of Rat Carotid Artery Models with Twice-Induced Arterial Balloon Injuries: Observation of Intimal Hyperplasia, Focusing on the Transformation of Vascular Smooth Muscle Tissue

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We induced arterial balloon injuries twice via a hyperdiastolic balloon catheter inserted into the carotid artery of Wister rats in order to observe the changes in the intima and the media. The thickness of the intima reached a maximum by 14 days after the injury. In a comparison of the thickness of the intima with samples having a once-induced arterial balloon injury, the samples with twice-induced arterial balloon injuries were found to have thickened more significantly. When the proliferating cells of the intima were observed, many proliferative smooth muscle cells were found. In the models of the twice-induced arterial balloon injuries, the area of the media decreased, while the area of the intima increased. In the models of the twice-induced arterial balloon injuries, intimal hyperplasia with positive remodeling<sup>1</sup> was observed, and findings were obtained that were substantially similar to lesions due to either intimal hyperplasia of arteriosclerosis lesions or intimal hyperplasia of restenotic lesions after PTA or stent placement.

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## Objectives

In recent years, the development of stent placement to treat the stenosis of arteries in the lower limbs or carotid arteries, such as in coronary artery disease, has been rapidly progressing. Accordingly, research on restenosis after stent placement has also been advancing. Consequently, it has become clear that restenosis after stent placement mainly depends on smooth muscle cell proliferation similar to stenosis after arterial balloon injuries caused by balloons.<sup>2,3</sup> Even though a drug eluting stent has emerged as a countermeasure against restenosis after stent placement, problems have been found, and therefore further research on restenosis after stent placement is thus considered to still be required.<sup>4</sup> By making use of restenotic models after treatment for actual vascular stenotic lesions, we decided that it would be necessary to create a restenotic condition that occurs in the presence of proliferative smooth muscle cells. We then induced an arterial balloon injury with a balloon in order to cause intimal hyperplasia to occur and then induced another arterial balloon injury before observation. We therefore obtained the

pathological findings of stenosis similar to those seen in restenotic lesions after actual stent placement or angioplasty.

## Method

We used 11-week-old specific pathogen free Wister rats. We induced an initial carotid artery injury and then induced another carotid artery injury 14 days later. The carotid artery injury was induced via the following method. After administering Isozol intraperitoneally with general anesthesia using Fluothane, we performed surgery while inserting a thermometer anally in order to monitor and maintain the body temperature at 37 °C. We made an approximately 4-cm-long midline incision that was approximately 5 mm from the bottom of the lower jaw to approximately 5 mm from the top of the sternum. When dividing the platysma muscle at the midline, the right and left submaxillary glands, the anterior sternohyoideus, and the right and left sternomastoids were observed. After retracting the internal submaxillary gland, we re-

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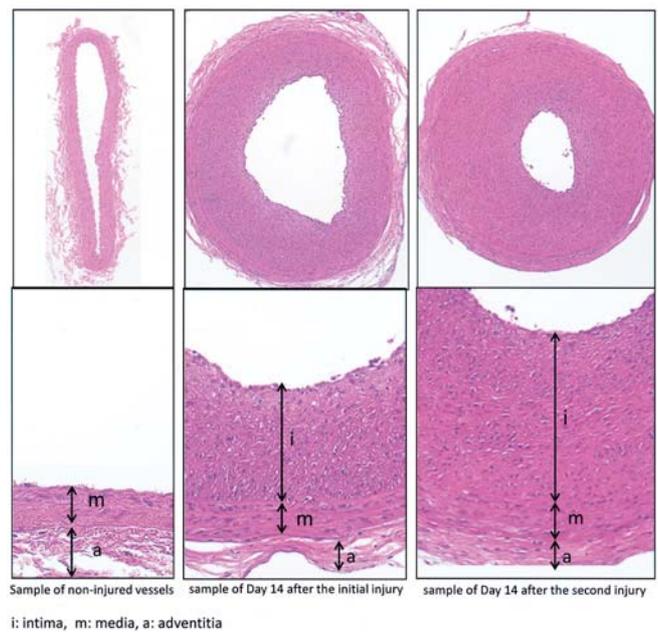
tracted the internal sternomastoid so that the common carotid artery and the pneumogastric could be observed through the exterior muscle of the sternohyoideus. We incised the skin in the right or left groin and exposed the iliac artery/iliac vein. After retracting the iliac artery, we ligated the distal end. While temporarily blocking the blood flow at the proximal artery, we inserted a balloon catheter. After changing the position of the rats and particularly the location of the head, we then guided a catheter into the left common carotid artery. We placed the catheter at the distal end of the left common carotid artery, inflated the balloon, and withdrew it via the proximal end. After deflating the catheter, we advanced it to the branch of the common carotid artery. After repeating this procedure three times, we then extracted the catheter. We created five ligations for the left femoral artery. For the second injury, we performed the same procedure from the opposite iliac artery. On Days 3, 5, 7, 10, and 14 after the first and second carotid artery injuries, we perfused heparin saline and extracted samples while the animals were under deep general anesthesia. We performed hematoxylin and eosin (H & E) staining for the extracted samples, measured the changes over time in the intimal hyperplasia by measuring the area of the intima/media with an NIH image, and performed a statistical assessment (*t*-test). Furthermore, in order to detect the proliferating cells, we performed PCNA (proliferating cell nuclear antigen) staining using anti-proliferating cell nuclear antigen (upstate biotechnology, New York) and then observed the localization of the positive cells. Moreover, in order to detect cells that were derived from the vascular smooth muscle cells and observe any localization, we performed smooth muscle-specific myosin heavy chain (MHC) isoform: SM1, SM2 staining and nonmuscle-type MHC (NMHC) isoform: SMemb /NMHC-B staining using SM1- or SM2- or SMemb/NMHC-B-specific monoclonal antibody (Yamasa Shouyu, Tokyo).

**Results**

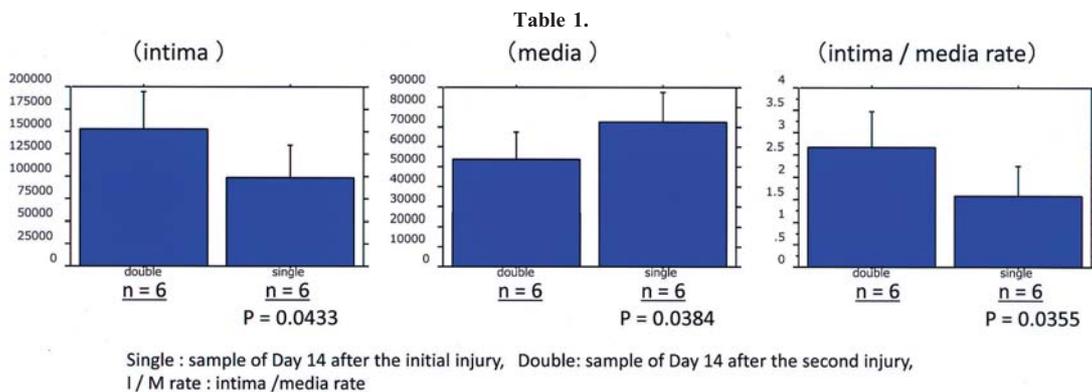
*H&E staining*

On Days 3, 5, 7, 10, and 14 after the first and second carotid artery injuries, we extracted samples and observed them by performing H&E staining on the samples. Specimens from six animals

were prepared for each sample. After the arterial balloon injury, a thickened intima appeared and reached a maximum thickness by Day 14 after the injury. In the first sampling of arterial balloon injury, the thickness of the intima gradually decreased by Day 14 after the injury. In the second sampling of the arterial balloon injury, the thickness was maintained even after Day 14. Figure 1 shows pictures of typical samples before the injury, on Day 14 after the initial injury, and on Day 14 after the second injury. The samples after both injuries showed a substantial thickness of the intima. We then measured the areas of the intima and media via NIH images and compared the two samples of Day 14 after the initial injury and Day 14 after the second injury. The area of the media showed a statistically significant high value in the once-injured vessels than in the twice-injured vessels. On the other hand, the area of the intima showed a statistically significant high value in the twice-injured vessels than in the once-injured vessels. The ratio of the intima/media also showed a statistically significant high value in the twice-injured vessels than in the once-injured vessels (Table 1).



**Figure 1.**



*Immunostaining*

*PCNA (proliferating cell nuclear antigen) staining*

Many positive cells were expressed in the injured intima. However, no such expression was observed in the non-injured vessels. We recognized that these were proliferating cells of the intimal hyperplasia after an arterial balloon injury (Figure 2).

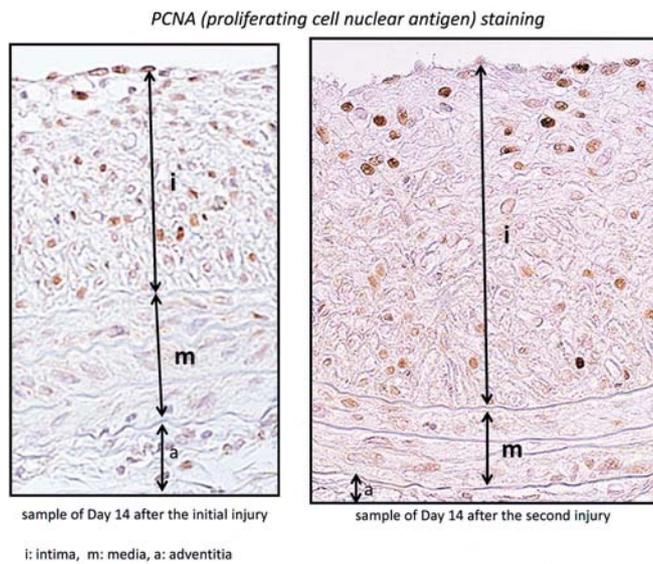


Figure 2.

*SM1 staining*

Both the non-injured vessels and the injured vessels always expressed SM1 in the media and the area of intimal hyperplasia (Figure 3). The adventitia was stained, but this was nonspecific.

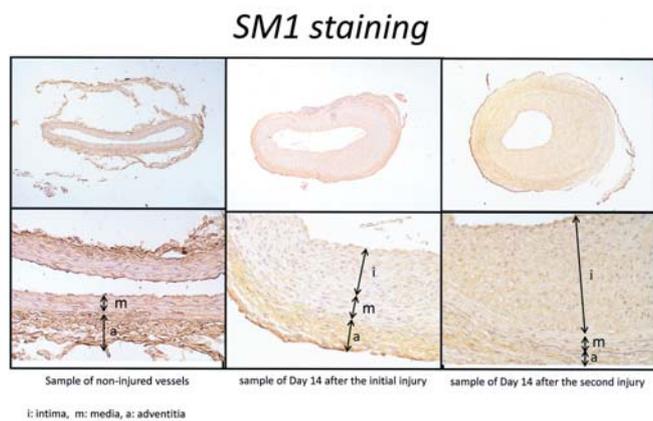


Figure 3.

*SM2 staining*

SM2 was expressed in the non-injured vessels but it was mostly negative in the once-injured vessels. On the other hand, it was slightly re-expressed in the intima closer to the media of the twice-injured vessels (Figure 4).

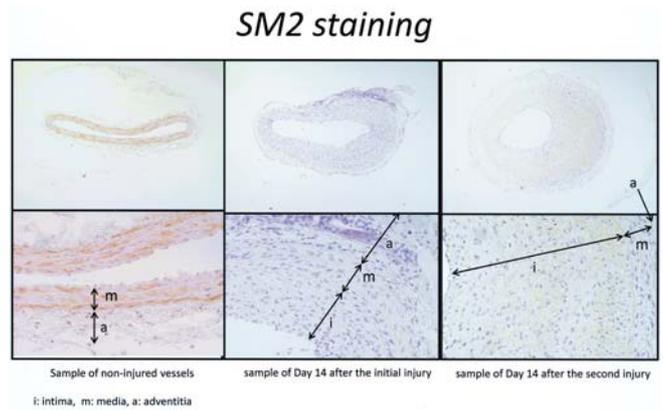


Figure 4.

*SMemb/NMHC-B staining*

SMemb/NMHC-B was negative in the non-injured vessels. It was expressed in the media and the intima for the once-injured vessels. The twice-injured vessels showed positive cells only in the intima (Figure 5). The adventitia was stained, but this was nonspecific.

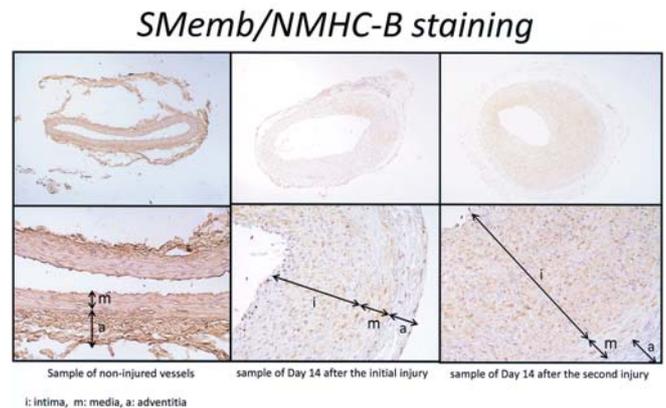


Figure 5.

**Discussion**

Today, it is clear that restenosis after stent placement mainly depends on the proliferation of smooth muscle cells.<sup>5,6</sup> We assessed smooth muscle cells that are proliferating cells of the intima in order to observe vascular injury models. Vascular smooth muscles are categorized as having a contractile state (adult type) and a synthetic state (embryonic type).<sup>7-9</sup>

SMC in the contractile state is observed in the normal media and it has the function of controlling the blood flow. However, it does not have a function of migrating, proliferating cells, or producing an extracellular matrix.

SMC in the synthetic state is observed in vessels in the embryonic period or during cultivation. Furthermore, it is observed in SMC in the arteriosclerosis intima or the newborn intima of the coronary restenotic lesion. Moreover, SMC in the synthetic state

does not have any contractility but it does have a migratory capacity and a proliferating capacity and thus it is able to produce many types of connective tissues, and it intakes denatured protein in order to produce foamy forms. In addition, it actively discharges protein and an extracellular matrix.

It is believed that the smooth muscle cells change into either type depending on changes in the cell cycle or physical and chemical changes. During the course of maturation, the smooth muscle cells (SMC) change from a synthetic state to a contractile state.<sup>9</sup> On the other hand, in a pathological condition such as arteriosclerosis or coronary restenosis, it conversely changes from a contractile state to a synthetic state.<sup>9-15</sup>

SMC changes the isoform (smooth muscle myosin heavy chain isoform) of molecular markers depending on the transformation.<sup>11</sup> Molecular markers are closely correlated with the morphology and specifically changes to respective characteristics. The smooth muscle myosin heavy chain isoform comprises SM1, SM2, and SMemb/NMHC-B.<sup>10, 12-18</sup>

SM1 exhibits a contractile state (adult type) and exists in vascular regions beginning from the development phase. This also exists in the presence of pathological conditions such as arteriosclerosis and coronary restenosis.<sup>10, 12-17</sup>

SM2 exhibits a contractile state (adult type) and does not exist during the embryonic phase, however, it does appear in the postnatal vessels. Some reports state that it decreases with aging and decreases or disappears in the presence of pathological conditions such as arteriosclerosis and coronary restenosis.<sup>10, 12, 14-17</sup>

SMemb/NMHC-B exhibits a synthetic state (embryonic type) and exists during the development phase as well as in the presence of pathological conditions such as arteriosclerosis and coronary restenosis.<sup>12, 14-16, 18</sup>

The results of this observation also revealed that SM1 and SM2 in normal vessels before injury and SMemb/NMHC-B were negative. As previously reported, SM1 is always expressed in normal vessels as well as vessels that have incurred an arterial balloon injury. On the other hand, most SM2 becomes negative after the first arterial balloon injury is induced.

However, the second arterial balloon injury continuously showed a negative conversion in the media, but positive cells reappeared in the intima. These positive cells were more lightly stained I in comparison to positive cells in the non injured vessel. The meaning of this finding is unclear at this moment and thus requires further examination.

Furthermore, the area of the media decreased in the twice-injured vessels than in the single arterial balloon injury samples. This result shows that samples of the twice-induced arterial balloon injuries correspond to the findings of positive remodeling in common arteriosclerosis lesions. SMemb/NMHC-B were observed in the media and the intima of the once-injured vessels. On the other hand, it was only observed in the intima for the twice-induced arterial balloon injury vessels. Furthermore, in the intima of the injured vessels, many PCNA staining positive cells were expressed. Moreover, the area of the intima was larger in the twice-injured vessels than in the

once-injured vessels. In conclusion, samples of the once-induced arterial balloon injury expressed simultaneous responses in the media and the intima. On the other hand, samples of the twice-induced arterial balloon injuries simultaneously showed a phenomenon of substantial cell proliferation in the intima as well as positive remodeling in the media. We specifically obtained findings that were substantially similar to the lesions of intimal hyperplasia, arteriosclerosis lesions, and intimal hyperplasia of restenotic lesions after PTA or stent placement. It is therefore suggested that these twice-induced arterial balloon injured vessel models will be useful for further research on intimal hyperplasia lesions.

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