Changes in the Proliferative Activities of Cells in Experimental Atherosclerotic Plaques During Remodeling

Yasuhiro Okamoto1, Kimio Satomura1, Haruo Nakamura1, Kyoko Takeuchi2, and Masahiko Yoshioka2

1First Department of Internal Medicine and 2First Department of Anatomy, National Defense Medical College, Saitama, Japan.

To investigate the relationship between cytologic alterations and cellular proliferation during atherosclerotic remodeling, we examined experimental atheromatous plaques by immunohistochemistry. Plaques were formed on rabbit aortas by cholesterol-enriched diets and mechanical stimulation over a period of 2 months. Plaques were examined 1 month and 6 months after induction. We used antibodies RAM-11, HHF-35, and monoclonal anti-proliferating cell nuclear antigen (PCNA) antibody for detection of macrophages (Mo), smooth muscle cells (SMC), and PCNA, respectively. One month after induction, the plaques revealed a thickened intima with a fibrofatty histologic pattern or accumulation of foam cells. With either histologic pattern, foam cells were found to be Mo and proliferative activity was mainly observed in Mo. Six months after induction, calcification and organization were seen on the induced plaques, suggesting progression of remodeling. There were fewer Mo and more SMC compared with plaques examined 1 month after induction. Proliferative activity was observed mainly in SMC. We have demonstrated that the proliferative activity of cell types changes during remodeling of atheromatous plaques. Our results suggest an important relationship between the proliferative activity of SMC and remodeling. J Atheroscler Thromb, 1998 ; 5 : 7-12.

Key words: Atherosclerosis, PCNA, Macrophage, Smooth muscle cell

Introduction

Several studies have analyzed the proliferative activity of cells in established atherosclerotic lesions or in such lesions after specific interventions (1-6). However, few reports have considered the natural course of remodeling of atherosclerotic lesions when there are no stimuli inducing progression of these lesions (7, 8).

We therefore investigated changes in cellular constituents during the natural course of atherosclerotic lesion remodeling without any stimuli, and asked what cell types proliferate during remodeling (9-12). We studied the cellular constituents of experimental atherosclerotic plaques and major cell types undergoing proliferation at different stages after lesion induction without any stimulus for progression.

Materials and Methods

Induction and observation of atherosclerotic plaque

White male rabbits, 2 or 3 weeks old, were used for the experiments. An atheromatous plaque was formed in the rabbit abdominal aorta by placing a polyethylene tube (internal diameter 0.6 mm; external diameter 1.2 mm) in the rabbit aorta for 8 weeks. The rabbits were fed commercially available pellets supplemented with 2% cholesterol while the tubes were in place to promote plaque formation (13, 14). After removing the tubes, the rabbits were fed with cholesterol-free pellets. Atheromatous
plaque were examined 1 month and 6 months after induction. Aortas from rabbits bred for the same period before observation and not receiving any stimulation were used as controls. There were five rabbits in the control group and in the groups examined 1 month and 6 months after induction.

**Histologic and immunohistochemical analysis**

Portions of aortic atherosclerotic lesions were excised after angioscopic confirmation (14). A part of each excised aorta was fixed with Bodian solution, embedded in paraffin, serially sectioned into 5 μm-thick sections, and stained with hematoxylin–eosin. Some of the serial sections were used for immunohistochemical analysis (15). The antibodies used were HHF-35, a mouse monoclonal antibody that reacts with α–actin in smooth muscle cells (SMC); RAM-11, a mouse monoclonal antibody that specifically reacts with rabbit macrophages (Mφ); and anti-proliferating cell nuclear antigen (PCNA) mouse monoclonal antibody. Biotinylated antiserum to mouse IgG was used as the secondary antibody. Labeled streptavidin (LSAB) and 3,3'-diaminobenzidine (DAB) were used to yield a brown color to identify antigen–antibody reactions (Table 1) (15-17). Sections from control aortas were treated similarly with the histologic and immunohistochemical procedures described above.

**Results**

**Control aorta**

In the control aortas, the media had thick, regularly arranged elastic fibers and SMC with no foam cells. PCNA was expressed in the SMC of the media (Fig. 1).

**Atheromatous lesions 1 month after induction**

One month after injury, prominent thickening of intimal lesions was noted. A fibrofatty histologic pattern or obvious accumulation of Mφ was the typical histologic pattern in these lesions. Both histologic patterns were observed in all five aortas. PCNA was diffusely expressed in the cells constituting the lesions showing a fibrofatty histologic pattern (Fig. 2). In lesions with obvious accu-

**Table 1. Immunohistochemistry procedure and working dilutions of antibodies.**

<table>
<thead>
<tr>
<th>Step</th>
<th>Procedure</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step 1</td>
<td>Deparaffinization</td>
<td></td>
</tr>
<tr>
<td>Step 2</td>
<td>Wash in PBS 5 min 3 times</td>
<td>×5000</td>
</tr>
<tr>
<td>Step 3</td>
<td>Reaction with primary Ab for 1 hr</td>
<td>anti-PCNA Ab ×200</td>
</tr>
<tr>
<td>Step 4</td>
<td>Wash in PBS 10 min for 3 times</td>
<td>B-2nd Ab ×200</td>
</tr>
<tr>
<td>Step 5</td>
<td>Reaction with biotinylated secondary Ab for 1 hr</td>
<td></td>
</tr>
<tr>
<td>Step 6</td>
<td>Wash in PBS 10 min 3 times</td>
<td></td>
</tr>
<tr>
<td>Step 7</td>
<td>×500 LSAB in PBS for 1 hr</td>
<td></td>
</tr>
<tr>
<td>Step 8</td>
<td>Wash in PBS 10 min for 3 times</td>
<td></td>
</tr>
<tr>
<td>Step 9</td>
<td>Reaction with DAB chromogen</td>
<td></td>
</tr>
<tr>
<td>Step 10</td>
<td>Wash in water for 10 min</td>
<td></td>
</tr>
<tr>
<td>Step 11</td>
<td>Counter staining</td>
<td></td>
</tr>
</tbody>
</table>

PBS, phosphate buffered saline, pH 7.4; Ab, antibody; LSAB, labeled streptavidin; DAB chromogen, 0.02% diaminobenzidine in Tris buffer with 3% H2O2; B-2nd Ab, biotinylated secondary antibody.

1. Antigen retrieved by autoclaving 120°C at 10 atm for 5 min was performed for detection of α–actin and PCNA.
Cell Proliferation in Atheromatous Plaques

Fig. 2. Lesion one month after induction without obvious Mø accumulation. SMC and Mø exist diffusely in the lesion without obvious accumulation. Proliferativity is revealed in both types of cells without distinct pattern. Magnification is ×120, bar shows 10 μm. Meanings of H.E., HHF-35, RAM-11 and PCNA are the same as in Fig. 1.

Fig. 3. Lesion one month after induction with obvious Mø accumulation. SMCs exist in the luminal side of the lesion and Mø is accumulated in the medial side of the lesion. Only a part of accumulating Mø express the proliferativity. Magnification is ×120, bar shows 10 μm. Meanings of H.E., HHF-35, RAM-11 and PCNA are the same as in Fig. 1.

mulation of Mø, however, PCNA was expressed only in the Mø (Fig. 3).

Atheromatous lesions 6 months after induction

Six months after induction, organization and calcification were seen on the induced plaques, suggesting progression of remodeling. There were fewer Mø and more SMC compared with plaques examined 1 month after induction. In such lesions, proliferative activity was observed mainly in SMC with or without Mø in the lesions (Figs. 4, 5).

The correlation between PCNA expression and observed histologic characteristics is summarized in Table 2.

Discussion

In animal models of atherosclerosis, regression, which is characterized by a reduction in lesion mass due to histologic alterations, is induced by decreases of serum cholesterol concentration with a cholesterol-free diet, while continued stimulation induces lesions (6, 9-12, 18). In the case of human atherosclerosis, the histology of atheromatous lesions can be altered and the lesions stabilized by controlling risk factors which promote atherosclerosis (8, 19). The histologic alterations which occur in animal models and human atherosclerosis are now referred to as remodeling, which includes histologic alterations in atheromatous plaques after specific interventions (especially percutaneous transluminal coronary angioplasty) (5, 20, 21).

Even before the concept of remodeling was introduced, the waxing and waning of atherosclerotic lesions under the influence of various risk factors had been noted (7). Although the occurrence of remodeling is now established, the histologic and cytologic alterations occurring naturally during remodeling without inciting stimuli have not been well described. We investigated the changing patterns of Mø and SMC, the major constituent cells of atherosclerotic lesions, during remodeling in the absence of promoting factors. In addition, as the proliferative activity of the atherosclerotic lesions we studied has already been investigated (22), we determined which cells show proliferative activity during such remodeling.

The lesions we observed 1 month after induction revealed histologic patterns classified as types III and IV (23). In lesions with a fibrofatty histologic pattern, which are classified as type III, proliferation of cells in the lesions...
occurred diffusely in both SMC and Mφ. The lesions with Mφ accumulation, which also showed extracellular lipid, were classified as type IV (23). In lesions with such histology, proliferation was seen in the accumulating Mφ. This finding suggests that development of such lesions is caused mainly by the accumulation and proliferation of Mφ. In lesions examined 6 months after induction, organization and calcification were seen. These lesions were classified as type Va, b, or c, without massive accumulation of Mφ or extracellular lipid (23). In these histologic patterns, the constituent proliferating cells were SMC, although Mφ were simultaneously present.

These findings suggest that histologic changes in atherosclerotic lesions during the natural course of remodeling involve a decrease in Mφ and an increase in SMC in the atherosclerotic lesions. In other words, SMC are the major cells responsible for remodeling.

The histologic changes observed during remodeling in our study are known to be alterations which increase plaque stability (24, 25). We suggest that the absence of factors promoting atherosclerosis may cause plaque stability, with a decrease in Mφ in lesions, inhibition of Mφ accumulation, and proliferation of SMC.

In clinical investigations of the histologic remodeling resulting in stable atherosclerotic lesions, as might be seen in endarterectomy specimens or other resected...
material, clinical factors contributing to this process could be identified. To understand the clinical implications of this process, interactions between \( \text{M0} \) and SMC (26, 27), phenotypic changes in these cells (28, 29), and changes in the extracellular matrix surrounding these cells during remodeling (30) should be investigated.

Acknowledgement: We wish to thank the staff of the First Department of Internal Medicine and the First Department of Anatomy of the National Defense Medical College.

References


Table 2. Summary of the results with semi-quantification of PCNA expression.

<table>
<thead>
<tr>
<th>Lesions 1 month after induction</th>
<th>Lesions 6 months after induction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histologic pattern</td>
<td>Fibrofatty Plaque III</td>
</tr>
<tr>
<td>Histological Classification</td>
<td>Foam Cell Accumulation IV</td>
</tr>
<tr>
<td>Cellularity (nuc/100 μm²)</td>
<td>With Mφ V a, b, c</td>
</tr>
<tr>
<td>(n=20)</td>
<td>Without Mφ</td>
</tr>
<tr>
<td>SMC rich area</td>
<td>36.0±2.5†</td>
</tr>
<tr>
<td>Mφ rich area</td>
<td>31.0±8.2</td>
</tr>
<tr>
<td>Number of PCNA expressing nuclei (percentage of total nuclei) (n=20)</td>
<td>9.5±2.1</td>
</tr>
<tr>
<td>SMC rich area</td>
<td>0.2±0.4</td>
</tr>
<tr>
<td>Mφ rich area</td>
<td>25.3±3.2†</td>
</tr>
<tr>
<td></td>
<td>25.3±3.2†</td>
</tr>
<tr>
<td></td>
<td>(66.2±4.5†)</td>
</tr>
</tbody>
</table>

*: average of four serial sections of representative lesions from each rabbit. 
**: unable to devide. The appropriate ratio of SMC/Mφ was 12/7 in these lesions.
Okamoto et al.


(22) Asada Y, Hayashi T, and Sumiyoshi A: Vascular injuries induced by materials released from platelet-rich thrombus in vivo. Atherosclerosis, 70: 1-6, 1988


