Role of Polymorphonuclear Leukocytes in Collar-Induced Intimal Thickening in the Rabbit Carotid Artery


Abstract—In this study, the involvement of polymorphonuclear leukocytes (PMNs) in the development of intimal thickening was investigated. A fibromuscular intima was induced by placing a silicone collar around the rabbit carotid artery for 3 days or 2 weeks; the contralateral artery was sham operated. Rabbits received placebo treatments (groups 1 and 3), granulocyte-colony stimulating factor (group 2; G-CSF, 20 \( \mu \text{g} \cdot \text{kg}^{-1} \cdot \text{d}^{-1} \), delivered by subcutaneous osmotic pumps), or an anti-CD18 monoclonal antibody (group 4; 1.5 mg/kg IV). The G-CSF treatment raised the peripheral PMN count 5- to 12-fold but had no effect on intimal thickening on day 3, 12, or 14. A single injection of anti-CD18 prevented PMN extravasation 6 hours after collar implantation without influencing intimal hyperplasia on day 14. Repeated daily administration of anti-CD18 strongly bound to CD18 on peripheral PMNs and inhibited both PMN-dependent plasma extravasation in the skin and accumulation of CD14-immunoreactive leukocytes in the intima and media. However, anti-CD18 did not suppress early intimal thickening or accumulation of \( \alpha \)-smooth muscle actin–immunoreactive cells by day 3. It thus appears that the PMN influx in the intima and media evoked by the perivascular collar is of little functional relevance to the subsequent smooth muscle cell migration and intimal thickening in this model. (Arterioscler Thromb Vasc Biol. 1998;18:915-921.)

Key Words: neointima ■ endothelium ■ smooth muscle cells ■ leukocyte adhesion molecules ■ granulocyte-colony stimulating factor

Intimal thickening is an early and essential stage in the development of atherosclerotic lesions.1 Experimental intimal hyperplasia can be evoked by an embolectomy balloon2-4 or placement of a rigid polyethylene collar,5 techniques that create extensive denudation of the endothelium, or by the perivascular placement of an endotoxin-soaked thread,6 a flexible silicone collar,7-10 or a flexible collar in combination with electrical injury.11 An influx of leukocytes precedes the migration and proliferation of the vascular SMCs in the intima in all of these models. However, the contribution of the different leukocyte types to intimal hyperplasia remains unclear. Because glucocorticoids inhibit intimal thickening as well as leukocyte activity13 in various models,2-6,12 it has been proposed that leukocytes stimulate intimal thickening.3,5,14 However, because this evidence was only circumstantial, the aim of the present study was to investigate the possible linkage between the transient appearance of PMNs and the subsequent intimal thickening evoked by a noneclusive, silicone collar placed around the rabbit carotid artery. The transient infiltration of leukocytes, which are predominantly neutrophils8-10 with a few monocytes,10 starts within 2 hours of collar placement, peaks at 12 hours, and has already decreased by 24 hours after collaring.5 The leukocytes probably migrate from the lumen and not from the adventitia, because they are first found under the endothelium and only later in the outer half of the media.5,9 On day 3, when the accumulation of subendothelial SMCs becomes detectable, most PMNs have vanished, but remnants of nuclei point to the prior presence of a larger contingent. Seven days after collar placement, even these traces of PMNs are not found.8

To study the role of PMNs, the animals were treated with either G-CSF to raise the number of circulating PMNs15 or an MoAb against CD18 (R15.7), the common subunit of the \( \beta_2 \)-integrins on leukocytes.15-17 Previously we documented that this MoAb abrogated CD18-dependent processes in rabbits, such as the diapedesis of neutrophils and the concomitant edema formation, in response to intradermal injection of chemotactic stimuli.18,19

Methods

Materials

Anti-CD18 MoAb R15.7 was kindly provided by Dr R. Rothlein (Boehringer Ingelheim, Ridgefield, Conn) and G-CSF by Dr B. Aguero (Amgen, Thousand Oaks, Calif). Mouse IgG, Evans blue, fMLP, prostaglandin E2, diaminobenzidine, and the MoAb to \( \alpha \)-SMC actin were obtained from Sigma Chemical Co; the MoAb to CD14 (MY-14) from Coulter; FITC-conjugated F(ab\(^9\))\(_2\) goat anti-mouse...
IgG/IgM antiserum (100 μg/mL) from Dako; the Vectastain ABC kit from Vector Laboratories; Alzet model 2 ML2 osmotic pumps from Astra Pharmaceuticals; and sodium pentobarbital (60 mg/mL) from Sanofi. The pentobarbital was diluted with 1 volume of sterile, pyrogen-free 0.9% NaCl before use. Silicone (Silastic E RTV and MDX4–4210, Dow Corning) and silicone glue (Silastic 732 RTV, Dow Corning) were obtained from the Compagnie Commerciale de Matières Premières.

**Experimental Protocol**

Male New Zealand White rabbits (2.3 to 3.5 kg) were fed standard laboratory chow during acclimatization, which lasted for at least 1 week, as well as during the experiment. After anesthesia was induced (sodium pentobarbital 30 mg/kg IV), both carotid arteries were exposed surgically. Around the left carotid artery a nonocclusive, flexible, silicone collar was placed and closed with silicone glue as described. The contralateral artery was used as a control and manipulated identically to the left carotid artery but was not enclosed by the collar. At the end of the study after anesthesia was induced in the rabbits, both carotid arteries were dissected 3, 12, or 14 days after collaring, and two segments (3 mm wide) were cut from the central ear artery, which fitted the carotid artery more closely.

**Blood Collection and Leukocyte Counts**

Arterial blood (100 μL) was incubated with a saturating concentration of FITC-conjugated F(ab'2)1 goat anti-mouse IgG/IgM, the secondary antibody, for 15 minutes at 4°C. Erythrocytes were lysed with NH4Cl. The cells were washed with PBS and fixed in 1% paraformaldehyde in PBS. Subsequently, flow cytometric analysis (FACScan, Becton-Dickinson) was evaluated as described earlier. To assess the number of potential R15.7 binding sites, a portion of the blood sample was incubated with a saturating concentration of anti-CD18 (50 μg/mL) for 15 minutes at 4°C before the addition of FITC-conjugated F(ab'2)1 goat anti-mouse IgG/IgM. The saturating concentration was determined by addition of serial dilutions (5 mg/mL to 0.5 μg/mL) of R15.7 to the samples. The degree of saturation with the antibody was expressed as a percentage of the available binding sites bound by anti-CD18 at the same time.

**PMN-Dependent Edema Formation in the Skin**

Functional activity of the PMNs was determined by measuring the PMN-dependent plasma leakage 0.5 hour before and 0.5 hour after each injection of anti-CD18 or mouse IgG and at 72 hours as described. First, Evans blue dye (2 mL, 0.5%, IV) was injected at −1, 23, 47, and 71 hours to visualize skin edema. The chemoattractant fMLP (5 × 10−10 mole/site) was then injected intradermally at two sites in the clipped back skin of the rabbit. The vasodilator prostaglandin E2 (3 × 10−10 mole/site) was co-injected to enhance local blood flow. The diameter of both blue spots was measured in two perpendicular directions 30 minutes later. Means were calculated for each time and expressed as a percentage of the diameter of the blue spot at t = 0, i.e., just before the first administration of IgG or anti-CD18.

**Histological Examination**

Segments of the carotid artery were fixed in methacarn (methylal 60%; 1,1,1-trichloroethane 30%; and glacial acetic acid 10%) or formaldehyde 4%. The tissues were dehydrated in a graded series of isopropanol (70% to 100%) followed by toluol and embedded in paraffin; transverse sections were stained with Sirius red–hematoxylin. The antibody to α-SMC actin was detected by an indirect peroxidase-antibody conjugate technique. The antibody to CD14 was demonstrated by using a Vectastain ABC kit (avidin/biotin, 0.5:1). For demonstration of the complex, diaminobenzidine was used as the chromogen, with H2O2 as the substrate. Sections were counterstained with hematoxylin–Carazzi.

In two distant segments of each carotid artery that were stained with Sirius red–hematoxylin, the cross-sectional areas of the intima and media were measured by using a digitizing tablet and software (Sigmascan, Jandel Scientific) and a microscope with low-power magnification. The intima was defined as that region between the luminal endothelial surface and the center of the IEL. Results were expressed as cross-sectional area of collared minus cross-sectional area of sham-operated arteries. The area containing α-SM actin-positive material in the intima was measured with a color image processing system (PCI image color, Foster Findlay Associates). Six randomly selected ROIs of one section of each carotid artery were analyzed. These six regions accounted for approximately half of the intimal thickness.
TABLE 1. Influence of G-CSF and Anti-CD18 on Collar-Induced Intimal Thickening (10⁻³ mm²)

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Vehicle</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>G-CSF, 3 days</td>
<td>6</td>
<td>12±5</td>
<td>11±3</td>
</tr>
<tr>
<td>G-CSF, 12 days</td>
<td>≥4</td>
<td>21±8</td>
<td>13±7</td>
</tr>
<tr>
<td>Anti-CD18, 3 days</td>
<td>3</td>
<td>9±3</td>
<td>10±3</td>
</tr>
<tr>
<td>Anti-CD18, 14 days</td>
<td>≥7</td>
<td>20±5</td>
<td>28±10</td>
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</table>

The cross-sectional area of the intima was calculated as the intimal area of collared arteries minus the intimal area of sham-operated arteries and expressed as mean±SEM. n represents the number of animals. Treatments were not different from placebo: P>0.05, Student’s t test.

Results

Cross-sectional Areas

Collar placement alone induced intimal thickening, because even for all placebo-treated animals, the intimal area of the collared arteries minus the intimal area of sham-operated arteries was positive (Table 1). Neither G-CSF nor anti-CD18 influenced the cross-sectional area of the intima after 3 or 12 days (Table 1). Because the G-CSF treatment showed a tendency to inhibit intimal thickening on day 12, the experiment was repeated. In the third experiment leukocyteosis was clearly induced as well, but again the intima of G-CSF–treated rabbits (0.14±0.04 mm², n=10) was not different from that of placebo-treated rabbits (0.13±0.05 mm², n=10) at day 14. Because the inlet and outlet of the collars used in the third experiment fitted the carotid artery more closely, the intimal thickness was more pronounced compared with that observed in the previous experiments.

In the segments of the 3-day experiments (groups 1b, 2b, 3b, and 4b), the α-SM actin–positive area was increased in collared arteries (placebo sham, 5±1; placebo collar, 66±23 10⁻³ mm²/mm IEL). There was, however, no significant effect of G-CSF or anti-CD18 treatment in sham-operated (G-CSF, 7±2; anti-CD18, 14±6 mm²/mm IEL) or collared (G-CSF, 55±25; anti-CD18, 77±28 10⁻³ mm²/mm IEL) arteries. None of the drug treatments influenced the cross-sectional area of the media. The media did not vary between collared or sham-operated arteries, except for groups 3 and 4 (G-CSF and placebo), in which the collar induced a thickening of the media (data not shown).

Leukocytes

Cell Counts

Treatment with G-CSF raised the number of circulating PMNs almost 5-fold on the day of collar implantation, and at dissection the number of peripheral neutrophils had increased 12- (3-day collar) to 9- (12-day collar) fold (Figure 1). The number of circulating lymphocytes was slightly increased after G-CSF treatment, whereas no differences were found for monocytes, eosinophils, and basophils (data not shown).

Treatment with anti-CD18 caused a doubling of circulating leukocytes after 3 days (group 4b day 0, 8.8±1.0×10⁶/mL; group 4b day 3, 18.4±1.5×10⁶/mL).

Presence of PMNs in the Vessel Wall

Six hours after collaring, scanning electron microscopy of arteries of the placebo group (group 3c) showed a luminal surface structure similar to the one previously described for collared arteries without IgG treatment. The luminal surface consisted of a nearly continuous endothelium with some small denuded areas. In these areas, platelets and occasional leukocytes were present on the basal lamina. Only a few leukocytes adhered to the luminal surface of the endothelium. The regular pattern of the elongated ECs was disturbed by many semispherical elevations with a diameter ranging from 7 to 30 μm (Figure 2A). A few of these spherical elevations showed thin, damaged ECs that partially covered globular cells (Figure 2A). On the endothelial surface of the sham-operated side of the placebo group, only a few leukocytes and no semispherical elevations (Figure 2B) were observed. In anti-CD18–treated animals (group 4c), the endothelial surface of the collared vessel had a morphology similar to that of the sham-operated vessel. A highly variable number of leukocytes adhered to the luminal surface of the endothelium, but the semispherical elevations were nearly completely absent (Figure 2C and 2D).

En face confocal microscopy of the placebo group (group 3c) 6 hours after collaring showed the presence of numerous
PMNs underneath the endothelium (Figure 3A). ECs were identified by the peripheral actin bands, which revealed their elongated cell shape, and by their flattened, oval nuclei. The longest axis of the ECs was oriented parallel to blood flow. PMNs were recognized by their irregularly shaped nuclei and by the “halo” of fluorescently labeled actin surrounding the nuclei. The localization of PMNs was determined by changing the depth of focus with the motorized focus control of the microscope. The subendothelial PMNs were frequently clustered (Figure 3A), forming bulging structures into the lumen, which appeared to correspond to the semispherical elevations observed by scanning electron microscopy. In contrast to the placebo group, PMNs were not present underneath the endothelium in anti-CD18–treated animals (group 4c). The endothelium was smooth and consisted of cells outlined by peripheral actin bands (Figure 3B).

In transverse sections of collared arteries of placebo-treated rabbits, small and rather variable numbers of CD14-immunoreactive cells were found on day 3. More CD14-positive cells were present in the media than in the intima (Table 2). Their numbers were not influenced by G-CSF treatment. However, CD14-positive cells were not detectable in the intima of anti-CD18–treated rabbits and were almost absent from the media (Table 2). The area of the intima did not display any correlation with the number of CD14-positive cells in either the intima or media (results not shown).

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### Table 2. Number of CD14-Immunoreactive Cells in Cross Sections of Collared Segments After 3 Days

<table>
<thead>
<tr>
<th></th>
<th>Intima</th>
<th>Media</th>
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<tbody>
<tr>
<td>Control (n=9)</td>
<td>7±3</td>
<td>30±13*</td>
</tr>
<tr>
<td>Anti-CD18 (n=3)</td>
<td>0±0†</td>
<td>1±1</td>
</tr>
<tr>
<td>G-CSF (n=6)</td>
<td>3±2</td>
<td>18±7*</td>
</tr>
</tbody>
</table>

The counts of three sections from two segments of each artery were first averaged. Values are mean±SEM. n represents numbers of rabbits.

*P<0.05, Wilcoxon test, media different from intima.
†P<0.05 Kruskal-Wallis test, anti-CD18 different from control.
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The present study confirmed that adhesion of neutrophils and monocytes to the intima and media. Even though the CD14 stain does not distinguish between monocytes and PMNs, very few monocytes enter the collared artery, unless lipoproteins are infused into the collar.

To investigate the functional significance of PMN infiltration, the number of circulating PMNs was raised by continuous treatment with G-CSF, starting 3 days before collaring. On the day of collar implantation the peripheral PMN count had drastically increased, as described previously, and rose even further as the G-CSF treatment was maintained. In spite of the elevated PMN count, intimal thickening was not augmented on day 12 or 14. It might be that the effect of the increased number of circulating PMNs was not visible at these late stages, since the PMN influx was restricted to the first day, whereas intimal thickening proceeds for another 2 weeks. The initial rate of intimal thickening might thus be accelerated without changing the final size of the intima. Therefore, experiments were repeated and the areas of the intima and of the zone with α-SM actin in the intima were measured at an early stage. At 3 days both areas were significantly increased in collared arteries but were still not influenced by the G-CSF treatment. Thus, the elevated number of PMNs was not associated with increased intimal hyperplasia at an early stage, when it is mainly due to SMC migration, nor after 2 weeks, when the process reaches a maximum. However, it remains possible that collar-induced PMN extravasation is not limited by normal numbers of circulating PMNs, thus explaining the lack of effect of the drastic increase on intimal thickening.

In additional experiments we therefore reduced the intramural invasion of PMNs by inhibiting the interaction between PMNs and ECs. Emigration from the vasculature of neutrophils and monocytes is regulated by three distinct molecular signals: selectin-carbohydrate, chemokine-receptor, and integrin-immunoglobulin family interactions, which act in sequence and not in parallel. Therefore, inhibition of any of these steps may yield essentially complete suppression of PMN extravasation. Indeed, treatment with the antibody R15.7 against the CD18 subunit of β2-integrins prevented the transendothelial PMN migration in the carotid artery during the first 6 hours after surgery. In spite of the clear inhibition of PMN emigration, the single treatment with the MoAb did not influence the intimal area on day 14. This result suggested that the transient PMN invasion during the first day is not an essential prerequisite for intimal proliferation in the collar model.

However, the experiment did not exclude the possibility that R15.7 only delayed the PMN diapedesis or that early inhibition of intimal thickening was masked by accelerated proliferation in the subsequent 2 weeks. Therefore, the arteries were investigated on day 3, and R15.7 was injected daily to assure inhibition of PMN diapedesis throughout the experiment. As a result, the CD18 integrins were permanently masked for 50% to 100%, the PMN-dependent edema formation in the skin was consistently suppressed, and most important, PMNs were not present in collared segments on day 3, as indicated by the absence of CD14 staining. This finding is in accordance with a recent report that a similar dose of R15.7 (1 mg/kg) completely prevented leukocyte influx in severely injured rabbit carotid arteries for 24 hours.

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**Figure 4.** Inhibitory effect of mouse IgG (O) and anti-CD18 (●) on neutrophil functions. Upper, Amount of free anti-CD18 binding sites, expressed as a percentage of available binding sites at the same time (mean ± SEM). Lower, Neutrophil-dependent edema formation, expressed as a percentage of response at t=0 (mean ± SEM). Arrows indicate injections of mouse IgG or anti-CD18.

**FACS Analysis**

In anti-CD18–treated animals the available epitopes on circulating PMNs were 50% to 100% saturated throughout the experiment. In each animal, binding sites were completely saturated 1 hour after each anti-CD18 injection, and the average was 75% 24 hours later (Figure 4). In one animal all anti-CD18 binding sites were occupied from the moment of collar implantation until the carotid arteries were collected.

**Edema Formation in the Skin**

The edema formation (mean diameter of the blue spot, 12 mm) in response to the intradermal injection of fMLP was always absent after anti-CD18 treatment (diameter 0 mm, Figure 4) and strongly suppressed 24 hours after each administration of anti-CD18. In the rabbit in which complete occupation of anti-CD18 binding sites on the PMNs persisted throughout the experiment, edema formation remained absent as well. The suppression of edema formation 24 hours after anti-CD18 application was related to the extent of anti-CD18 saturation in the FACS analysis. PMN-dependent plasma leakage was not influenced by the placebo mouse IgG (Figure 4).

**Discussion**

The mechanisms responsible for the collar-induced fibromuscular hyperplasia in rabbit carotid arteries are still debated. Recently we proposed that the combination of medial damage and hindrance of transmural fluid transport triggered collar-induced SMC migration and proliferation, rather than hypoxia due to occlusion of the vasa vasorum, destruction of perivascular innervation, kinking of the artery, or increased blood flow velocity. As in other models employing perivascular manipulation, and in accordance with the acute injury of the media, intimal hyperplasia is preceded by a transient influx of leukocytes, predominantly PMNs, in the collar model. The present study confirmed that adhesion and extravasation are in full progress at 6 hours and that most PMNs have disappeared by day 3, as indicated by the small and variable numbers of CD14-immunoreactive cells in the intima and media. Even though the CD14 stain does not
In spite of the clear inhibition of PMN influx by R15.7, areas of the intima and α-SM actin did not even show a tendency toward reduced SMC migration. It might be argued that because the experimental groups were small, this study carried the risk of a type II statistical error. However, this possibility seems unlikely. If invading PMNs were essential for SMC migration, each animal should have demonstrated a reduced intimal thickening, if PMN extravasation was to be completely suppressed. PMN function, as indicated by edema formation and masking of the CD18 integrins, was fully inhibited throughout the entire experiment in one animal. Even in this rabbit the intima increased to the same extent as in mouse IgG-treated animals. Finally, the CD14 staining ruled out the possibility that there was a rebound invasion of PMNs into the artery after 24 hours.

Previously we showed that this dose of anti-CD18 completely prevented the CD18-dependent extravasation of PMNs in response to intradermal application of classic chemottractants (eg, FMLP, C5a desArg, leukotriene B4) and the CXC chemokine interleukin-8 in rabbits. However, inhibition of PMN accumulation was incomplete when the cytokine interleukin-1 was used as the inflammatory stimulus. This implies that neutrophils could enter collared segments via receptor-ligand systems unrelated to the CD11b/CD18–intercellular adhesion molecule-1 interaction, eg, via CD31–CD31 interactions. The initial tethering and rolling are mediated by selectins in the sequential multistep model of neutrophil emigration. P-selectin, formerly known as PADGEM or GMP-140, is colocalized with vWF in Weibel-Palade bodies of ECs and is redistributed to the plasma membrane when vWF is secreted. We previously described an increased subendothelial deposition of vWF during collar-induced intimal hyperplasia. Initially, the area of these vWF deposits was correlated with the area of the intima, but as intimal thickening “stagnated,” the vWF deposits vanished. Moreover, the earliest extracellular vWF deposits are found in regions infiltrated by PMNs. This raises the possibility that the collar-induced adhesion of PMNs to the endothelium, which was not prevented by the anti-CD18 treatment, was mediated by P-selectin. However, the absence of CD14-positive cells in collared arteries of anti-CD18–treated rabbits on day 3 makes it unlikely that important numbers of PMNs or monocytes entered the vessel wall through alternative pathways, eg, via sequential interactions between P-selectin/carbohydrate and CD31/CD31.

It is therefore concluded that the diapedesis of PMNs does not contribute substantially to SMC migration and intimal thickening in the collar model. This also implies that mechanisms other than inhibition of PMN emigration, eg, inhibition of plasma extravasation, of SMC proliferation, or of inflammatory injury of the adventitia, must be involved in the strong suppression of intimal thickening by the glucocorticoid dexamethasone in the collar model. The conclusion that PMN diapedesis is without effect on collar-induced intimal hyperplasia is in accordance with the finding that total blockade of PMN extravasation with another MoAb (60.3) against the CD18 subunit of β2-integrins failed to influence SMC migration into the subendothelial space in response to electric injury of rabbit carotid arteries.

In the latter model ECs remained present as well, but substantial numbers of monocytes accumulated in the vessel. The mononuclear cell influx was inhibited by only 50% by MoAb 60.3. Only complete blockade of leukocyte infiltration by the combined treatment with MoAbs against CD18 and very late antigen 4 on mononuclear cells resulted in 70% inhibition of intimal thickening. This indicates that mononuclear leukocytes promote lesion development by stimulating early SMC migration in that model. A recent angiographic study in humans also suggested that monocytes promote luminal narrowing after percutaneous transluminal coronary angioplasty, whereas activated blood granulocytes may even prevent lumen loss. The contribution of mononuclear cells to SMC migration and proliferation seems to be less likely in the collar model, because mononuclear cells are virtually absent. The lack of effect of anti-CD18 treatments in models in which massive endothelial denudation is avoided (this study and Kling et al) is at variance with a recent report that daily administration of R15.7 for 5 days inhibited intimal thickening evoked by crushing the rabbit carotid artery. The crushing injury is likely to cause direct damage and pronounced desquamation of the ECs, as described for rigid perivascular collars. Indeed, both models contain important thrombotic components, which contribute to neointima formation, as illustrated by the inhibition by the antiplatelet drugs ticlopidine and auranofin by auranofin. Monocytes are the most common leukocytes invading mural thrombi in rabbit carotid arteries and are known to promote SMC migration. In collared arteries mural thrombi were not formed, and monocytes were virtually absent. Therefore, a tentative explanation for the discrepancy with the collar model could be that inhibition of monocyte accumulation contributed to the suppression of intimal thickening by R15.7 in the crushed artery model.

In conclusion, the transient accumulation of PMNs in collared arteries is in accordance with the hypothesis that the collar induces injury of the media as well as local trapping of chemokines and cytokines. However, the PMN infiltration appears to be without effect on the subsequent SMC migration and proliferation, because neither elevating the number of circulating PMNs nor inhibiting their extravasation with a MoAb against CD18 influenced early or late intimal hyperplasia in the collar model.

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References


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