# Real-time imaging of mechanically injured femoral artery in mice reveals a biphasic pattern of leukocyte accumulation

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# Real-time imaging of mechanically injured femoral artery in mice reveals a biphasic pattern of leukocyte accumulation

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**Osaka M, Hagita S, Haraguchi M, Kajimura M, Suematsu M, Yoshida M.** Real-time imaging of mechanically injured femoral artery in mice reveals a biphasic pattern of leukocyte accumulation. *Am J Physiol Heart Circ Physiol* 292: H1876–H1882, 2007. First published December 15, 2006; doi:10.1152/ajpheart.00708.2006.—Wire injury of an artery has been recognized as a standard model of vascular inflammation and atherosclerosis; however, the mechanism of leukocyte recruitment has not been studied in this model. In this study, we documented the recruitment of leukocytes to the murine femoral artery after a wire injury. A transluminal mechanical injury was generated by insertion of a wire into the femoral artery of male C57BL/6J mice. The mice were anesthetized and ventilated after tracheotomy and protected from hypothermia by a warming lamp. Body temperature and blood pH did not significantly change during the experiment. The interaction between rhodamine 6G-labeled leukocytes and the injured femoral artery was monitored using an epifluorescent microscope, and the images were evaluated using a computer-assisted image analysis program. In the absence of injury, virtually no leukocyte adhesion was observed. In contrast, the number of adherent leukocytes increased 4 and 24 h after injury and declined 72 h after injury. The rolling flux of leukocytes increased 4 h after injury and remained high up to 7 days, but it was faster 72 h after injury. We identified another peak of leukocyte adhesion 7 days after injury. Injection of anti-P-selectin antibody significantly reduced leukocyte adhesion at the early and later phases. In conclusion, we have established a novel experimental system for direct observation of leukocyte recruitment to the injured femoral artery. Our system revealed a previously undetected, unique profile of leukocyte recruitment during vascular injury.

vascular injury; leukocyte adhesion; intravital microscopy; inflammation

LEUKOCYTE-ENDOTHELIAL INTERACTIONS are critical in the progression of inflammation and atherosclerosis (4). The recruitment of leukocytes to the sites of inflammation or atherosclerosis-prone vasculature involves multistep, complex cascades of adhesion events, including selectins, integrins, the Ig superfamily, and chemokines. The importance of these adhesion molecules and soluble factors has been studied using cultured vascular endothelium with leukocytes in vitro and in vivo (1, 7). Application of physiological flow to endothelial monolayers in vitro significantly expands our knowledge of the dynamic cell adhesion phenomenon (20, 21), but this model cannot completely recapitulate the leukocyte-endothelial interaction in vivo, because numerous systemic and local factors are involved in the modulation of adhesive interactions. Intravital microscopic (IVM) analysis in experimental animal models is used to observe rolling and adhesion of leukocytes in venules and arterioles (9, 10, 18). Although these vasculatures represent important aspects of endothelial-leukocyte adhesion, their anatomic and physiological characteristics are not identical to those of arteries where atherosclerosis develops (6). Leukocyte-endothelial interaction has not been extensively studied in large arteries during vascular injury, such as clinical angioplasty. It is known that leukocyte recruitment is observed at sites of vascular injury where the endothelial lining was denuded (15). Experimental arterial injury in mice has been widely used to elucidate the cellular mechanisms underlying the development of intimal hyperplasia and restenosis (2, 19). Injury to the arterial wall triggers smooth muscle cell proliferation and subsequent intimal hyperplasia, a common histological finding in restenosis after angioplasty and other vascular diseases. Previous studies with histological examination of this model claimed that leukocytes in the initial phase of recruitment could be similar to those observed in intact, yet inflamed, vasculature coated with endothelial cells. Histological techniques on fixed tissue sections are not able to document dynamic interactions that have occurred at the site of injury. There is no direct demonstration to document the initial phase of leukocyte recruitment after vascular injury in vivo.

In this study, we used an IVM technique to study the dynamics of leukocyte recruitment in the injured femoral artery of mice in vivo. Leukocyte rolling and adhesion were observed in the injured artery, whereas leukocyte adhesion was virtually absent in sham-operated mice. We were able to document dynamic leukocyte recruitment up to 7 days after injury. These data revealed, for the first time, the unique features of leukocyte recruitment in mechanically injured blood vessels.

## MATERIALS AND METHODS

*Animals.* Male C57BL/6 mice (7 wk of age; Oriental Yeast, Tokyo, Japan) were fed a standard diet (CLEA Japan, Tokyo, Japan), and food and water were provided ad libitum. The experiments adhered to the American Physiological Society "Guiding Principles in the Care and Use of Animals" and were approved by the Ethical Committee for Animal Experimentation of Tokyo Medical and Dental University.

*Wire injury in mice.* All mice were healthy before the experiments. The operative time for femoral injury ranged from 20 to 30 min. Clamping of the femoral artery did not exceed 1 min, thereby allowing introduction of the wire after arteriotomy, without significant blood loss. The mechanical injury of the femoral artery was induced by insertion of a large (0.38-mm-diameter) wire (catalog no. C-SF-15-15,

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Cook, Bloomington, IN), as previously described (15). Briefly, the left or right femoral artery was exposed by blunt dissection. A small branch artery between the rectus femoris and vastus medialis muscles was isolated, looped proximally, and ligated distally with 6-0 silk suture. A straight spring wire was carefully inserted through the ligated muscle branch artery to the femoral artery for 1 cm toward the iliac artery. The wire was left in place for 1 min to denude and dilate the artery and then removed, and the silk suture loop at the proximal portion of the branch artery was secured. This procedure led to complete endothelial denudation. Each experimental group consisted of ≥10 mice, and they survived until the time of microscopic examination.

*IVM.* IVM of injured arteries was carried out 4 h, 24 h, 72 h, and 7 days after injury. Each group consisted of 10 mice. Mice subjected to the same procedure, except femoral wire injury, were also included as the control group (0 h, $n = 10$). Mice were anesthetized by pentobarbital sodium, and a catheter was placed in the contralateral femoral vein. Blood pressure was measured through a tail cuff during the procedure. Mice were intubated and ventilated to maintain normal acid-base balance. Rectal temperature was kept at 37°C with a heating pad and an infrared heat lamp. The exposed tissue was superfused with a thermostated (37°C) bicarbonate-buffered saline solution. The mice were injected via the left femoral vein with rhodamine 6G chloride (Molecular Probes; 0.3 mg/kg in 200–300 μl of PBS) to label leukocytes in vivo. The injured femoral artery was found within 30 min after injection of rhodamine 6G chloride and visualized with a microscope (model BX51WI, Olympus, Tokyo, Japan) equipped with a water immersion objective (×20). Epifluorescence was illuminated by a 100-W fluorescent lamp source, and images were directly captured to a personal computer via an ultrasensitive charge-coupled device camera (Cool SNAP HQ, Olympus). In some experiments, a rat anti-mouse P-selectin antibody (RB40.34, Pharmingen) and isotype-matched control IgG (rat IgG1, Pharmingen) were intraperitoneally injected (30 μg/mouse) 2 h before IVM analysis.

*Image analysis.* Adhesion of labeled leukocytes was clearly visualized on the anterior half of the vessels facing the objective. All images were recorded using a computer-assisted image analysis program (Meta Morph) according to the manufacturer's protocol. The parameters used to characterize the adhesive interactions of leukocytes have been described in detail previously (8). The number of adherent leukocytes (i.e., those that did not move for ≥3 s during the 1-min recording period) was counted along a region of interest (ROI), a 100 × 100 μm segment of the vessel, and expressed as the number of adherent cells per $10^4$ $\mu m^2$ of the vessel surface. The frequency of rolling interactions was determined by counting fluorescent cells that moved (i.e., passed a reference line perpendicular to the vessel axis). The results are expressed as the number of rolling cells per minute per $10^3$ μm of the vessel perimeter to adjust the differences in vessel size. We calculated the mean rolling velocity of rolling leukocytes from the total distance measured by tracking individual rolling leukocytes over a distance of 10–50 mm and their elapsed time.

*Immunohistochemical analysis.* After IVM examination, the injured vessels were perfusion fixed through the left ventricle with 2% paraformaldehyde in phosphate buffer. The arterial segment was dehydrated with 8% sucrose in phosphate buffer for 1 h and then in 18% sucrose in phosphate buffer at 4°C overnight. After the samples were embedded in OCT compound, snap frozen in liquid nitrogen, cut into 5-μm-thick sections, and washed in PBS, unspecific binding was blocked with 10% normal goat serum in PBS for 30 min at room temperature. The sections were stained for leukocytes (rat anti-mouse CD11b IgG; Serotec), platelets (rat anti-mouse gpIIb/IIIa IgG; Santa Cruz Biotechnology), or preimmune rat IgG overnight at 4°C and then incubated with biotinylated sheep anti-rat IgG (1:300 dilution; CALTAG) for 45 min at room temperature. After the primary antibody-specific signals were detected using an ABC kit (Vector Laboratories, Burlingame, CA) and developed with an AEC kit (Vector Laboratories), the sections were stained with Mayer's hematoxylin.

*Quantification of serum IL-6 by ELISA.* An anti-mouse IL-6 monoclonal antibody (catalog no. 32C11, Endogen) in PBS (0.4 μg/well) was adsorbed onto microtiter-plate wells overnight at room temperature. Plates were washed with 0.02% Tween 20 in PBS and blocked

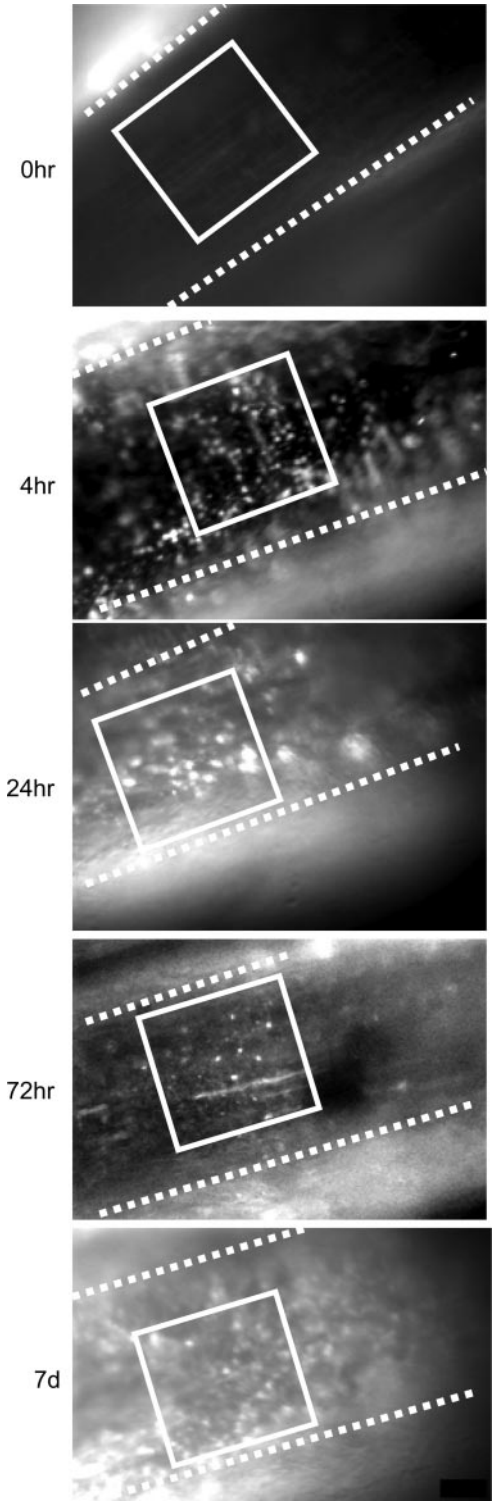


Fig. 1. Serial snapshots from intravital video microscopy records illustrating chronological differences in the frequency of leukocyte-adhesive interactions in mechanically injured femoral arteries of mice. White spots are fluorescent leukocytes illuminated by intravenous injection of rhodamine 6G. Dashed lines indicate margin of vessels. Leukocyte adhesion was calculated from regions of interest (100 × 100 μm squares).

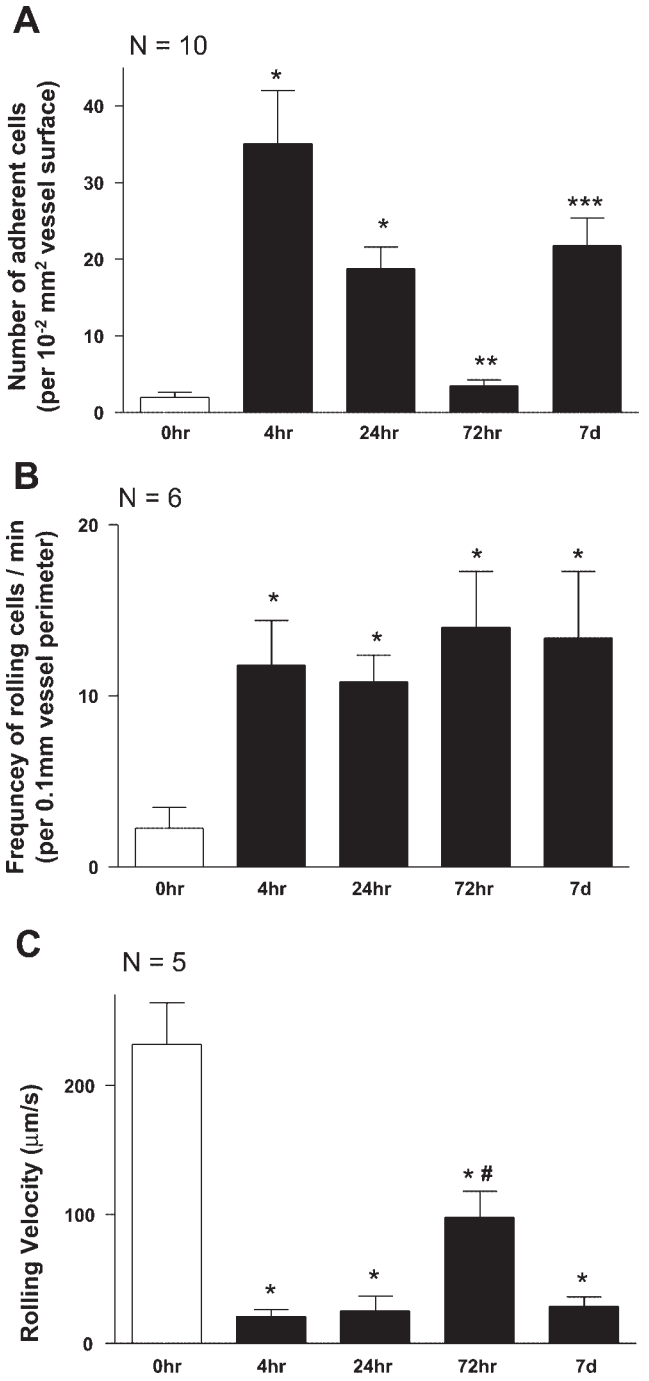


Fig. 2. Time-dependent analysis of leukocyte adhesive interactions in injured arteries, as determined by intravital video microscopy. Experiments with sham operation without mechanical injury are represented by 0 h. *A*: number of cells that exhibit firm adhesion. *B*: frequency of rolling leukocytes as a function of time. *C*: rolling velocity of leukocytes. Values are means ± SE; *n*, number of mice at each time point. **P* < 0.05 vs. 0 h. ***P* < 0.005 vs. 24 h. ****P* < 0.005 vs. 72 h. #*P* < 0.05 vs. 4 h, 24 h, or 7 days.

with 5% normal goat serum in PBS for 1 h at room temperature. Plates were then washed three times with 0.02% Tween 20 in PBS. The samples were added to the wells and incubated for 1 h at room temperature. After the plates were washed three times, rat anti-mouse IL-6 biotinylated monoclonal antibody (catalog no. 20F3, Endogen) was diluted to 0.5 μg/ml with PBS, and 50 μl were added to each well. After 1 h of incubation at room temperature, horseradish peroxidase-streptavidin (100 μl/well, 1:10,000 dilution) was added, and the plates were incubated at room temperature for 30 min. After this wash, the immunoreactive IL-6 was developed by addition of tetramethylbenzidine peroxidase substrate (100 μl/well). After 30 min, the reaction was stopped by addition of 0.6 N $H_2SO_4$, and the absorbance was measured at 450 nm. A sample concentration was obtained by comparison with a standard curve.

*Statistical analysis.* Values are means ± SE. One-way ANOVA with Tukey's post hoc test or two-tailed unpaired *t*-test was used to analyze statistical significance. $P < 0.05$ was considered statistically significant.

## RESULTS

*Physiological parameters.* Body temperature, systolic blood pressure, and blood pH were maintained in the normal range during IVM experiments: 36.5 ± 0.25°C, 117.67 ± 8.45 mmHg, and 7.385 ± 0.054, respectively.

*Real-time observations of leukocyte recruitment to injured artery.* The dynamic adhesive interaction of leukocytes in the injured femoral artery was observed via IVM 4 h, 24 h, 72 h, and 7 days after mechanical injury. Representative snapshot video images are shown in Fig. 1 (also see supplemental movie files I–V in online version of this article). The number of adherent leukocytes per ROI was calculated by image analysis software, and ≥10 ROIs were analyzed for each time point. As clearly shown in Fig. 2*A*, the number of leukocytes that adhered to the mechanically injured artery peaked 4 h after injury (36.3 ± 6.96 cells/$10^4$ $\mu m^2$ vessel surface, $P < 0.005$ vs. 0 h, $n = 10$). The number of adherent leukocytes remained high 24 h after injury (19.5 ± 2.84, $P < 0.005$, $n = 10$). Interestingly, the number of adherent leukocytes decreased 72 h after injury (3.5 ± 0.82, $P < 0.005$ vs. 24 h, $n = 10$). Moreover, when the arteries were examined 7 days after injury, the number of adherent leukocytes had increased significantly compared with the number observed 72 h after injury (22.8 ± 3.64, $P < 0.005$ vs. 72 h, $n = 11$). Because of the increased thickness of the arterial wall, visualization of the vascular lesion after 7 days was difficult. The rolling flux of leukocytes was also examined. As shown in Fig. 2*B*, virtually no rolling was observed in sham-operated arteries (0 h). In contrast, rolling flux of leukocytes in the injured arteries significantly increased 4 h after injury and remained high up to 7 days after injury. Interestingly, rolling velocity of leukocytes was significantly faster 72 h after injury than 4 h, 24 h, or 7 days after injury (Fig. 2*C*).

*Systemic leukocyte counts.* To confirm that the enhanced adhesion of leukocytes to the injured vessel was based on local

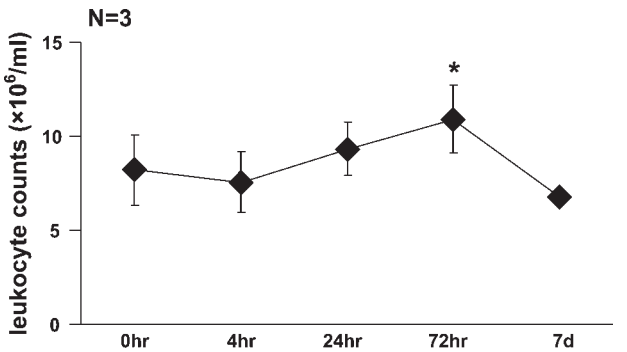


Fig. 3. Systemic leukocyte counts in blood samples from the femoral vein at 0 h, 4 h, 24 h, 72 h, and 7 days. Values are means ± SE. **P* < 0.05 vs. 0 h.

inflammation independent of systemic leukocytosis, we measured systemic leukocyte counts at various times after mechanical injury. As shown in Fig. 3, the systemic leukocyte count did not change significantly until 72 h after injury. Thus the induction of leukocyte adhesion 4 and 24 h after injury was not related to systemic leukocytosis.

*Histological analysis.* The specimens prepared from injured arteries at each time point were analyzed histologically. The presence of CD11b-positive leukocytes was documented 4 h (Fig. 4*D*), 24 h (Fig. 4*G*), and, to a lesser extent, 72 h after injury. The presence of platelets (MWReg30 staining) was noticed in the injured area 4 h (Fig. 4*E*) and 24 h (Fig. 4*H*) after injury. The presence of monocytes/macrophages (MOMA2 staining) was not clearly appreciated until 72 h after injury (Fig. 4, *L* and *O*). We also confirmed that endothelial lining (CD31 staining) was completely lost after 4 h (Fig. 5*C*) and

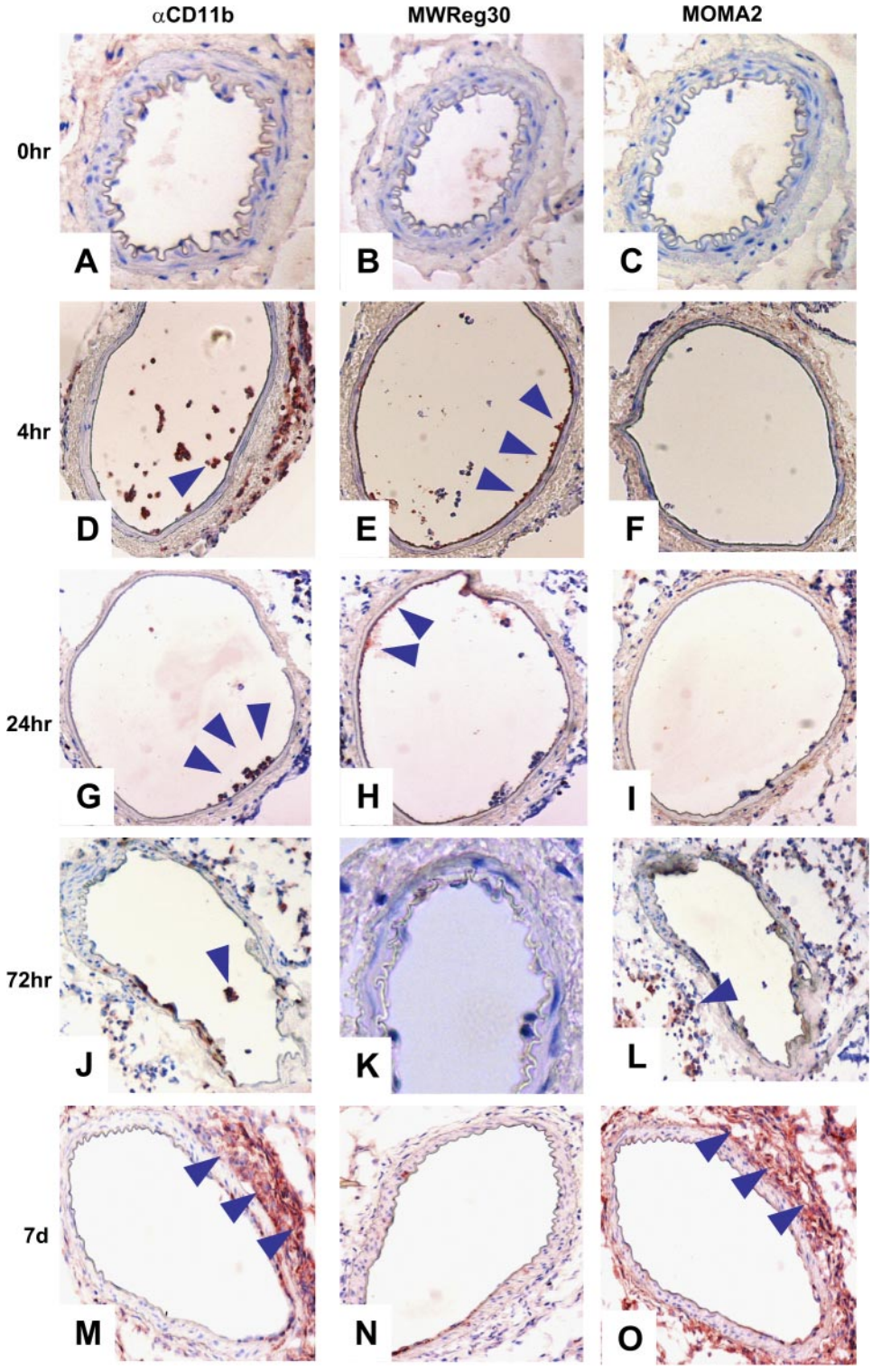


Fig. 4. Histological examination of injured vessels. Injured femoral artery of C57BL/6 mouse was perfusion fixed before injury (0 h) and 4 h, 24 h, 72 h, and 7 days after injury. Samples were then embedded in OCT compound, snap frozen in liquid nitrogen, and cut into 5-μm-thick sections and stained for leukocytes (CD11b; *A*, *D*, *G*, *J*, and *M*), platelets (MWReg30; *B*, *E*, *H, K*, and *N*), and monocytes/macrophages (MOMA2; *C*, *F*, *I*, *L*, and *O*) using the avidin-biotin complex technique and Vector Red as substrate. Sections were counterstained with hematoxylin. Arrowheads point to platelets.

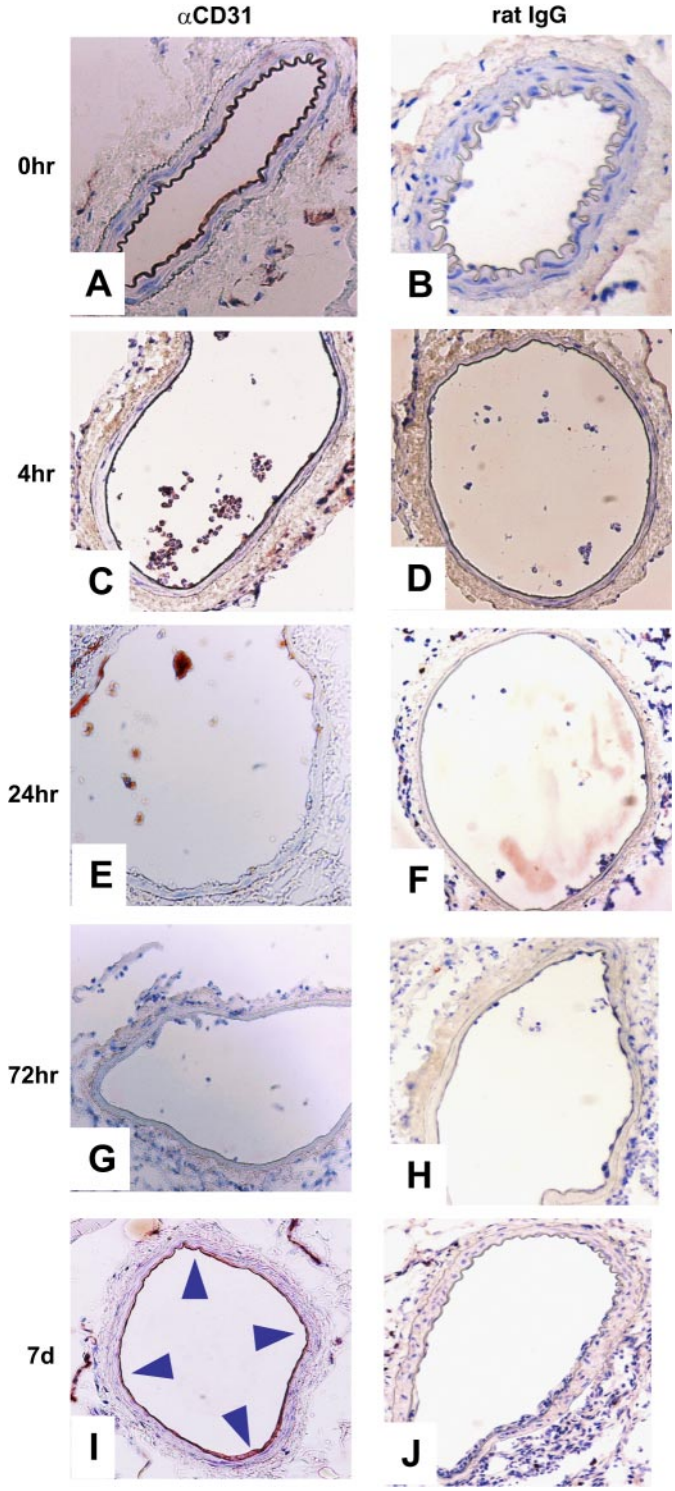


Fig. 5. Histological examination of injured vessels. Injured femoral artery of the C57BL/6 mouse was perfusion-fixed before injury (0 h) and 4 h, 24 h, 72 h, and 7 days after injury. Samples were then embedded in OCT compound, snap frozen in liquid nitrogen, and cut into 5-μm-thick sections and stained for endothelial cells (CD31; *A, C, E, G*, and *I*) and control (rat IgG; *B*, *D*, *F*, *H*, and *J*) using the avidin-biotin complex technique and Vector Red as substrate. Sections were counterstained with hematoxylin.

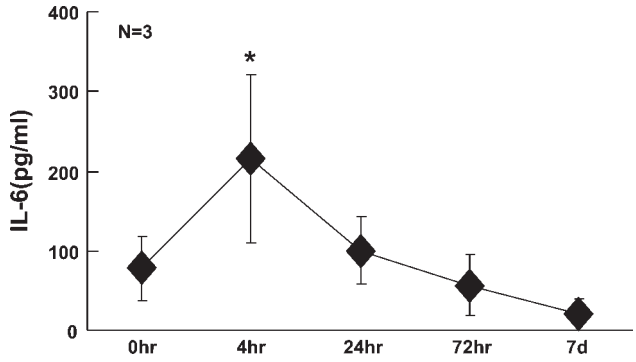


Fig. 6. Plasma level of IL-6 measured by ELISA 0 h, 4 h, 24 h, 72 h, and 7 days after mechanical injury. Values are means ± SE. *$P < 0.05$ vs. 0 h.

was recovered after 7 days (Fig. 5*I*). As previously reported, massive intimal hyperplasia was observed 28 days after injury (data not shown).

*Inflammatory markers.* To estimate the systemic inflammation after a mechanical injury, the serum level of IL-6 was measured by ELISA. The level of IL-6 was significantly increased 4 h after injury and gradually decreased as a function of time (Fig. 6).

*Role of P-selectin in wire injury-induced leukocyte recruitment.* To identify adhesion molecules responsible for the initial and later phases of leukocyte recruitment, antibodies against anti-P-selectin were injected before IVM assay. The anti-P-selectin antibody significantly reduced leukocyte adhesion at the early phase, i.e., 4 h (Fig. 7). The antiadhesive effect of anti-P-selectin antibody was more prominent ($P < 0.05$) in the later phase.

## DISCUSSION

We have conducted a novel real-time imaging study that directly monitors leukocyte recruitment to mechanically injured femoral arteries in mice. Although adhesion assays conducted under flow conditions in vitro significantly extended our understanding of the dynamic interaction of cell adhesion, the lack of systemic factors limited its interpretation in vivo.

Mechanical injury of the murine artery has been widely used to examine the process of vascular inflammation and its modeling to understand the mechanisms of restenosis after angioplasty and atherosclerosis (15, 19). Critical observation of the

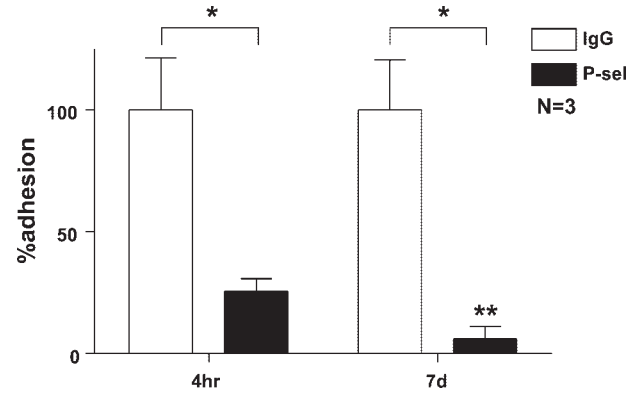


Fig. 7. Effects of anti-P-selectin antibody (P-sel) in leukocyte adhesive interactions in arteries 4 h and 7 days after injury. Values are means ± SE. * $P < 0.01$ vs. IgG. **$P < 0.05$ vs. 4 h P-sel.

early phase of the injured vessels was not extensively performed, except for comparison of its long-term effect, manifested as intimal thickening of the injured vessels.

Examination of the atherosclerosis-prone large arteries, such as the femoral artery, has a striking advantage over previously established observations utilizing much smaller vessels, such as mesenteric arteries (11, 13) or cremasteric postcapillary venules (12, 16). Although these relatively small vessels have been utilized in IVM analysis, their anatomic structures and physiological characteristics are quite different from those of the large arteries, such as the aorta, carotid artery, and femoral arteries. Moreover, the biomechanical shear force applied to the arterial vessel wall by oscillatory blood flow is a critically important factor for regulation of the pathophysiological conditions of the vessel (3, 5). Only a few studies have attempted to examine the leukocyte-endothelial interaction in large arteries (14), and those have not done so in any extensive way. Since mechanical injury (such as coronary angioplasty) would physically dilate a vessel as well as trigger a complex cascade of inflammation and coagulation (19), it is critical to demonstrate mechanical injury utilizing large arteries, such as femoral arteries.

In the present study, we have found that the frequency of leukocyte-vessel interactions dramatically increases at the time of mechanical injury. We found that leukocyte interactions peaked at 4 h, remained increased up to 24 h, and decreased at 72 h. As previously reported, the endothelial lining was completely denuded at 4 and 24 h after injury. Massive inflammation and thrombogenic cascade induced by mechanical injury lowered the shear stress of the injured vessels, which facilitates leukocyte recruitment. As previously reported, the accumulation of platelets and neutrophils plays an important role in the progression of neointimal hyperplasia after angioplasty (17). As we demonstrated in Fig. 7, P-selectin plays a pivotal role in the early and later phases of leukocyte recruitment to the injured vessels. We speculated that P-selectin mediated leukocyte rolling and subsequent adhesion to activated platelets covering denuded injured vessels in the early phase. In contrast, in the later phase, P-selectin mediated the adhesive interaction between leukocytes and newly covered endothelial cells. The detailed molecular mechanisms responsible for the temporal difference in leukocyte recruitment are not completely understood. Several mechanisms, including oxidative stress (unpublished observation) and a potential role of bone marrow-derived vascular progenitor cells, require further investigation. Our model will provide critical information regarding the initial phase of the mechanical injury that leads to neointimal hyperplasia. Systemic inflammation, measured as plasma IL-6 concentration, peaked 4 h after injury, which coincides with the first peak of leukocyte adhesion, but not with the second peak, implying that the systemic level of inflammation may not be directly reflected by local inflammatory responses in the later phase of vascular injury.

In contrast to the drastic temporal change in the number of adherent leukocytes, the number of rolling cells remained similar throughout the experiments, suggesting a distinct molecular mechanism for rolling interactions. Although we found that the rolling velocity was higher 72 h after injury (Fig. 2*C*), additional investigation is necessary.

In summary, we have developed a novel IVM system for observation of leukocyte recruitment to vessel walls in mechanically injured femoral arteries of mice. We have identified early-phase (4 and 24 h after injury) as well as later-phase (7 days after injury) leukocyte accumulation in the injured vessels.

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