Reduced Neointima Hyperplasia of Vein Bypass Grafts in Intercellular Adhesion Molecule-1–Deficient Mice

Yiping Zou, Yanhua Hu, Manuel Mayr, Hermann Dietrich, Georg Wick, Qingbo Xu

Abstract—Recently, we established a new mouse model of vein graft arteriosclerosis through the grafting of vena cava to carotid arteries. In many respects, the morphological features of this murine vascular graft model resemble those of human venous bypass graft disease. With this model, we studied the role of intercellular adhesion molecule-1 (ICAM-1) in the development of vein graft arteriosclerosis in ICAM-1–deficient mice. Neointimal hyperplasia of vein grafts in ICAM-1 −/− mice was reduced 30% to 50% compared with that of wild-type control animals. Immmunofluorescent analysis revealed that increased ICAM-1 expression was observed on the endothelium and smooth muscle cells (SMCs) of the grafted veins in wild-type, but not ICAM-1 −/−, mice. MAC-1 (CD11b/18)–positive cells that adhered to the surface of vein grafts in ICAM-1 −/− mice were significantly less as identified with en face immunofluorescence, and these positive cells were more abundant in the intimal lesions of vein grafts in wild-type mice. Furthermore, aortic SMCs cultivated from wild-type mice exhibited high ICAM-1 expression in response to tumor necrosis factor-α. When tumor necrosis factor-α–stimulated SMCs were incubated with mouse spleen leukocytes, the number of cells that adhered to ICAM-1 −/− SMCs was significantly lower than the number that adhered to ICAM-1 +/+ SMCs, which was markedly blocked through pretreatment of leukocytes with the anti–MAC-1 antibody. Taken together, our findings demonstrate that ICAM-1 is critical in the development of venous bypass graft arteriosclerosis, which provides essential information for therapeutic intervention for vein graft disease in patients undergoing bypass surgery. (*Circ Res*. 2000;86:434-440.)

Key Words: veins ∎ neointima ∎ adhesion molecules ∎ mice ∎ arteriosclerosis

Since Garrett and colleagues[1] performed the first aortocoronary saphenous vein graft implantation in a human in May 1967, many patients have received such surgery for vascular reconstruction. Although the treatment is highly successful in the relief of symptoms and prolongs survival in patients with severe coronary artery disease, nearly all veins implanted into the arterial circulation develop intimal hyperplasia within 4 to 6 weeks, which may reduce the lumen by as much as 25%. This process in itself rarely produces significant stenosis, but it represents the foundation for the later development of venous bypass graft atheroma.[2] The pathogenesis of this disease remains poorly understood, and no successful clinical treatments are available.

The neointimal lesion has an inflammatory nature characterized by mononuclear cell infiltration in the early stage of vein bypass grafts, followed by smooth muscle cell (SMC) proliferation.[3,4] Activated monocytes and macrophages, which produce mitogenic, fibrogenic, and angiogenic factors that can influence tissue remodeling, are central to inflammation[5,6] and may play a crucial role in the development of neointimal hyperplasia in the grafted veins. The molecular

mechanism by which monocytes/macrophages are continuously recruited to the neointima of vein bypass grafts is currently unknown.

Intercellular adhesion molecule-1 (ICAM-1), a surface glycoprotein of the immunoglobulin superfamily, contains 5 immunoglobulin-like motifs in its extracellular domain, followed by a single transmembrane region and a short cytoplasmic tail.[7,8] The major known functions of ICAM-1 relate to its role in cell adhesion and migration. ICAM-1 is a counterreceptor for the β_2 leukocyte integrins MAC-1 ($\alpha_M\beta_2$, CD11b/CD18) and lymphocyte function-associated antigen-1 (LFA-1) ($\alpha_L\beta_2$, CD11a/CD18), and their engagement results in leukocyte adhesion and transmigration through endothelium.[9] Several lines of evidence have suggested that ICAM-1/MAC-1–dependent cellular interaction is involved in a number of inflammatory processes and in arteriosclerosis via mononuclear cell adhesion and migration.[10–13] However, it remains unknown whether ICAM-1 plays a causal role in the development of vein bypass graft arteriosclerosis.

In our previous study, we established and characterized a new model for the study of neointima formation of venous

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bypass grafts in mice and demonstrated the presence of abundant MAC-1^{+} monocytes/macrophages in the early stages of lesions in vein grafts.[14] In the present study, we evaluated the role of ICAM-1 in the development of vein graft lesions with the use of ICAM-1–deficient mice and demonstrated that the local adherence of circulating monocytes to the endothelium of grafted veins is one of the earliest cellular events. Herein, we provide the first evidence that ICAM-1 plays an important role in the pathogenesis of venous bypass graft arteriosclerosis.

Materials and Methods

Mice and Vein Graft Procedure

ICAM-1–deficient mice of the C57BL/6J strain[15] were purchased from Jackson Laboratories. Three genotypes of ICAM-1 ($-/-$, $+/-$, and $+/+$) mice were identified.

The vein grafts were performed with animals of the same genotype as donors and recipients. The procedure used for vein grafts was similar to that described previously.[14] Briefly, the vena cava vein was harvested, and the right common carotid artery was mobilized, with a cuff placed at the end. The artery was turned inside out over the cuff and ligated. The vein segment was grafted between the 2 ends of the carotid artery.

Tissue Preparation

Vein grafts were harvested at 1 day and 1, 4, and 8 weeks postoperatively (4 to 8 mice at each time point per group) by cutting the implanted segments from the native vessels at the cuff end.

Histology and Lesion Quantification

Histological sectioning began at the center of the graft to avoid the effects of the cuff. The thickness of the vessel wall was determined by measuring 4 regions of a section along a cross and was recorded in micrometers (mean±SD) as described elsewhere.[14] For neointimal area measurement, sections were reviewed with a BX60 microscope equipped with a Sony 3CCD camera and television monitor. The neointimal area was determined by subtracting the area of the lumen from the area enclosed by the border of the adventitia.

Immunofluorescent Staining

The procedure used for immunofluorescent staining was similar to that described previously.[16] Briefly, serial 5-μm-thick frozen sections were labeled with a rat monoclonal antibody against mouse MAC-1 (CD11b/CD18) leukocytes, a rabbit polyclonal antibody against mouse ICAM-1 (Santa Cruz Biotechnology), or a mouse monoclonal antibody against α-actin conjugated with FITC.

En Face Immunofluorescence

The procedure used in this experiment was similar to that described previously.[17] In brief, each vein graft segment was cut longitudinally, mounted endothelium side up onto a glass slide (2.6×7.5 cm), and air dried for 1 to 2 hours at room temperature. The segments were incubated with appropriately diluted rat monoclonal antibody to MAC-1 and visualized with FITC-labeled rabbit anti-rat Ig. MAC-1–positive cells were counted at 10×40 magnification with water immersion objectives in 10 fields of each segment.

SMC Culture and Treatment and Leukocyte/SMC Adhesion Assay

Vascular SMCs from ICAM-1 $-/-$ and $+/+$ mice were cultivated from their aortas as described previously.[18] Briefly, mouse thoracic aortas were removed, and the intima and media were carefully dissected from the vessel under an anatomic microscope, cut into pieces (1×1×0.1 mm), and implanted onto a gelatin (0.02%)-coated plastic bottle. SMCs were passaged 7 to 10 days after implantation. They were treated with 100 ng/mL tumor necrosis factor-α (TNF-α)
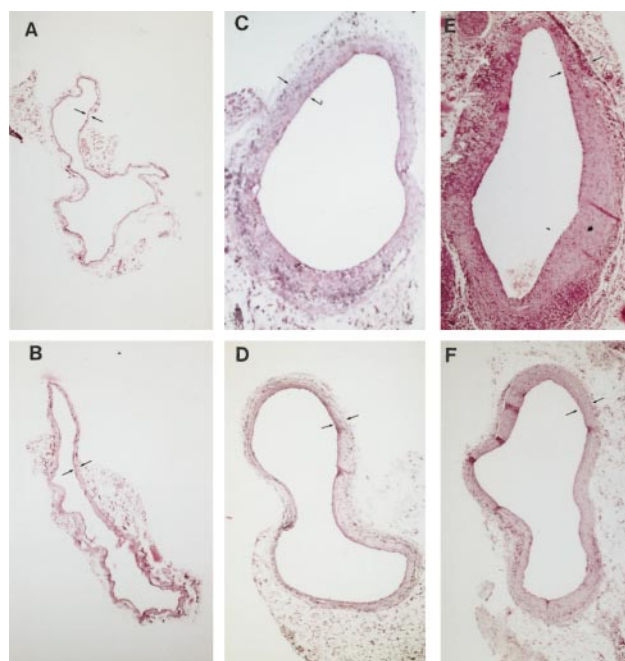

Figure 1. Hematoxylin-eosin–stained sections of mouse control vein and vein grafts. Under anesthesia, vena cava veins of ICAM-1 $+/+$ (A, C, and E) or $-/-$ (B, D, and F) mice were removed and isografted into carotid arteries. Animals were sacrificed 0 (A and B), 4 (C and D), and 8 (E and F) weeks after surgery, and the grafted tissue fragments were fixed in 4% phosphate-buffered (pH 7.2) formaldehyde, embedded in paraffin, sectioned, and stained with hematoxylin-eosin. Arrows indicate the control vessel wall (A and B) and neointima (C to F) (original magnification ×40).

and assessed for immunofluorescent staining or cell adhesion assays. The procedure for splenocyte isolation and leukocyte adhesion assays was similar to that described elsewhere.[17]

Statistical Analysis

Statistical analyses were performed on a Macintosh computer with the Mann-Whitney *U* test and ANOVA, respectively.

An expanded Materials and Methods section is available online at http://www.circresaha.org.

Results

Reduced Neointima Hyperplasia in ICAM-1 $-/-$ Mice

The venous wall of ICAM-1 $-/-$ and wild-type mice is composed of intima, a monolayer of endothelium, media, 1 or 2 layers of SMCs, and adventitia, a small amount of connective tissues (Figures 1A and 1B). Vein grafts of wild-type mice at 4 and 8 weeks (Figures 1C and 1E) showed neointimal hyperplasia (ie, thickening of the vessel wall up to 10 or 20 layers of cells). Interestingly, neointimal lesions of vein grafts in ICAM-1 $-/-$ mice showed a marked reduction at 4 as well as at 8 weeks (Figures 1D and 1F).

Figure 2 summarizes data for neointima thickness and area measured microscopically. Thickening of the vein grafts began as early as 2 weeks after surgery (data not shown). A significant difference was found between groups of ICAM-1 $-/-$ and wild-type mice ($P<0.001$). Neointimal hyperplasia of vein grafts in ICAM-1 $-/-$ was reduced 30% to 50% compared with that of wild-type control animals (Figure 2).
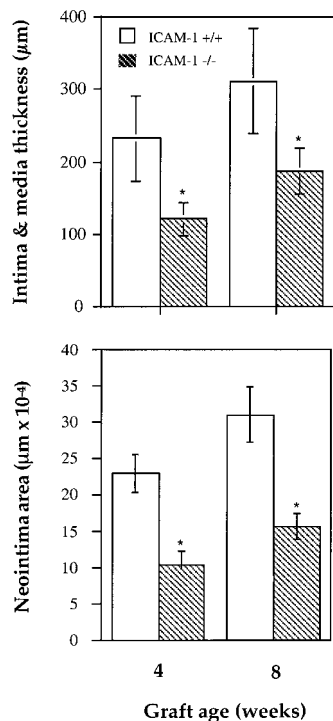
Figure 2. Reduced neointima hyperplasia of vein grafts in ICAM-1 −/− mice. The procedure for animal models and the preparation of hematoxylin-eosin–stained sections are same as described in the legend to Figure 1. Neointimal thickness and area were measured as described in detail in Materials and Methods. Data are mean±SD values for 6 animals per group. *P<0.001 vs ICAM-1 +/+ group.

Figure 3. Immunofluorescent labeling for ICAM-1 in vein grafts. Sections derived from vein grafts of ICAM-1 +/+ (A through C) and ICAM-1 −/− (D) at 1 day (A), 1 week (B), and 4 weeks (C and D) were labeled with a rabbit anti–ICAM-1 antibody and visualized with the use of swine anti-rabbit Ig conjugated with FITC. Arrows indicate the surface of the intima (original magnification ×250).

ICAM-1 Expression in Vein Grafts

ICAM-1 is thought to be involved in the firm adhesion step in leukocyte infiltration and has been shown to be highly expressed in atherosclerotic lesions in humans and hypercholesterolemic animals.[19–22] It would be interesting to assess whether ICAM-1 is also expressed in the grafted veins. Serial cross sections of vein specimens from both ICAM-1 −/− and wild-type mice were immunologically stained with rabbit anti–ICAM-1 antibodies. There was scant immunostaining for ICAM-1 on the endothelium of freshly excised veins but significantly increased staining on the surface of vein grafts 1 day after surgery (Figure 3A). ICAM-1–positive staining was also observed in sections of vein grafts of wild-type mice at 1 and 4 weeks (Figures 3B and 3C). The pattern of ICAM-1 staining in the vein grafts shown in Figure 3 was different. In the 1-day graft, most surface areas in the intima had become more intensively stained (Figure 3A), whereas vein grafts at 1 and 4 weeks displayed elevated ICAM-1 content in the endothelial and subendothelial regions (ie, not only in the intima, but also in the media and adventitia; Figures 3B and 3C). As expected, there was no positive staining for ICAM-1 in the vein grafts of ICAM-1 −/− mice (Figure 3D).

Decreased Leukocyte Adhesion to and Infiltration in Vein Grafts of ICAM-1 −/− Mice

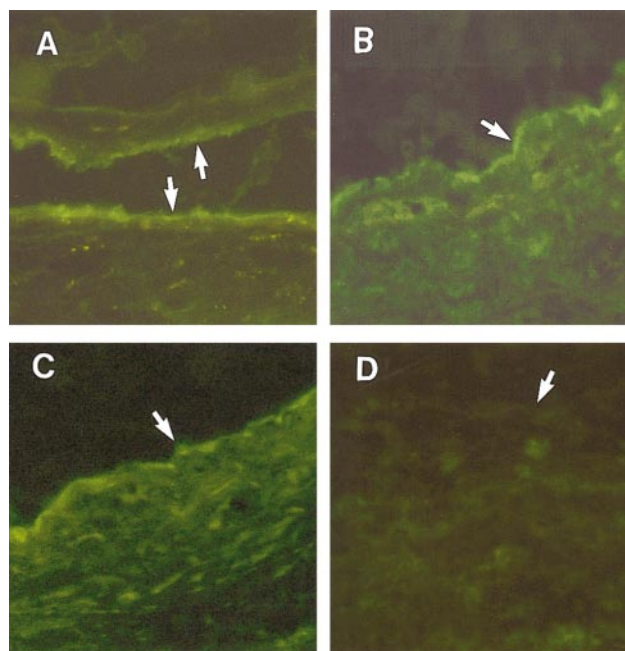We previously adopted the vessel en face immunofluorescence method[17] for semiquantification of cells that adhere to the endothelium of vascular segments. This method was generally useful in the clarification of the kinetics and phenotypes of cells that adhere to vascular endothelial surface in vivo. Nonspecific reactivity was minimal in the negative control labeled with normal rat serum (Figure 4A), and cells that adhered to the endothelial surface were positively stained with a rat monoclonal antibody recognizing MAC-1+ leukocytes (CD11b/CD18; Figures 4B through 4D). A large number of MAC-1+ leukocytes were observed adherent to the endothelium of vein graft segments of wild-type mice 1 day after surgery (Figure 4C), whereas cells adherent to the surface of vein grafts from ICAM-1 −/− mice were much less profound (Figure 4D). Occasionally, MAC-1+–stained cells were also seen on the surface of freshly harvested vein segments (Figure 4B). Figure 4E shows statistical data from 5 animals of each group and indicates a significant difference in adherent cells between ICAM-1 −/− and ICAM-1 +/+ vein grafts. These results indicate that leukocyte adhesion to the endothelium is one of the earliest cellular events in vein graft disease.

There is evidence of increased expression of monocyte chemotactic protein-1 in vein grafts associated with the development of vein graft intimal hyperplasia.[23] Cells that tether to the endothelium can be followed with transmigration and localization in the vein graft. Using immunofluorescent techniques, we found MAC-1–positive cells in vein grafts at 4 and 8 weeks. MAC-1+ cells are monocytes/macrophages, natural killer cells, and granulocytes. The majority of infiltrating cells in neointima were mononuclear cells (ie, monocytes/macrophages). Abundant infiltration of these positive cells was found in the intima, media, and adventitia of 4-week vein grafts (Figure 5A; 50 to 200 positive cells/×400 field), whereas small numbers of
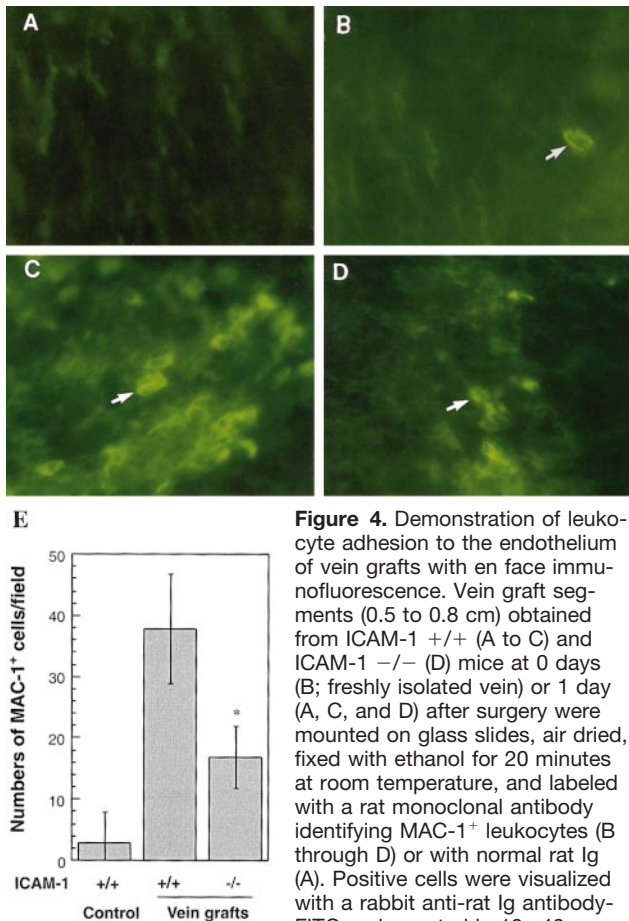
Figure 4. Demonstration of leukocyte adhesion to the endothelium of vein grafts with en face immunofluorescence. Vein graft segments (0.5 to 0.8 cm) obtained from ICAM-1 +/+ (A to C) and ICAM-1 −/− (D) mice at 0 days (B; freshly isolated vein) or 1 day (A, C, and D) after surgery were mounted on glass slides, air dried, fixed with ethanol for 20 minutes at room temperature, and labeled with a rat monoclonal antibody identifying MAC-1$^+$ leukocytes (B through D) or with normal rat Ig (A). Positive cells were visualized with a rabbit anti-rat Ig antibody-FITC and counted in 10×40 magnification. Ten fields from each vein segment were randomly selected. E, Statistical mean±SEM values for 5 animals of each group. Arrows indicate examples of positive cells (original magnification ×250). *$P<0.05$ vs wild-type mice.
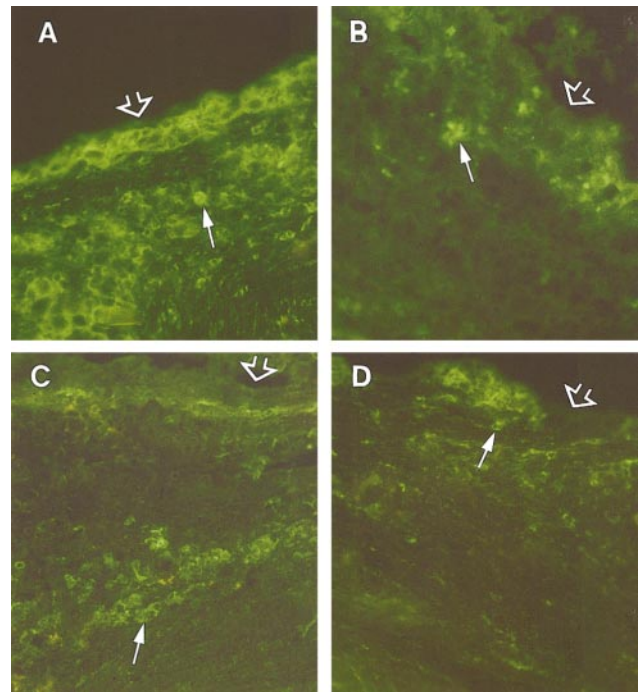

Figure 5. Immunofluorescent staining demonstrates MAC-1$^+$ mononuclear cell infiltration in vein grafts. Sections derived from vein grafts of ICAM-1 +/+ (A and C) and ICAM-1 −/− (B and D) mice at 4 (A and B) or 8 (C and D) weeks were labeled with the rat monoclonal antibody (CD11b/CD18) against MAC-1$^+$ leukocytes and visualized with a rabbit anti-rat Ig antibody-FITC. Arrows indicate examples of positive cells (original magnification ×250).

MAC-1$^+$ cells were seen in the vein grafts of ICAM-1 −/− mice (Figure 5B; 20 to 96 positive cells/×400 field). MAC-1$^+$ monocytes/macrophages were also detected at the luminal surface at 8 weeks after engraftment in both ICAM-1 −/− and +/+ mice (Figures 5C and 5D), but these positive cells were rarely seen in the neointima of 8-week grafts in ICAM-1 −/− mice (Figure 5D).

Figure 6 shows histological data for 4- and 8-week vein grafts from both ICAM-1 −/− and wild-type mice. Mononuclear cell infiltration in vein segments of wild-type mice was more predominant than those of ICAM-1 −/− mice. When cell nuclei in the intima and media of neointima of grafted vessels were counted in 100-μm lengths, total cell numbers were significantly higher in 4- and 8-week grafts of wild-type mice (Figure 6E). These observations indicate that ICAM-1 is important in the mediation of leukocyte adhesion and transmigration in vein grafts.

SMCs Expressed ICAM-1 and Leukocyte Adhesion

We previously demonstrated the presence of abundant SMCs in venous bypass graft lesions 4 and 8 weeks after surgery.[14] Figure 3 shows ICAM-1–positive staining not only localized on the surface but also inside the neointima. To test whether SMCs express ICAM-1 in vein grafts, a double immunofluorescent labeling was performed. Strong staining for ICAM-1 was observed in sections from vein segments of wild-type mice 4 weeks postoperatively (Figure 7a) but not of ICAM-1–deficient mice (Figure 7b). Importantly, a large portion of positive-stained cells were α-actin–positive SMCs (Figure 7a).

To study ICAM-1 expression and the role of this molecule in cell adhesion, aortic SMCs from both ICAM-1 −/− and +/+ mice were cultivated and treated with TNF-α, and ICAM-1 was examined with immunofluorescence with the specific antibody against ICAM-1. TNF-α–stimulated ICAM-1 induction was observed in ICAM-1 +/+, but not ICAM-1 −/−, SMCs (Figures 8A to 8C). Untreated ICAM-1 +/+ SMCs had very weak staining (Figure 8B). Furthermore, we investigated SMC/leukocyte adhesion in vitro, where ICAM-1 −/− and +/+ SMCs were treated with TNF-α and incubated with spleen leukocytes prepared from ICAM-1 +/+ mice. Leukocyte adhesion to the ICAM-1 +/+ SMC surface increased 2- to 3-fold after treatment with TNF-α and was significantly lower in ICAM-1 −/− SMCs. Partial block of adhesion could be achieved through preincubation of spleen leukocytes with the monoclonal antibody directed to MAC-1, and this block was less effective on ICAM-1 −/− SMCs. These findings suggest that ICAM-1 expressed in SMCs is responsible, at least in part, for leukocyte adhesions.
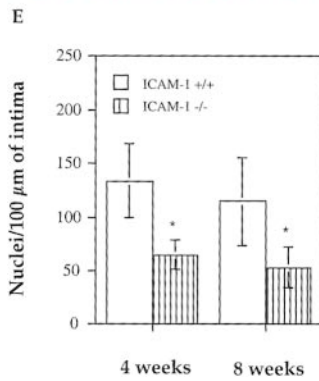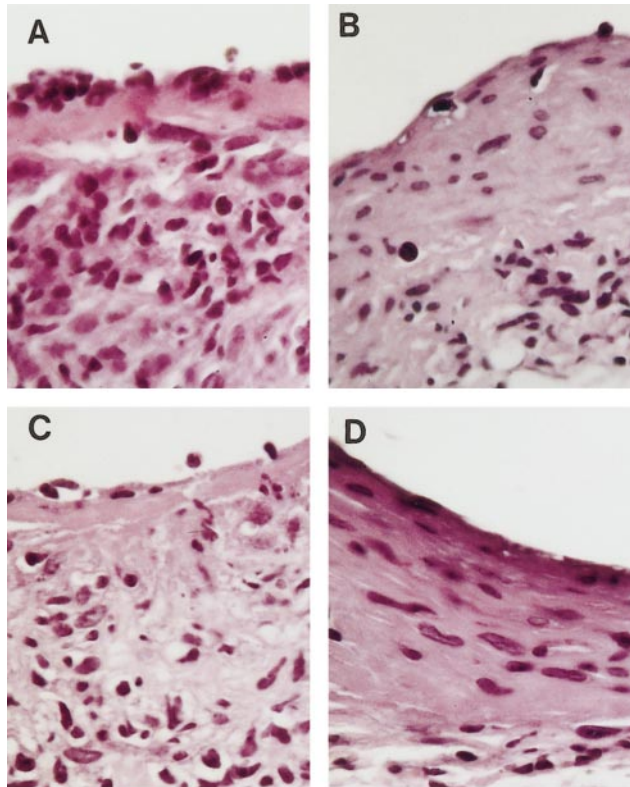
E, Statistical data for total number of cells per 100 μm of vein grafts. Nuclei in 100-μm lengths of neointima/media of grafted veins were counted manually. Two opposite areas from each section were counted, and 5 sections per animal were selected. Values are mean±SD for 6 animals per time point. *$P<0.05$ vs control.

Figure 6. Hematoxylin-eosin–stained sections of vein grafts from ICAM-1 +/+ (A and C) or ICAM-1 −/− (B and D) mice. Animals were sacrificed 4 (A and B) and 8 (C and D) weeks after surgery, and the grafted tissue fragments were fixed in 4% phosphate-buffered (pH 7.2) formaldehyde, embedded in paraffin, sectioned, and stained with hematoxylin-eosin (original magnification ×250).

Figure 7. Immunofluorescent double labeling of vein graft sections. Cryostat sections from mouse vein grafts 8 weeks after surgery were fixed with cold 5% acetone/methanol for 30 minutes, air dried, and incubated with a polyclonal rabbit anti-ICAM-1. The reaction was visualized with anti-rabbit Ig/FITC-conjugated swine Ig. After washing, sections were incubated with a mouse monoclonal antibody against α-actin conjugated with TRITC for 30 minutes at room temperature. The sections were examined with a laser confocal microscope. Note ICAM-1–positive staining in the section from the wild-type mice (a) but not ICAM-1–deficient mice (b). The arrows indicate examples of double-positive cells (bar 20 μm).

Discussion

Recently, we established and characterized a new model for the study of neointima formation of venous bypass grafts in mice.[14] In the present study, we demonstrated that this mouse model is useful for investigation of the role of adhesion molecules in vein graft disease with knockout mice. When vein isografts were performed in ICAM-1 −/− mice, intimal lesions were reduced up to 50% compared with wild-type control animals. The mechanism of reduced neointimal hyperplasia is due to the lack of ICAM-1 expression, resulting in reduction of leukocyte adhesion to the endothelium of vein grafts at the early stage and a lower rate of accumulation of monocytes/macrophages in neointimal lesions at the late stage.
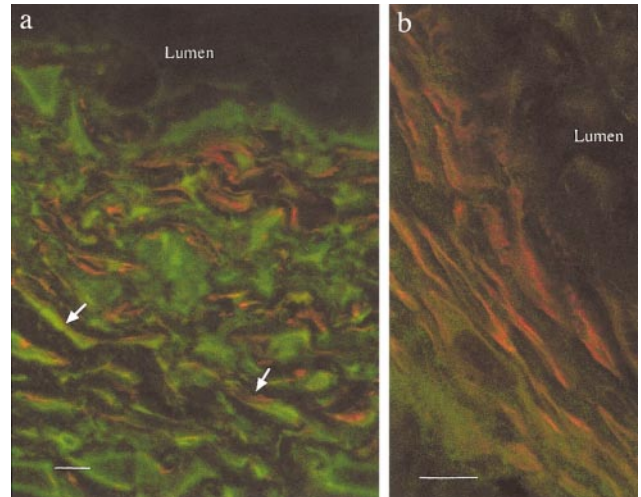
In the present study, we demonstrate that a hallmark of vein grafts in wild-type mice is ICAM-1 expression between 1 day to 8 weeks postoperatively. What is the initial factor that results in adhesion molecule expression on the endothelial cells of grafted veins? Surgical or traumatic and ischemic injury to the vein segments may be in part responsible for ICAM-1 induction in the vein grafts, but we posit that mechanical stress plays a role in ICAM-1 gene expression via signal transduction pathways leading to NF-κB activation. In grafted veins, mechanical force on the vessel segment suddenly increases >10-fold (arterial versus venous blood pressure), which provides a strong stimulus to vascular endothelial and SMCs. We previously demonstrated that acutely elevated blood pressure and mechanical stress activate growth factor receptor/mitogen-activated protein kinase signal pathways,[24–27] which are closely related to NF-κB activation. Other reports have established that the exposure of endothelial cells to shear (mechanical) stress results in increased expression of ICAM-1 and monocyte chemotactic protein-1 via the activation of transcription factor NF-κB and activator protein-1.[28–34] These molecules are essential for leukocyte/endothelial cell interaction and, subsequently, cell infiltration, which is characteristic of the early lesions of vein grafts that undergo elevated blood pressure. Thus, our observations, together with others, suggest that mechanical stress is one of the most important factors in the initiation of ICAM-1 expression in vein grafts.

Although the importance of ICAM-1 in the mediation of cell adhesion to the endothelium has been established, little is known about the role of ICAM-1 expressed in vascular SMCs.[35,36] Given the facts that SMCs express ICAM-1
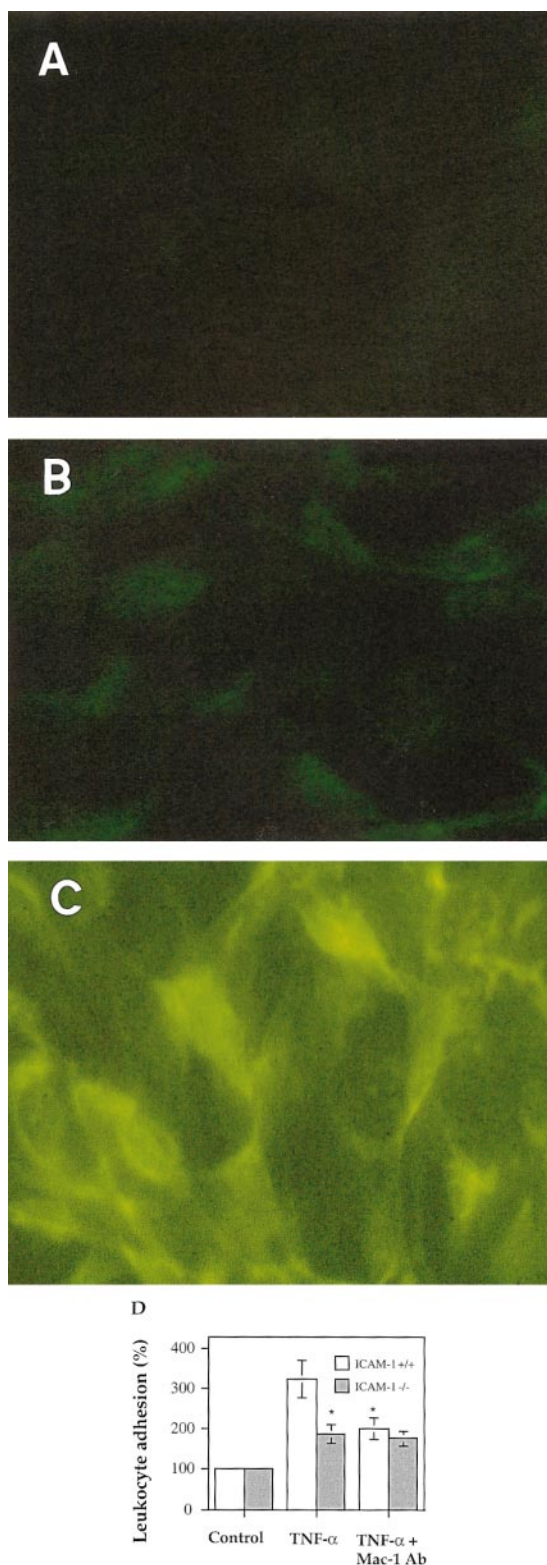
associated with monocyte/macrophage accumulation in vein grafts and that SMCs of ICAM-1 $-/-$ mice do not express ICAM-1 correlated with reduced neointimal lesions, we postulate the role of ICAM-1 expression on SMCs in the development of intimal hyperplasia via 3 ways. First, the interaction of MAC-1 and ICAM-1 expressed on SMCs may initiate intracellular signaling necessary for cytokine secretion by monocytes/macrophages. Support for this notion comes from the fact that macrophage inflammatory protein-1α production was induced in monocytes cultured on ICAM-1–coated plates.[37] Second, the binding of MAC-1 to ICAM-1 expressed on SMCs may be responsible for monocyte retardation in the vessel wall. Third, it has been reported that expression of ICAM-1 on SMCs may be relevant to the phenotypical change of SMCs,[38] which is considered to be essential to the migration and proliferation of SMCs in the pathogenesis of atherosclerosis.[39] Therefore, the binding of MAC-1 to ICAM-1 on SMCs might result in intracellular signaling within SMCs, which initiates the gene expression needed for phenotypical change.

In summary, nearly all veins implanted into the arterial circulation in patients develop intimal hyperplasia within 4 to 6 weeks, which represents the foundation for later development of venous bypass graft atheroma.[2] We demonstrated that ICAM-1 is critical in the accumulation of monocytes/macrophages responsible for neointimal hyperplasia in early grafted vessels. If ICAM-1 expression of vein grafts in patients is inhibited by locally applied drugs, such as aspirin, or neutralized by anti–ICAM-1 antibodies, reduced intimal lesions may be seen.

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Figure 8. ICAM-1 expression on SMCs and leukocyte adhesion. Mouse SMCs from ICAM-1 $-/-$ (A) and $+/+$ (B and C) mice were incubated with TNF-α (100 ng/mL; A and C) for 24 hours. Cells were fixed with cold 5% acetone/methanol ($-20°C$) for 10 minutes, air dried, and incubated with the anti–ICAM-1 antibody (A to C) for 1 hour. The reaction was visualized with anti-rabbit Ig/FITC-conjugated swine Ig (original magnification $\times250$). E, Leukocyte adhesion to SMCs. Mouse spleen leukocytes were obtained by passing spleen tissue through a 120-mesh stainless steel net and removing red blood cells through lysis with NH$_4$Cl.

Mouse SMCs were preincubated with or without TNF-α for 24 hours at 37°C. After washing with RPMI, the spleen cells were added to the culture and incubated for 1 hour at 37°C. The adherent cells were enumerated under the microscope. Four fields ($\times250$) were evaluated from each well. Values are mean$\pm$SEM of 3 experiments. *$P<0.05$ vs ICAM-1 $+/+$ SMCs treated with TNF-α.

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