Absence of CD40 Signaling Is Associated With an Increase in Intimal Thickening After Arterial Injury

Mojca Remskar, Hongyan Li, Kuang-Yuh Chyu, Prediman K. Shah, Bojan Cercek

Abstract—Immune-mediator CD40 ligand (CD40L), a member of the tumor necrosis factor family, and its receptor CD40 of the nerve growth factor/tumor necrosis factor receptor family were originally considered to be restricted to activated T and B cells. Recently they have been localized to a variety of cell types involved in the immune system (B cells, T cells, monocytes, and dendritic cells), fibroblasts, endothelial cells, and vascular smooth muscle cells. CD40-CD40L interaction is associated with T- and B-cell activation and expression of a variety of chemokines, cytokines, matrix metalloproteinases, tissue factor molecules, and leukocyte-adhesion molecules. In vascular tissues, inhibition of CD40-CD40L signaling has been associated with a reduction of experimental atherosclerosis and transplant-associated vasculopathy. Immune response also plays a cardinal role in intimal thickening after acute arterial wall injury. After carotid artery injury in CD40 ligand knockout (CD40L^−/−) mice, the intimal thickening was increased 3-fold compared with the thickening in background B6/129 mice. The extent of thickening was similar to the thickening in B6/129 mice depleted of T lymphocytes with anti-CD4 and anti-CD8 antibodies. Injection of splenocytes from B6/129 mice into the CD40L^−/− mice reduced the intimal thickening to the level comparable to the thickening in background B6/129 mice. These data suggest that CD40 signaling plays a significant role in the inhibitory effect of T lymphocytes on intimal thickening after arterial injury. (Circ Res. 2001;88:390-394.)

Key Words: CD40 □ CD40 ligand □ T lymphocytes □ intimal thickening □ arterial wall injury

Materials and Methods

Animals

The Institutional Animal Care and Use Committee of Cedars-Sinai Medical Center approved the study. Animals were housed in conditions of 12-hour light and dark periods in an animal facility accredited by the American Association of Accreditation of Laboratory Animal Care.

Male B6/129 mice and CD40L^−/− mice of the same genetic background (weight 33.3 to 51.1 g) were obtained from Jackson Laboratories (Bar Harbor, Maine) and fed standard diet and water ad libitum. At age 25 to 26 weeks, they underwent collar-induced injury to the right carotid artery (see below).

Mice were divided into the following groups: immunocompetent B6/129 injected with saline (n=6) or IgG isotype (n=6) as control; T lymphocyte–depleted B6/129 mice by intravenous injections of anti-CD4 and anti-CD8 antibodies (n=7); CD40L^−/− mice (n=7); and CD40L^−/− mice reconstituted with intravenous injections of 50 to 60×10^6 splenocytes obtained from B6/129 mice (n=6).

Carotid Artery Injury

Animals were anesthetized with 0.015 mL/g of 2.5% Avertin (2,2,2-tribromoethyl alcohol in tertiary-amyalcohol; Aldrich) injected intraperitoneally. The right carotid artery was exposed under sterile conditions, and a nonocclusive, flexible silicone collar (length 3 mm; internal diameter 0.51 mm; Cole-Parmer Instrument Co) was placed around the carotid artery proximal to the carotid artery bifurcation, and secured in place with a suture across the cut in the collar. The skin was closed with a series of sutures. After that time point, the mice were housed individually. Mice were killed 21 days after collar placement for morphometric analysis.

Immunosuppression

Rat anti-mouse CD4 monoclonal antibody GK 1.5 and anti-mouse CD8 monoclonal antibody GK 2.43 (PharMingen), 125 μg each,
were injected into the tail vein of B6/129 mice 1, 3, and 6 days before
and 5, 11, and 17 days after collar placement. The extent of the
depletion of T lymphocytes was determined by flow cytometry performed on the day of injury and before every injection of monoclonal antibodies after injury. Control B6/129 mice were injected with 250 \( \mu \)L of purified mouse IgG \( \kappa \) immunoglobulin isotype (PharMingen). All other mice were injected with 250 \( \mu \)L of saline.

**Flow Cytometry**

Peripheral blood mononuclear cells (PBMCs) were isolated from 300 \( \mu \)L of peripheral venous blood obtained from retro-orbital plexus. Blood was collected into 200 \( \mu \)L of 0.5 mol/L EDTA, and red blood cells were removed by lysis with 0.83% ammonium chloride and 5 mmol Tris at pH 7.2. PBMCs were stained with 100 \( \mu \)g/mL of the following antibodies: phycoerythrin-conjugated rat anti-mouse CD4 monoclonal antibody, antifulfrusein isothiocyanate–conjugated anti-mouse CD8 monoclonal antibody, phycoerythrin-conjugated hamster anti-mouse CD154 (CD 40L) monoclonal antibody, and antifulfrusein isothiocyanate–conjugated anti-mouse CD3e monoclonal antibody (PharMingen). After incubation for 25 minutes at 4°C in the darkroom and 3 washes in PBS, PBMCs were analyzed in Becton-Dickinson FACScan flow cytometer. The concentration of various subtypes was expressed as percent of total PBMCs.

Flow cytometry was performed after the first 3 injections of antibodies before injury and before each injection after the injury. PBMCs of a separate group of B6/129 and CD40L \(-/-\) mice were analyzed for CD40L expressing CD3-positive T lymphocytes before and 24 hours after injury.

The expression of CD40L on splenocytes obtained from B6/129 mice \((n=2)\) was determined by staining the splenocytes with phycoerythrin-conjugated anti-mouse CD40L antibody (PharMingen) followed by analysis in Becton-Dickinson FACScan flow cytometer. Concentration of CD40L-positive cells was expressed as percent of PBMCs.

**Splenocyte Reconstitution**

B6/129 mice were killed with an overdose of methoxyflurane (Metoflurane, Schering-Plough) in an inhalation chamber. Medial laparotomy was performed in aseptic conditions, and spleens were removed from the animals. Spleens were gently scraped with sterile scissors to obtain splenocytes. Larger tissue particles were removed by filtration. Red blood cells were removed by lysis buffer, as described above. Spleenocytes were counted and checked for viability by Trypan blue staining. Spleenocytes, 50 to 60 \( \times 10^8 \) cells in a volume of 350 \( \mu \)L of sterile saline, were then injected into a tail vein of a CD40L \(-/-\) mouse 1 day before injury.

**Immunohistochemistry and Immunofluorescence**

Twenty-one days after the injury, the mice were killed, as described above, and the arteries were flushed with saline at physiological pressure. Carotid arteries were harvested, immediately embedded in OCT, and stored at -70°C. Sections from the middle half of the injured segment were collected. Serial 6-\( \mu \)m-thick sections, an average of 6 \( \mu \)m apart, were collected. Twenty to 25 slides with 3 sections each were sequentially divided into 3 groups, and the first 3 slides of each group were stained with H&E for morphometric analysis. Total intimal area (area within internal elastic lamina minus luminal area) and medial area (area within external elastic lamina minus area within internal elastic lamina) were determined with computer-assisted analysis using Image Pro-plus 4.0 (Media Cybernetics). The measurements were performed in a blinded manner.

For immunohistochemical staining, the sections were incubated with anti-mouse vascular cellular adhesion molecule-1 (VCAM-1) antibody (PharMingen) followed by biotinylated secondary antibody and avidin-biotin complex (Sigma), as described previously.10

Collagens type I and III were stained by Picrosirius red stain, as described previously.7 Frozen sections were incubated for 90 min-

<table>
<thead>
<tr>
<th>Group</th>
<th>Intima, mm^2</th>
<th>Media, mm^2</th>
<th>I/M Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>B6/129+saline</td>
<td>0.009±0.001</td>
<td>0.024±0.005</td>
<td>0.36±0.05</td>
</tr>
<tr>
<td>B6/129+IgG isotype</td>
<td>0.012±0.001</td>
<td>0.039±0.002</td>
<td>0.4±0.15</td>
</tr>
<tr>
<td>B6/129+anti-CD4/CD8</td>
<td>0.028±0.005*</td>
<td>0.036±0.008</td>
<td>0.84±0.16</td>
</tr>
<tr>
<td>CD40L(-/)</td>
<td>0.029±0.02*</td>
<td>0.040±0.016</td>
<td>0.64±0.29</td>
</tr>
<tr>
<td>CD40L(-/) rec</td>
<td>0.014±0.007</td>
<td>0.034±0.007</td>
<td>0.39±0.17</td>
</tr>
</tbody>
</table>

*\( P<0.05 \) vs B6/129+saline and vs CD40L\(-/\) rec.

Table 1. Intimal Area, Medial Area (mm^2), and Intima/Media (I/M) Ratio 21 Days After Injury

The extent of intimal thickening in CD40L \(-/-\) mice was similarly exuberant as in T lymphocyte–depleted mice (0.029±0.02 mm^2). In mice reconstituted with CD40L-containing splenocytes, the intimal area after injury was substantially decreased and was comparable to the findings in the immunocompetent B6/129 mice (0.014±0.007 mm^2). Because the medial area was similar in all groups of mice, the intima/media ratio paralleled changes in the intimal area. The representative cross sections of carotid arteries are shown in Figure 1.

**T-Lymphocyte Depletion**

There were on average only 2% of positive CD4/CD8 PBMCs in mice injected with anti-CD4/CD8 antibodies, compared with 10% to 20% in mice injected with saline or

utes in 0.1% Sirius red (Polysciences) dissolved in saturated picric acid, rinsed in 0.01 mol/L HCl and distilled water, and dehydrated in 70% ethanol. Staining was analyzed by polarizing microscopy.

For immunofluorescence, 6-\( \mu \)m-thick serial frozen sections were incubated with primary rat anti-mouse CD40 (3/23) antibody and hamster anti-mouse CD40L monoclonal antibody (PharMingen) at 4°C overnight. After incubation with biotinylated secondary antibody (mouse anti-hamster IgG, IgG\(_\alpha\), and goat anti-rat IgG) (PharMingen) and avidin-biotin complex, the reaction was visualized by use of dichlorotriazinylamino fluorescein–conjugated streptavidin (Jackson ImmunoResearch Laboratories) under fluorescence microscope.

**Statistical Analysis**

Results for each group of animals are presented as mean±SD. Groups were compared by ANOVA. \( P<0.05 \) was considered statistically significant.

**Results**

The results of morphometric measurements of intimal and medial areas 21 days after collar-induced carotid artery injury in all groups of mice are shown in Table 1. Depletion of T lymphocytes was associated with 3.1-fold increase in intimal thickening compared with the intimal thickening in immunocompetent B6/129 mice injected with saline and with a 2.3-fold increase in mice injected with IgG isotype (0.028±0.005 versus 0.009±0.001 versus 0.012±0.001 mm^2, \( P=0.0086 \)).

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IgG isotype (Table 2). The depletion of circulating T lymphocyte was at least 80% complete.

### CD40L Expression

In B6/129 mice, 11±4% of CD3-positive T cells expressed CD40L before injury compared with 38±17% 24 hours after injury. As expected, the PBMCs of CD40L−/− mice did not express CD40L on their surfaces. CD40L was expressed on 2% of splenocytes of B6/129 mice before injury.

### VCAM-1 Immunohistochemistry

Twenty-one days after injury, VCAM-1 stain was present in the intima and media. In B6/129 mice, 16±5% of intima and media stained positive for VCAM-1 compared with 24±12% in CD40L−/− mice. In CD40L−/− mice reconstituted with splenocytes, 15±5% of the vessel area stained positive for VCAM-1 (P=0.39).

### CD40 Immunofluorescence

Twenty-one days after injury, CD40 was expressed on the luminal side of the intimal thickening, as shown in Figure 2.

### Collagen Stain

The extent of collagen deposition detected in the intima was similar in both groups of mice. Picrosirius stain covered 16.8±2.5% of intimal area in CD40L−/− mice compared with 18.9±4% of intimal area in B6/129 mice (P=0.3, n=5 each).

## Discussion

The present study demonstrates the inhibitory effect of CD40-CD40L signaling on intimal thickening after arterial injury.
injury. Placement of a nonocclusive collar around the carotid artery of CD40L−/− mice resulted in a significant increase in intimal thickening compared with the intimal thickening in control B6/129 mice. The extent of thickening was similar to the thickening in B6/129 mice that were depleted of T lymphocytes. Intimal thickening in CD40L−/− mice was decreased to the level comparable to thickening in immunocompetent mice when reconstituted with splenocytes from normal mice. These data suggest that the absence of CD40 signaling is at least in part responsible for the exuberant intimal thickening after injury in immune-deficient animals. Because the intimal thickening was comparable in immunocompetent mice and CD40L−/− mice reconstituted with splenocytes, it seems that CD40L expression on splenocytes and not expression on the cells of the vascular tissue is of importance in regulation of this process. The potential mechanism of the inhibitory effect of injected splenocytes on intimal thickening is the activation of the T lymphocytes, but other mechanisms cannot be excluded.

The mechanism for increased intimal thickening in CD40L−/− mice is unclear. Activation of CD40–CD40L signaling is associated with upregulation of proinflammatory pathways. T cells and macrophages produce several cytokines, interferon (IFN)-γ, interleukin-6, and interleukin-8. T lymphocytes are not activated in the CD40L−/− mice. Because IFN-γ has consistently been shown to have antiproliferative and antidifferentiating effects on SMCs, the reduction of IFN-γ may be at least in part responsible for increased intimal thickening in CD40L−/− mice in our study. CD40/CD40L interaction leads to increased adhesion molecule expression on endothelial cells. Interestingly, in our study, VCAM-1 expression was higher in injured carotid arteries of CD40L−/− mice compared with control mice, although the difference was not statistically significant. The mechanism of at least similar medial and intimal VCAM-1 expression in the absence of CD40/CD40L interaction is unknown. It is possible that reduction of IFN-γ results in more pronounced SMC phenotypic modulation and VCAM-1 expression. These findings are opposite the findings of decreased VCAM-1 expression in the atherosclerotic plaques in mice treated with anti-CD40L.

Anti-CD40L antibody treatment of LDL receptor–deficient mice resulted in increased atherosclerotic plaque SMC cellularity and collagen deposition, favoring plaque stabilization. In our study, there was no difference in intimal collagen content, as detected by Picrosirius in CD40L−/− mice compared with immunocompetent B6/129 mice. The collagen content detected in our study is somewhat lower, ~18%, compared with 30% detected with the same technique previously. This might be explained by the use of different animal models and different time points of measurements in the two studies. In the study by Karim et al., the intimal collagen content increased from 18% to 32% when measured at 1 week and 4 weeks after balloon angioplasty, respectively.

Previous studies have implicated T lymphocytes to exert modifying effects on intimal thickening after injury. The results of our study confirm that T lymphocytes exert an inhibitory effect on intimal thickening. Depletion of T cells with specific monoclonal antibodies resulted in a several-fold increase in intimal thickening, similar to the results of Hansson and Holm in a rat model of arterial injury. These effects were specific to T-cell depletion, because injections of IgG isotype had no effect. The extent of augmentation of intimal thickening with T-cell depletion was similar to the effect of absence of CD40 signaling. The increased intimal thickening in CD40L−/− mice was reversed with reconstitution with normal splenocytes. It thus seems that CD40 signaling is a crucial step in the inhibitory effect of T cells on intimal thickening. Our results additionally indicate that in contrast to the process of atherosclerosis, CD40L expressed on SMCs, endothelial cells, and macrophages may not play a significant role in regulation of the intimal thickening after acute arterial injury. We conclude that CD40 signaling has an inhibitory effect on intimal thickening after acute arterial injury.

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References


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