Inhibition of CD40 signaling limits evolution of established atherosclerosis in mice

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Interruption of inflammatory pathways may provide a novel approach to the therapy of atherosclerosis. Recently, we and others have implicated the immune mediator dyad CD40/CD40L (CD40 ligand), which is expressed on endothelial and smooth muscle cells, macrophages, and T lymphocytes within human atherosclerotic lesions, in aspects of atherogenesis and the acute coronary syndromes, including regulation of matrix metalloproteinases, procoagulant activity, cytokines, etc. In vivo, interruption of CD40 signaling reduced the initiation and early phases of atheroma formation in hypercholesterolemic mice. However, whether interruption of CD40 signaling can retard the progression or even regress established lesions remains unknown. We report here that anti-CD40L antibody treatment of randomly assigned low-density lipoprotein receptor-deficient mice during the second half of a 26-week regimen of high-cholesterol diet did not regress, but did significantly reduce further evolution of established atherosclerotic lesions within the aortic arch and particularly the thoracic and abdominal aorta, as compared with control treatment (application of rat-IgG or saline; 13 weeks, continued high-cholesterol diet). In addition to limiting lesion progression, anti-CD40L treatment changed the composition of atheroma in manners thought to favor plaque stability, e.g., reduced relative content of macrophages and lipid, as well as increased relative content of smooth muscle cells and collagen. These data implicate CD40/CD40L as crucial mediators not only in the initial events of atherogenesis but also during the evolution of established atheroma. This study lends further support to the importance of this specific inflammatory signaling pathway in atherosclerosis and its complications.

Atherosclerosis bears many hallmarks of a chronic inflammation (1). However, our understanding of the precise molecular mechanisms underlying the inflammatory and immune responses implicated in atherogenesis remains sparse. Recent studies have suggested a role for the immune mediator CD40 ligand (CD40L, CD154, previously also termed gp39) and its receptor CD40 in regulation of processes associated with this prevalent human disease in vitro and in vivo.

Expression and biological function of CD40 and CD40L originally were considered restricted to activated CD4- positive T cells and B lymphocytes (2–9). More recent studies have revealed expression of both CD40 and CD40L on a broad variety of cells, including vascular endothelial cells (ECs), smooth muscle cells (SMCs), and macrophages (10–14). In situ studies have further localized CD40L and CD40 on these cells at sites of chronic inflammation, including atherosclerotic lesions (12, 14–16).

Considerable evidence supports the importance of CD40 signaling in control of processes thought crucial in various stages of atherogenesis: initiation, evolution, and acute complications after the rupture of atherosclerotic lesions. Ligation of CD40 induces the expression of adhesion molecules, e.g., E-selectin, VCAM-1 (vascular cell adhesion molecule 1), or ICAM-1 (intercellular adhesion molecule 1), on human vascular ECs (17–19) and stimulates the release of chemokines, such as IL-8, RANTES (regulated upon activation, normal T cell expressed and secreted), or macrophage inflammatory protein 1α (20–22). These molecules probably participate in the initiation of atheroma. CD40 ligation also induces the expression of cytokines, such as IL-1, IL-6, IL-12, IFN-γ, and tumor necrosis factor α, in various cell types, including ECs, SMCs, and macrophages in vitro (14, 20, 23–25). Those mediators likely play a role in ongoing local inflammatory reactions during the evolution of the fatty streak into the differentiated lesion. With regard to the later complications of atheroma, recombinant or native CD40L induces the expression of matrix metalloproteinases in atheroma-associated cells (26–31). These enzymes can weaken the fibrous skeleton of the plaque, rendering it susceptible to rupture and hence thrombosis. Interestingly, CD40 and CD40L colocalize with interstitial collagenases at sites of collagenolysis within human atheroma in situ, supporting a role for CD40 signaling in plaque rupture (29). Finally, we and others have demonstrated that ligation of CD40 induces the expression of procoagulant tissue factor on monocytes, ECs, and SMCs, probably promoting procoagulant activity potential within atheroma (28, 32–34).

Recently, we tested whether CD40 signaling alters atherogenesis in vivo. We have established that this pathway modulates the early phase of atherogenesis. Anti-CD40L treatment drastically reduced the de novo formation of atherosclerotic lesions in 8-week-old low-density lipoprotein receptor (Ldlr)-deficient mice, fed a high-cholesterol diet and treated in parallel with either anti-CD40L antibody, control IgG, or saline (35). Similar findings recently have been reported with ApoE/CD40L compound mutant mice (36).

The present study tests the additional and highly clinically relevant hypothesis that treatment with anti-CD40L antibody might further affect the evolution of already established atherosclerotic lesions, e.g., by regression and/or stabilization of existing lesions.

Materials and Methods

Treatments of Mice. Ldlr-deficient mice were obtained from the Jackson Laboratory. At the age of 8–10 weeks, 30 mice were fed a high-cholesterol diet (product #D12108, Research Diets, New Brunswick, NJ; 1.25% cholesterol, 0% cholate). After 13 weeks, eight randomly assigned mice were killed. The heart, aorta, and certain organs, e.g., lung, liver, spleen, kidney were removed and analyzed as described below. The arch and abdominal portions of the aorta were separated, and the aortic arches were perfused with PBS and snap-frozen in OCT (OCT compound, Tissue-Tek, Torrance, CA), as described (35). This study group was used to determine the extent of established lesions in these hypercholesterolemic mice at the baseline of 13 weeks. The remaining 22 mice...
were randomly assigned for treatment with either saline (n = 8; 250 μl/mouse twice weekly via i.p. injection; Baxter, Deerfield, IL), rat IgG (n = 8; 250 μg/mouse twice weekly; Sigma), or anti-CD40L antibody (M158; n = 6; 250 μg/mouse twice weekly). The anti-murine CD40L antibody (kindly provided by Immunex, Seattle, WA) was raised as described (37). During the 13 weeks of treatment, the mice were continuously fed the high-cholesterol diet. Subsequent serum analysis revealed no significant differences in the lipoprotein profile or total cholesterol content. After 26 weeks of treatment, the mice were killed and the respective tissue was harvested as described above. During the second half of the study two mice in the saline and three mice in the rat IgG-treated group died. Autopsy did not reveal an obvious cause of death and showed no evidence of internal bleeding. These mice, however, did have severe stenosis (>90%) of the artery. However, these findings could not be directly linked to the cause of death.

Immunohistochemistry. For immunohistochemical analysis, serial cryostat sections (6 μm) of the aortic arch were cut, fixed in acetone (−20°C, 5 min), air-dried, and stained with the respective antibody (anti-α-actin polyclonal antibodies, 1:100 (Santa Cruz Biotechnology); anti-mouse Mac-3 mAbs (to analyze content of macrophages), 1:1,000 (PharMingen); anti-CD4, 1:100 (PharMingen) as described (14). Briefly, tissue sections were treated with 0.3% hydrogen peroxide to inhibit endogenous peroxidase activity and were incubated with primary antibodies diluted in PBS supplemented with 4% of the species respective normal serum, followed by the respective biotinylated secondary antibodies and avidin-biotin complex (Vector Laboratories). The reaction was visualized by using 3-amino-9-ethyl carbazole as substrate (Sigma), and the sections were counterstained with Gill’s hematoxylin solution (Sigma). For specificity control we performed staining with the respective nonimmune IgG subclass (Dako).

Collagens type I and III were stained by Picrosirius red as described (29). Briefly, frozen tissue sections were incubated for 90 min in 0.1% Sirius red F3BA (Polysciences) dissolved in saturated picric acid. After rinsing twice in 0.01 M HCl, and in distilled water, sections were briefly dehydrated with 70% ethanol and coverslipped. Staining with Sirius red was analyzed by polarization microscopy.

Deposition of lipids within the thoracic and abdominal aorta

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**Fig. 1.** Anti-CD40L antibody administration reduces evolution of established atherosclerotic lesions. Ldlr-deficient mice consumed a high-cholesterol diet for 13 weeks (diet alone; white bars) before treatment with either rat IgG (light gray bars), saline (dark gray bars), or anti-CD40L antibody (α-CD40L; black bars) while continuing a high-cholesterol diet (13 weeks). Photomicrographs of longitudinally cut tissue sections of mouse aortic arches were analyzed by computer-assisted image quantification. The maximal wall, medial and intimal area as well as the maximal thickness of the inner aortic arch wall for each mouse was measured extending 3 mm distal from the right carotid. Calculation of total areas, thickness, and percent positive areas was performed independently by two blinded observers. Data are presented as mean ± SD, and comparison between the respective study groups used Student’s t test.

**Fig. 2.** Evidence for enhancement of features associated with plaque stability by anti-CD40L antibody treatment. Ldlr-deficient mice consumed a high-cholesterol diet for 13 weeks before treatment with either rat IgG, saline, or anti-CD40L antibody (α-CD40L) for 13 weeks during continued high-cholesterol diet. Aortic arches were perfused with PBS and snap-frozen, and sequential (6 μm) tissue sections were examined for the expression of α-actin (SMCs), CD68 (macrophages, MØ), or interstitial collagen by Picrosirius red as described (29). Briefly, frozen tissue sections were incubated for 90 min in 0.1% Sirius red F3BA (Polysciences) dissolved in saturated picric acid. After rinsing twice in 0.01 M HCl, and in distilled water, sections were briefly dehydrated with 70% ethanol and coverslipped. Staining with Sirius red was analyzed by polarization microscopy.
(fixed with 10% buffered formalin) was determined by Oil red O staining. Subsequently, the aortas were opened longitudinally to the iliac bifurcation and pinned out on black wax surface with 0.2-mm steel pins. Lipid deposition within the aortic arch was determined by similar staining techniques applied to the serial cryostat sections of the tissue.

**Tissue Analysis.** To quantify the extent of atherosclerotic lesions, longitudinal sections (about 30 per mouse) of the aortic arch as well as cross-sections of the longitudinally opened abdominal aortas at defined locations were analyzed microscopically in all mice. In the aortic arch, a 3-mm segment of the lesser curvature was defined proximally by a perpendicular dropped from the right side of the innominate artery origin, and the total aortic arch wall area, as well as the medial and intimal area, subtended by this 3-mm stretch of intima were separately calculated for each section of all mice by computerized image analysis, using IMAGEPRO PLUS software (Media Cybernetics, Silver Spring, MD). The aortic arch wall thickness was determined on this same segment of the lesser curvature. For the abdominal aorta, serial cross sections (6 μm; n = 10) distal of the origin of the renal artery were used to measure the areas of interest, e.g., total aortic wall, medial, and intimal area. To compute averages per group maximal areas of the total aortic wall, medial, and intimal area as well as the maximal thickness of the total aortic wall (media + intima) of each mouse were used. The percentage of the total area of the aortic arch stained for SMCs, macrophages, collagen, or lipid was determined by using computer-assisted image quantification (IMAGEPRO PLUS).

*En face* analysis of the abdominal aorta before sectioning included measurement of the percentage of the surface area (15 mm from the iliac bifurcation to the thoracic section of the aorta) stained by Oil red O by using computer analysis (IMAGEPRO PLUS).

**Statistical Analysis.** Calculation of total areas, thickness, and percent positive areas was performed independently by two blinded observers. Data are presented as mean ± SD and were compared between study groups treated with anti-CD40L antibody and those treated with either rat IgG or saline by using the Student’s *t* test. A value of *P* ≤ 0.05 was considered significant.

**Results**

** Interruption of CD40/CD40L Interaction Limits Progression of Established Aortic Arch Atherosclerotic Lesions in Mice.** To determine whether interruption of CD40/CD40L signaling affects the evolution of established atherosclerotic lesions, Ldlr-deficient mice were fed a high-cholesterol diet for 13 weeks, and the extent of atherosclerotic lesions of the inner aortic arch was determined. The data obtained for the total aortic arch (0.49 ± 0.06 mm²), medial (0.14 ± 0.02 mm²), and intimal (0.36 ± 0.09 mm²) area, as well as the aortic arch wall thickness (373 ± 68 μm) (Fig. 1 Left), agreed well with those previously published for Ldlr-deficient mice treated for 13 weeks with a combination of the identical high-cholesterol diet and control IgG (0.46 ± 0.05 mm²) total aortic arch area, 320 ± 37 μm aortic arch wall thickness, respectively (35). Under a continued regimen of high-cholesterol diet, application of control rat IgG or saline for an additional 13 weeks yielded significantly enlarged atherosclerotic arch lesions (Fig. 1), as defined by an approximately 1.6-fold larger total and intimal aortic arch wall area, and an approximately 1.4-fold enhanced aortic arch wall thickness. In contrast, the medial area did not vary significantly between the different treatment groups. However, in mice fed for 26 weeks with high-cholesterol diet, treatment during the last 13 weeks with anti-CD40L antibody significantly reduced both total (0.52 ± 0.08 mm²; *P* < 0.02) and intimal (0.38 ± 0.07 mm²; *P* < 0.01 and *P* < 0.03, respectively) aortic arch area as well as aortic arch wall thickness (421 ± 45 μm; *P* < 0.01 and *P* < 0.03, respectively) compared to the rat IgG and saline control groups, respectively (Fig. 1). Lesion size did not differ from the 13-week baseline lesions in mice receiving anti-CD40L antibody, indicating that interfering with CD40 signaling did not regress, but did inhibit the progression of already established lesions.

** Interruption of CD40/CD40L Interaction Promotes Plaque Stabilizing Processes in Established Atheroma in Mice.** Detailed histological studies analyzing plaque composition beyond the mere extent of lesions revealed substantial differences between the various groups. We evaluated in particular the cellular composition, e.g., macrophages vs. SMCs, as well as variables thought in humans to govern plaque vulnerability, e.g., interstitial collagen content and lipid deposition (Fig. 2). Anti-CD40L treatment (11.6 ± 5.1%) significantly (*P* < 0.02) reduced the percent positive area for macrophages, compared with the baseline group (21.2 ± 4.3%) (Fig. 3). In contrast, both control groups demonstrated a significantly elevated percentage of macrophage-positive areas [35.2 ± 7.1% (*P* < 0.01) and 34.7 ± 10.5% (*P* < 0.001) for rat IgG and saline, respectively], ultimately yielding an approximately 3-fold reduction in the content of macrophages because of interruption of CD40/CD40L signaling. Analysis of sudanophilic areas showed similar findings, demonstrating an approximately 3.5-fold reduction of lipid deposition within aortic arch lesions of anti-CD40L treated animals, as compared with control-treated animals. Furthermore, lipid deposition...
deposition declined significantly ($P < 0.001$) in anti-CD40L treated mice when compared with the baseline group (13 weeks high-cholesterol diet). In contrast to macrophage content and lipid deposition, the content of SMCs and collagen significantly increased during anti-CD40L treatment. Compared with the rat IgG (SMC: 9.1 ± 3.1%; collagen: 16.4 ± 3.0%) or saline-treated groups (SMC: 10.7 ± 4.3%; collagen: 14.7 ± 2.6%), analysis of aortic arch lesions of mice treated with anti-CD40L antibody revealed a 2.8-fold higher content of SMC (26.7 ± 7.3%; $P < 0.01$) and collagen (40.8 ± 9.3%; $P < 0.01$) (Fig. 3). Indeed, anti-CD40L-treated animals had significantly greater SMC and collagen content ($P < 0.02$ and $P < 0.03$, respectively) in lesions than did the baseline group. These findings, in addition to the diminished macrophage and lipid content, suggested that interruption of CD40/CD40L interaction alters features of lesions, associated in humans with stable plaques. Fibrous cap thickness varied substantially within the same treatment group and even within the same lesion, rendering quantitation of this variable noninformative. The degree of calcification did not vary significantly between the different study groups (rat-IgG: 8.6 ± 11.1%; saline: 3.9 ± 4.1%; anti-CD40L: 2.0 ± 2.6%), but showed wide variation among individuals within groups (0–15.9%).

![Fig. 4. Anti-CD40L antibody treatment reduces lipid deposition in the abdominal aorta of hypercholesterolemic mice. Ldlr-deficient mice consumed a high-cholesterol diet for 13 weeks (A and B; white bar, diet only) before treatment with either rat IgG (A and C; light gray bar), saline (A and C; dark gray bar), or anti-CD40L antibody (A and C; black bar, α-CD40L) for 13 weeks during continued regimen of the diet. Photomicrographs of tissue sections of longitudinally cut abdominal aortas pinned out on black wax surface and stained for lipid deposition with Sudan IV (B and C), were analyzed by computer-assisted image quantification (shown are mean ± SD analysis, calculation of percent positive areas was performed independently by two blinded observers, comparison between the respective study groups used Student’s t-test) (A). The thoracic section of the aorta is on the right. Representative specimens from each group are shown.](image1)

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![Fig. 5. Anti-CD40L antibody treatment reduces the extent of atherosclerotic lesions in the abdominal aorta of hypercholesterolemic mice. Ldlr-deficient mice consumed a high-cholesterol diet for 13 weeks before treatment with either rat IgG, saline, or anti-CD40L antibody (α-CD40L) for 13 weeks during continued high-cholesterol diet. Cross sections of the longitudinally cut abdominal aortae from three mice (proximal to the renal artery bifurcation) are shown.](image2)

Fig. 5. Anti-CD40L antibody treatment reduces the extent of atherosclerotic lesions in the abdominal aorta of hypercholesterolemic mice. Ldlr-deficient mice consumed a high-cholesterol diet for 13 weeks before treatment with either rat IgG, saline, or anti-CD40L antibody (α-CD40L) for 13 weeks during continued high-cholesterol diet. Cross sections of the longitudinally cut abdominal aortae from three mice (proximal to the renal artery bifurcation) are shown.
treatment, however, prevented this progression of atherosclerotic lesions in hypercholesterolemic mice ($P < 0.0002$ and $P < 0.00001$, compared to the rat IgG and saline control groups, respectively). In addition to en face area, anti-CD40L treatment diminished the thickness of the aortic wall by approximately 3-fold, compared with the control groups ($P < 0.003$ and $P < 0.001$, respectively). Neither treatment affected the medial area. Immunohistochemical analysis could not be performed on these sections because of the prior staining with oil red O.

**Discussion**

Interest in the immune and inflammatory aspects of atherosclerosis recently has burgeoned (1) and the CD40/CD40L receptor/ligand dyad has received some attention in this regard (1, 38, 39). Atherosclerotic lesions of the abdominal aorta. (Fig. 6). Anti-CD40L antibody treatment over this time period reduced the extent of the inner aortic arch lesion approximately 1.5-fold. However, the results obtained regarding plaque composition were even more striking. Lesions of anti-CD40L-treated mice contained more SMCs and fewer macrophages, a finding that correlated with the enhanced concentration of interstitial collagen (a product of SMCs) (42, 43), and had smaller lipid-rich areas. Macrophages serve as a major depot for lipids within atherosclerotic lesions (44). The content of SMCs vs. macrophages as well as the extent of collagen within the lesion and the size of the lipid core correlated in human lesions with the liability for rupture and thrombosis (45, 46). Indeed, composition rather than just the size of the lesion serves as a major determinant of plaque stability (48). Indeed, we recently provided direct evidence for collagenolysis at the shoulder region of the aortic wall (49). For more detailed quantitation of the extent of atherosclerotic lesions within the abdominal aorta, we analyzed serial cross sections of the longitudinally opened abdominal aorta, proximal to the renal artery bifurcation, which served as a fiducial mark (Fig. 5). When mice were treated with either control rat IgG or saline, the extent of total and intimal lesions area increased 4- to 5-fold, compared with that found in baseline study animals (13 weeks high-cholesterol diet) (Fig. 6). Anti-CD40L antibody

**Fig. 6.** Reduced evolution of established atherosclerotic lesions of the abdominal aorta by anti-CD40L antibody treatment. Photomicrographs of cross sections of formalin-fixed and longitudinally opened abdominal aorta (as exemplified in Fig. 5) were analyzed by computer-assisted image quantification. The maximal wall, medial, and intimal area as well as the maximal thickness of the inner aortic arch wall for each mouse was measured. Data are mean ± SD for mice treated with either irrelevant rat IgG (light gray bars), saline (dark gray bars), or anti-CD40L antibody (black bars). These groups are compared with mice fed 13 weeks with high-cholesterol diet (white bars; diet alone). Comparison between the respective study groups used Student's t test.
human atherosclerotic lesion, the predominant site of plaque rupture (29). CD40 strongly colocalizes with the expression of the interstitial collagenases matrix metalloproteinase (MMP)-1 and MMP-13 at those sites of collagenolysis (14, 27–29). Moreover, CD40 ligation induces both matrix degrading enzymes in vitro (27–29). Thus, interruption of CD40 signaling likely limits this expression of these interstitial collagenolytic enzymes, preventing further impairment of plaque integrity. Consequently, the enhanced collagen content might result from shifting the balance toward conservation of interstitial collagen. Alternatively, interruption of CD40/CD40L interactions might inhibit collagen synthesis by SMC, diminish SMC proliferation, or reduce the expression of these interstitial collagenolytic enzymes, promoting atheroma formation (54, 55), anti-CD40L treatment, which slows the initiation of atherosclerosis (35), may affect preferentially earlier than more fully established lesions.

In conclusion, our study demonstrates that interruption of CD40-signaling in mice not only inhibits progression of established atheroma, but also promotes features associated in humans with plaque stability. Our findings illustrate the importance of evaluating composition rather than merely the size of lesions when intervening experimentally in atherogenesis. The present data support the concept that interruption of the chronic inflammatory cycle, as indicated by fewer leukocytes and diminished inflammatory mediator expression, mitigates atherogenesis. These results highlight the importance of inflammation as a potential therapeutic target in atherosclerosis.

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