Both early and delayed anti-CD40L antibody treatment induces a stable plaque phenotype

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In the present study, we investigated the role of the CD40L-CD40 pathway in a model of progressive atherosclerosis. ApoE−/− mice were treated with an anti-CD40L antibody or a control antibody for 12 wk. Antibody treatment started early (age 5 wk) or was delayed until after the establishment of atherosclerosis (age 17 wk). In both the early and delayed treatment groups, anti-CD40L antibody did not decrease plaque area or inhibit lesion initiation or age-related increase in lesion area. The morphology of initial lesions was not affected, except for a decrease in T-lymphocyte content. Effects of anti-CD40L antibody treatment on the morphology of advanced lesions were pronounced. In both the early and delayed treatment groups, T-lymphocyte content was significantly decreased. Furthermore, a pronounced increase in collagen content, vascular smooth muscle cell/myofibroblast content, and fibrous cap thickness was observed. In the delayed treatment group, a decrease in lipid core and macrophage content occurred. Interestingly, advanced lesions of anti-CD40L antibody-treated mice exhibited an increased transforming growth factor β1 immunoreactivity, especially in macrophages. In conclusion, both early and delayed treatment with an anti-CD40L antibody do not affect atherosclerotic lesion initiation but do result in the development of a lipid-poor collagen-rich stable plaque phenotype. Furthermore, delayed treatment with anti-CD40L antibody can transform the lesion profile from a lipid-rich to a lipid-poor collagen-rich phenotype. Postulated mechanisms of this effect on plaque phenotype are the down-regulation of proinflammatory pathways and up-regulation of collagen-promoting factors like transforming growth factor β.

Increasing evidence suggests a central role for the CD40L-CD40 signaling pathway in several immunogenic and inflammatory processes, including atherosclerosis. The interaction between CD40L (CD154, gp39) and CD40, members of the tumor necrosis factor (TNF) and TNF-receptor family, respectively, was originally thought to be restricted to B and T lymphocytes (1). However, this interaction is now found to play an important role in several autoimmune diseases, including the X-linked hyper-IgM syndrome (2), collagen-induced arthritis (3), allergic encephalitis and multiple sclerosis (4), and acute and chronic graft vs. host disease (5, 6).

An important role for CD40L-CD40 signaling in atherosclerosis has been reported (7–9). In atherosclerotic plaques of mice and humans, CD40L and CD40 are present on vascular smooth muscle cells (VSMCs), endothelial cells, macrophages, and T lymphocytes (8, 9). *In vitro* stimulation of CD40L-CD40 signaling in atheroma-derived cells (10) results in the activation of proatherogenic pathways, like the production of chemokines (10), cytokines (10), matrix metalloproteinases (9, 11), tissue factor (11), and leukocyte adhesion molecules (12–14).

Recently, we reported an important role for CD40L-CD40 interactions in the progression of atherosclerosis by using mice deficient in CD40L and apoE. We showed a dramatic decrease in plaque area in CD40L−/−/apoE−/− mice compared with normal apoE-deficient animals. Moreover, advanced atherosclerotic lesions of these mice showed a lipid-poor collagen-rich stable plaque phenotype, with reduced macrophage and T-lymphocyte content (7). Furthermore, administration of an anti-CD40L antibody to LDL-R−/− mice, when started early in the development of atherosclerosis, inhibited lesion initiation (8).

In this study, we investigated further the role of the CD40L-CD40 pathway in atherosclerotic plaque development and progression. An anti-CD40L antibody was administered to apoE−/− mice for 12 wk, either at the onset of atherosclerosis (early treatment) or after the development of advanced plaques (delayed treatment). Anti-CD40L antibody treatment affected neither plaque area nor the age-related increase in plaque area. The most prominent effect of anti-CD40L antibody treatment in both treatment groups was the development of a lipid-poor collagen-rich stable plaque phenotype, a phenotype similar to that in CD40L−/−/apoE−/− mice (7).

Because most acute complications of atherosclerosis, like myocardial infarction and cerebrovascular accidents, are the result of a rupture of an unstable lipid-rich collagen-poor lesion (15), anti-CD40L antibody treatment may prevent the acute complications of advanced atherosclerosis.

Methods

Mice. ApoE−/− mice (Iffa Credo), on a normal chow diet, received either a hamster anti-CD40L antibody or a hamster control IgG, generously provided by Biogen, at 500 μg per mouse by i.p. injection once per week for 12 wk. The early treatment group started at 5 wk of age ($n = 9$ anti-CD40L, $n = 8$ control), when hardly any atherosclerotic lesions were present. The delayed treatment group ($n = 8$ anti-CD40L, $n = 9$ control) started at 17 wk of age, the time point at which advanced atherosclerotic plaques have developed.

Lipid Profile. Plasma cholesterol and plasma triglyceride levels were determined in duplicate by using colorimetric assays (CHOD-PAP 1442341 and GPO-PAP 701912, respectively; Boehringer Mannheim).

Histomorphometry. Atherosclerotic plaques were divided into initial and advanced lesions. Initial lesions were defined as fatty streaks containing macrophage-derived foam cells with intracellular lipid accumulation (AHA type II) or pools of extracellular lipid (AHA type III), whereas advanced lesions contained

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Abbreviations: TGF, transforming growth factor; ASMA, α-smooth muscle actin; VSMC, vascular smooth muscle cell.

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extracellular lipid, a lipid core (AHA type IV), and/or a fibrous cap (AHA type Va-c) (16).

Tissue processing, histological classification, and morphometry were performed as described previously (7, 17).

Immunohistochemistry. Sections were immunolabeled with α-smooth muscle actin [$(ASMA)^{FITC}$ monoclonal, 1:3,000; Sigma] as a marker for vascular smooth muscle cells and fibroblasts, ED-1[20] (1:10) for the detection of macrophages, CD3 (CD3 polyclonal, 1:200; Dako A0452) for the detection of T lymphocytes, antitransforming growth factor β1 (Bionostics, Wyboston, Bedfordshire, U.K.) for the detection of transforming growth factor (TGF)β1, BrdUrd (Mas 250b, Harlan Laboratories, Haslett, MI) for the detection of DNA-synthesizing cells, and terminal deoxynucleotidyltransferase-mediated UTP end labeling (Boehringer Mannheim) for the detection of apoptosis, as described previously (7, 17).

Statistical Analysis. Data are expressed as mean ±SEM. Anti-CD40L antibody-treated apoE−/− mice were compared with control-treated apoE−/− mice. Anti-CD40L antibody-treated apoE−/− mice of the delayed treatment group were also compared with control-treated 17-wk-old apoE−/− mice to investigate plaque progression after treatment. For all analyses, a nonparametric Mann–Whitney *U* test was used. The level of statistical significance was set at $P < 0.05$.

Results

General. No significant differences in age, body weight, cholesterol, or triglyceride levels were observed between anti-CD40L antibody- and control-treated animals. Macroscopic and histological analysis of lungs, liver, intestine, spleen, and kidneys revealed no pathology in either anti-CD40L antibody- or control-treated mice. One mouse of the early anti-CD40L antibody treatment group and one mouse of the delayed treatment group died during the experiment.

***(i)* Early Treatment.** In total, 57 atherosclerotic lesions in the aortic arch of the anti-CD40L antibody-treated ($n = 8$) and 49 lesions of control-treated animals ($n = 8$) were analyzed. Total plaque area (24,625 ± 11,290 μm^2/per aortic arch, anti-CD40L vs. 32,485 ± 15,061 μm^2/per aortic arch, control) and individual initial and advanced plaque area did not differ between the treatment groups (Fig. 1*a*). Furthermore, no differences between the treatment groups could be observed in the number of initial (4.6 ± 0.7 anti-CD40L and 4.0 ± 0.8 control) or advanced (2.5 ± 0.5 vs. 2.4 ± 0.7) plaques, or in the number of lipid cores (0.8 ± 0.4 vs. 0.9 ± 0.3) or chondrocyte-containing plaques (0.4 ± 0.1 vs. 0.3 ± 0.1) per aortic arch.

Initial Lesions. Detailed histomorphological analysis revealed that no parameters except T-lymphocyte content differed between anti-CD40L antibody and control-treated groups. The relative T-lymphocyte content was 0.4 ± 0.2% in the anti-CD40L antibody-treated group, whereas it was 4.4 ± 1.3% in the control-treated group ($P < 0.05$) (Fig. 1*b*). Relative macrophage content and collagen content, as well as ASMA content, did not differ between the treatment groups (Fig. 1 *c–f*). Also, no differences in cell number (7,941 ± 1,416 mm^{-2} in anti-CD40L vs. 5,718 ± 1,016 mm^{-2} in control), DNA synthesis (3.7 ± 1.6% vs. 13.6 ± 5.8%), or apoptosis (0.3 ± 0.2% vs. 0.4 ± 0.3%) could be observed ($P > 0.05$). The ratio between type II and type III lesions did not differ between both treatment groups (6.4 ± 2.4 vs. 3.6 ± 1.8) ($P > 0.05$).

To substantiate further the lack of effect of early treatment on lesion initiation, lesions of descending thoracic and abdominal aorta were also analyzed. As expected, only small initial lesions

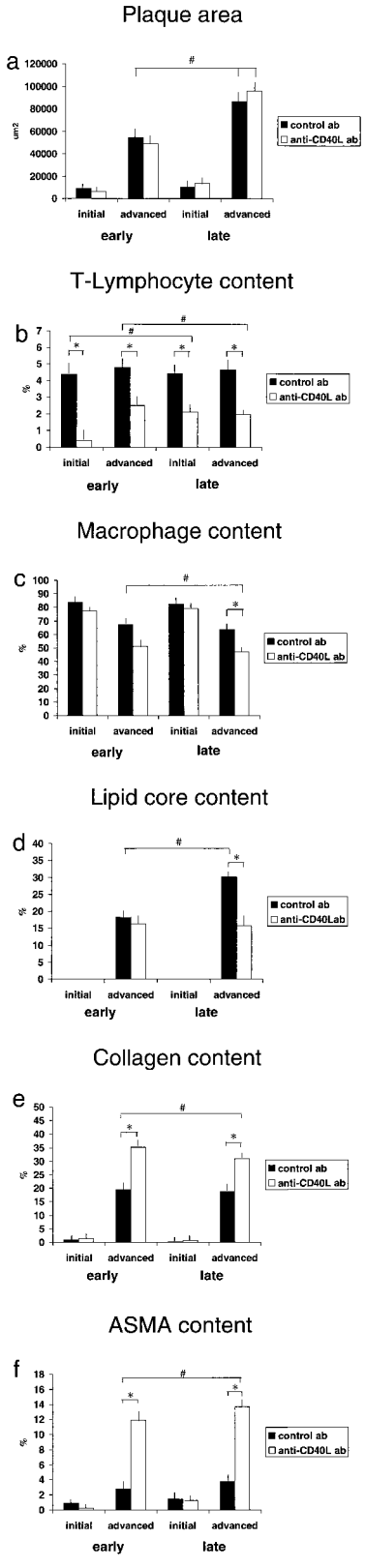

Fig. 1. Quantification of plaque characteristics of both early and delayed anti-CD40L antibody- and control-treated apoE−/− mice. (*a*) Individual plaque area; (*b*) T-lymphocyte content; (*c*) macrophage content; (d) lipid core content; (e) collagen content; (f) ASMA content. *, $P < 0.05$, anti-CD40L antibody vs. control; #, $P < 0.05$, 17-wk control treatment group (age at which delayed treatment started) vs. delayed anti-CD40L antibody treatment group or delayed control treatment group.

were present. No differences in initial lesion development were found between the treatment groups.

Advanced Lesions. In contrast to initial lesions, analysis of advanced atherosclerotic lesions revealed several differences in plaque composition between the treatment groups. First of all, a 48% reduction in T lymphocytes was observed in the anti-CD40L antibody-treated mice ($P < 0.05$)(Fig. 1*b*). Furthermore anti-CD40L antibody treatment resulted in an 81.2% increase in collagen content and a 325% increase in ASMA content ($P < 0.05$) (Fig. 1 *e* and *f*). Thus, fibrous caps were significantly thicker in anti-CD40L-treated animals (37.9 ± 7.2 vs. 15.2 ± 0.6 μm) ($P < 0.05$). No differences could be observed in macrophage content, lipid core content (Fig. 1 *c* and *d*), and chondrocyte content. Also, total cell number ($4{,}161 \pm 485$ vs. $5{,}886 \pm 2{,}632$ mm^{-2}), as well as the fraction of DNA-synthesizing ($2.9 \pm 1.2\%$ vs. $3.2 \pm 0.9\%$) or apoptotic ($1.2 \pm 0.3\%$ vs. $3.2 \pm 0.9\%$) cells did not differ.

The ratio between type IV and V lesions did not differ between anti-CD40L antibody- and control-treated mice (1 ± 0.3 vs. 1 ± 0.5) ($P > 0.05$). However, subdivision of type V lesions into either type Va (lipid rich) and type Vc (fibrous), revealed that in the anti-CD40L antibody-treated group, 50% of type V lesions were confined to type Vc (fibrous), whereas in the control-treated group, most of type V lesions were type Va (lipid-core rich) (87.5%) ($P < 0.05$).

***(ii)* Delayed Treatment.** Seventy-six atherosclerotic lesions of the anti-CD40L antibody-treated group ($n = 8$ mice) and 87 lesions of the control-treated group ($n = 8$) were analyzed. Concordant with the results of the early treatment group, no differences could be observed in total plaque area ($430{,}947 \pm 64{,}377$ μm^2 vs. $414{,}133 \pm 64{,}158$ μm^2) (Fig. 2 *a* and *b*), individual initial or advanced plaque area (Fig. 1*a*), the number of either initial (5.0 ± 0.5 vs. 6.0 ± 0.5 per aortic arch) or advanced (4.3 ± 0.7 vs. 4.6 ± 0.7 per aortic arch) plaques, and the number of chondrocyte-containing lesions (0.3 ± 0.3 vs. 0.6 ± 0.4) between anti-CD40L antibody- and control-antibody-treated animals ($P > 0.05$). However, delayed treatment with anti-CD40L antibody decreased the number of lipid cores (2.7 ± 0.7 vs. 3.9 ± 0.5 per aortic arch) ($P < 0.05$).

Initial Lesions. As in the early treatment group, detailed histomorphological analysis showed a reduction (67%) in T-lymphocyte content after anti-CD40L antibody treatment ($P < 0.05$) (Fig. 1*b*). Macrophage content (Fig. 1*c*), collagen content (Fig. 1*e*), total cell number ($4{,}355 \pm 677$ vs. $4{,}337 \pm 872$ mm^{-2}), DNA synthesis ($4.5 \pm 1.8\%$ vs. 5.2 ± 1.2), apoptosis (0.1 ± 0.1 vs. 0.5 ± 0.2), and the ratio between type II and III lesions were not affected (7.8 ± 1.0 vs. 2.6 ± 0.9) after anti-CD40L antibody treatment ($P > 0.05$).

Advanced Lesions. As in advanced lesions of the early treatment group, lesions of the anti-CD40L antibody-treated mice of the delayed treatment group also exhibited a decrease in T-lymphocyte content (58%), and increases in collagen content (64.2%), ASMA content (269.2%), and fibrous cap thickness (54.4%) were observed ($P < 0.05$) (Fig. 1 *b–f*, Fig. 2 *c–h*). However, in the delayed treatment group, changes in plaque phenotype were more pronounced. Macrophage and lipid core content had decreased significantly compared with the control treated animals (26% and 48%) ($P < 0.05$) (Fig. 1 *c–d*, Fig. 2 *c, d, i,* and *j*). Also, the chondrocyte positive area of chondrocyte containing lesions was significantly enlarged ($19.4 \pm 4.6\%$ in anti-CD40L vs. $6.8 \pm 1.2\%$ in control) ($P < 0.05$). The ratio between type IV and type V lesions did not differ between both treatment groups (0.4 ± 0.2 in anti-CD40L antibody vs. 0.9 ± 0.4 in control) ($P > 0.05$). However, in anti-CD40L antibody treated animals, 79.2% type V lesions were type Vc (fibrous), compared with 20.8% in control-treated animals.

Plaque Progression. To investigate the effects of delayed anti-CD40L antibody treatment on plaque progression, we also compared the delayed anti-CD40L antibody treatment group (age 29 wk) with the 17-wk-old control-treated group (the age at which delayed antibody treatment started). This revealed that anti-CD40L antibody treatment did not prevent the age-related increase in plaque area (Fig. 1*a*) or the increase in the number of initial (4.0 ± 0.8 to 6 ± 0.5) or advanced (2.4 ± 0.8 to 4.6 ± 0.7) plaques ($P > 0.05$).

However, several phenotypical changes occur with anti-CD40L antibody treatment. The percentages of T lymphocytes decreased in both initial (56%) and advanced lesions (59%) ($P < 0.05$) (Fig. 1*b*), as did the macrophage content (30%) ($P < 0.05$) (Fig. 1 *b* and *c*). Relative to the 17-wk-old animals, anti-CD40L antibody treatment also completely inhibited lipid core expansion (Fig. 1*d*). During anti-CD40L antibody treatment, collagen content, ASMA content, and fibrous cap thickness increased as compared with the 17-wk-old control-treated group (59%, 392%, and 183%, respectively) ($P < 0.05$) (Fig. 1 *e–f*). These data reveal that anti-CD40L antibody treatment can modify the lesion profile from lipid-rich collagen-poor lesions into a lipid-poor collagen-rich stable plaque phenotype.

TGFβ. Because TGFβ is known to reduce inflammation and induce tissue fibrosis and extracellular matrix synthesis (18), we hypothesized that anti-CD40L antibody treatment induces up-regulation of TGFβ, thereby contributing to the stable atherosclerotic plaque phenotype. In support of that hypothesis, anti-CD40L antibody-treated mice showed an increased TGFβ1 immunoreactivity in macrophage-rich lesions of advanced lesions, whereas fibrous regions did not stain for TGFβ1 (Fig. 3 *A* and *B*).

Discussion

The present study describes the effects of both early and delayed treatment with an anti-CD40L antibody in a mouse model of atherosclerosis. First, it reveals that interruption of the CD40L-CD40 pathway does not inhibit lesion initiation or age-related increase in lesion area, especially because anti-CD40L antibody treatment affected neither initial lesion area nor phenotype in early and delayed treatment groups. Secondly, anti-CD40L antibody treatment results in the development of a lipid-poor collagen-rich advanced stable plaque phenotype, with a reduced T-lymphocyte and macrophage content. The observation that the change of phenotype was most profound in advanced lesions of the delayed treatment group stresses the importance of CD40-CD40L signaling in late atherosclerotic changes.

The lipid-poor collagen-rich atherosclerotic plaque phenotype is concordant with the phenotype we observed after complete genetic disruption of CD40L-CD40 signaling (7). In the present manuscript, we show that anti-CD40L antibody treatment results in the development of a similar stable plaque phenotype. Moreover, we show that anti-CD40L antibody treatment is able to transform lipid-rich established atheromata into the lipid-poor collagen-rich stable plaque phenotype, implying a beneficial role for anti-CD40L antibody in the treatment of human atherosclerosis.

Activation of CD40L-CD40 signaling is associated with up-regulation of proinflammatory pathways, important in the progression of atherosclerosis and plaque destabilization. First, after activation of CD40L-CD40 signaling, T lymphocytes and macrophages produce several proatherogenic chemokines (MCP-1, IL-8, RANTES) and cytokines (TNFα, IL-6) (10). Second, activation of the CD40-CD40L pathway in endothelial cells results in the up-regulation of intercellular adhesion molecule, vascular cell adhesion molecule, and E-selectin (12–14), and third, CD40-CD40L signaling induces the up-regulation of matrix metalloproteinases (MMP 1, 3, 6, 9) (9, 11). These substances are associated with plaque macrophage recruitment, expansion of the lipid core, inhibition of collagen synthesis, and

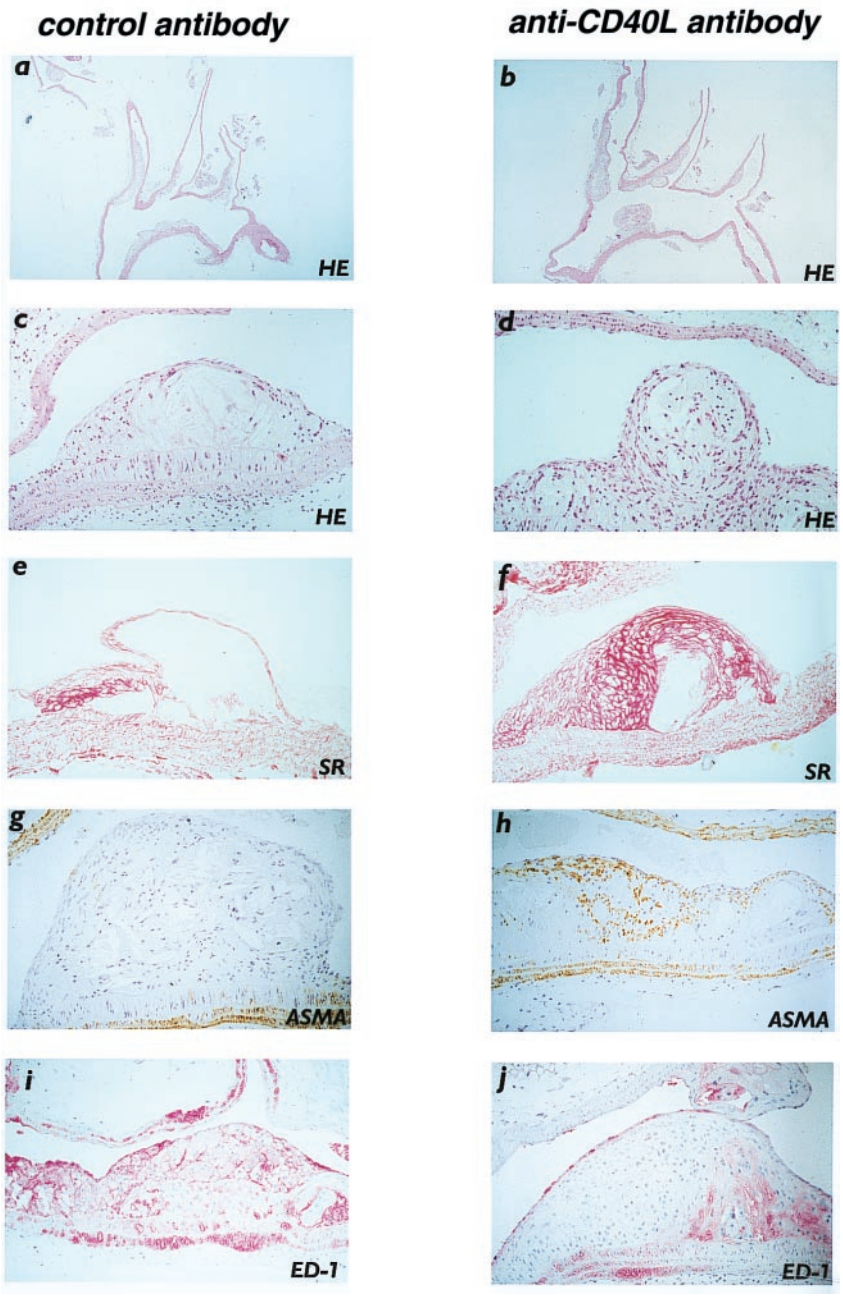

Fig. 2. Histological characteristics of delayed anti-CD40L antibody treatment. (*a* and *b*) Hematoxylin-and-eosin-stained longitudinal section of the aortic arch, including the brachiocephalic trunk, left carotid, and left subclavian artery (×25). Neither lesion area nor the number of lesions differed between anti-CD40L antibody- and control-treated mice. (*c* and *d*) Advanced atherosclerotic lesion, containing a lipid core and a fibrous cap. The relative lipid core area is less, and relative fibrous cap thickness is increased in anti-CD40L antibody- (*d*) compared to control-treated mice (*c*). (*e* and *f*) Sirius red staining of advanced atherosclerotic lesions, showing a higher relative collagen content in the anti-CD40L antibody-treated mouse (*f*) than in the control-treated mouse (*e*). (*g* and *h*) ASMA staining of advanced atherosclerotic lesions, showing a higher relative VSMC/myofibroblast content in the anti-CD40L-treated mouse (*h*) than in the control-treated mouse (*g*). (*i* and *j*) ED-1 staining of advanced atherosclerotic lesions, showing a decreased relative macrophage content in the anti-CD40L-treated mouse (*j*) compared to the control-treated mouse (*i*).

degradation of the fibrous cap *in vivo* (19). Inhibition of proinflammatory pathways might therefore have caused the observed lipid-poor collagen-rich plaque phenotype. Furthermore, both activated platelets and fresh thrombi express high levels of CD40L (20), and T-cell-mediated activation of the CD40L-CD40 system results in the up-regulation of tissue factor in macrophages and VSMCs (11, 21). Patients suffering from unstable angina show elevated levels of degranulated platelets and sCD40L plasma levels (22). Therefore, CD40L-CD40 signaling may induce a procoagulant stage, a feature favoring the development of plaque rupture and thrombosis (23).

Another postulated mechanism responsible for the change into a more stable plaque phenotype may be a different pattern of cell turnover. The influence of CD40L-CD40 signaling on cell turnover is, however, contradictory. It stimulates proliferation of B cells and Burkitt's lymphoma cells, whereas it inhibits proliferation of B-cell lymphoma cells (24–26). Furthermore, CD40 regulates apoptosis in a dual fashion, depending on the up-regulation of apoptosis resistance factors (24). In the present study, anti-CD40L antibody treatment did not affect cell turnover, because total cell number and the level of DNA synthesis and apoptosis did not differ from control-treated animals. Alternatively, anti-CD40L antibody treatment might cause cell turnover of specific cell types (VSMC), thereby resulting in a different plaque phenotype. Selectivity of both DNA synthesis and apoptosis in atherosclerosis was shown before in APOE*3Leiden mice, where both DNA synthesis and apoptosis are confined to macrophage-derived foam cells (17). However, in the present study, too, DNA synthesis and apoptosis were confined to the macrophage,

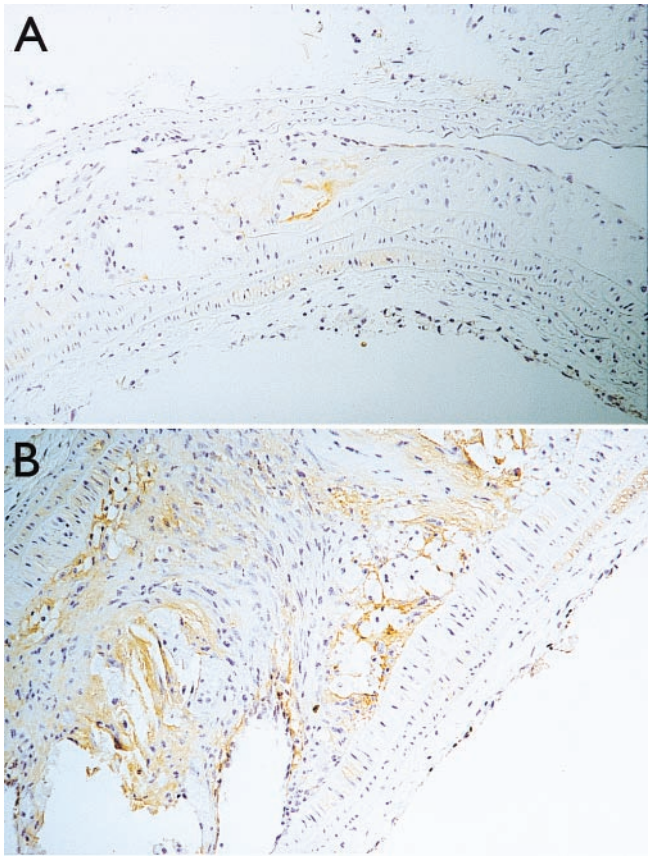

Fig. 3. Anti-CD40L antibody treatment induces increased immunoreactivity of TGFβ1. (*A* and *B*) TGFβ1 immunostaining of advanced atherosclerotic lesions, showing an increased immunoreactivity of TGFβ1 in macrophages of the anti-CD40L antibody-treated mouse (*B*) compared with the control-treated mouse (*A*).

making involvement of the CD40L-CD40 system in cell turnover during atherogenesis unlikely.

Interestingly, our study showed an increased immunoreactivity of TGFβ1 in lesions of anti-CD40L antibody-treated mice. Effects of TGFβ on atherosclerosis are, however, contradictory. On the one hand, TGFβ seems to have antiatherogenic effects, because it prevents atherosclerosis in animals (27, 28) and because patients with severe coronary artery disease express low levels of TGFβ (29). In addition, TGFβ can stimulate the synthesis of several extracellular matrix proteins (30, 31), inhibit the expression of matrix metalloproteases, enhance the expression of tissue inhibitors of matrix metalloproteinases (TIMPs) (32), and induce tissue fibrosis (33). Therefore, up-regulation of TGFβ might be responsible for the development of collagen-rich stable atherosclerotic plaques after anti-CD40L antibody treatment by stimulation of collagen production and inhibition of collagen degradation (down-regulation of MMPs and up-regulation of TIMPs). On the other hand, TGFβ and its receptors were abundantly expressed in human fatty streaks and fibrofatty lesions (34), and TGFβ induces the production of lipoprotein-trapping proteoglycans (35), indicating a stimulatory role of TGFβ in the progression of atherosclerosis.

Although we and others report an increase of plaque collagen content after inhibition of CD40-CD40L signaling (7, 36), it has been shown that anti-CD40L antibody treatment prevents lung fibrosis after irradiation-induced injury (37). Although we have no clear explanation for this phenomenon, the different tissue characteristics (artery vs. lung) and the different pathogenesis of the two models might play a role. In both models, inhibition of CD40-CD40L signaling results in a down-regulation of inflammation. However, in atherosclerosis, anti-CD40L antibody treatment results in the up-regulation of TGFβ, a molecule known to induce collagen synthesis, whereas in irradiation-induced lung injury, a down-regulation of TGFβ is suggested (37).

In conclusion, we have shown that inhibition of CD40L-CD40 signaling in mice with advanced atherosclerotic lesions results in the development of a lipid-poor collagen-rich stable plaque phenotype. Therefore, treatment with an anti-CD40L antibody in humans might be beneficial, not by inhibiting lesion initiation or progression, but by transforming unstable plaques into stable atherosclerotic lesions, thereby reducing the acute complications of atherosclerosis.

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