Leukocyte Transglutaminase 2 Expression Limits Atherosclerotic Lesion Size

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Objective—Transglutaminase 2 (TG2), a broadly expressed regulator of protein cross-linking, wound healing, and tissue fibrosis, mediates apoptotic cell ingestion and transforming growth factor-β release by macrophages and thereby can limit leukocyte-mediated inflammation. In atherosclerosis, oxidative stress and accumulation of unesterified cholesterol stimulate atherosclerotic lesion cell apoptosis. Cell death in advanced atherosclerotic lesions promotes lesion expansion and vulnerable plaques prone to rupture. Hence, we tested the hypothesis that leukocyte TG2 expression limits atherosclerosis.

Methods and Results—We transplanted TG2+/− or TG2+/+ bone marrow into lethally irradiated low-density lipoprotein receptor (LDLR)−/− mice and evaluated diet-induced atherosclerosis after 16 weeks. We subsequently studied cultured TG2−/− and congenic TG2+/+ mouse macrophages for selected atherogenesis regulatory functions. Atherosclerotic aortic valve lesions in LDLR−/− recipients of TG2−/− bone marrow were larger and more subintimal lesional macrophage penetration than in TG2+/+ macrophage recipients. Lesion intimal TG2 expression appeared robust in TG2+/+ but not TG2−/− macrophage recipients. Cultured TG2−/− macrophages demonstrated diminished phagocytosis of apoptotic leukocytes, unaltered endocytosis, and degradation of oxidized LDL but decreased retinoic acid induction of the reverse cholesterol transport and apoptotic cell uptake mediator ABCA1.

Conclusions—We conclude that macrophage TG2 expression promotes both apoptotic cell clearance and ABCA1 expression in vitro and limits atherosclerotic lesion size in vivo. (Arterioscler Thromb Vasc Biol. 2006;26:563-569.)

Key Words: apoptosis ■ macrophage ■ ABCA1

Activated lesion macrophages are central mediators of inflammation driving atherogenesis. Growing evidence implicates transglutaminase 2 (TG2) in macrophage differentiation and functions including adhesion, migration, and phagocytosis. Oxidative stress and certain proatherogenic inflammatory mediators stimulate upregulation of TG2 in vitro. Furthermore, increased expression of TG2 and the TG isoenzyme Factor XIIIa (FXIIIa) occur in established atherosclerotic lesions. TGs catalyze calcium-dependent transamidation that produces covalent cross-linking of proteins (EC 2.3.2.13). TGs thereby stabilize extracellular matrix collagens, fibronectin, and other substrate proteins and mediate wound repair and tissue fibrosis. Transamidination by TG2 also increases phospholipase A2 activity and promotes activation of transforming growth factor-β (TGF-β) from latency. In addition, GTPase/ATPase, phospholipase C81 binding, fibronectin, and integrin binding activities of TG2 are implicated in TG2 effects on cell differentiation and function.

Apoptosis increases in developing atherosclerotic plaques and reflects exposure of cells in established atherosclerotic lesions to oxidative stress and accumulation of unesterified cholesterol. Increased endothelial cell and macrophage apoptosis modulate early atherogenesis, and apoptotic vascular smooth muscle cells (VSMCs) and macrophages and macrophage-derived matrix metalloproteinases contribute substantially to necrotic core formation and thinning of lesional fibrous caps. Extensive macrophage and VSMC apoptosis, large necrotic cores, thinned fibrous caps, and inflammation within the necrotic core and fibrous cap contribute to formation of vulnerable plaques.

TG2 regulates phagocytic clearance of apoptotic cells. In macrophages exposed to apoptotic cells, TG2 deficiency prevents generation of active TGF-β1, thereby decreasing the efficiency of apoptotic cell uptake but without suppressing binding of the apoptotic cells to macrophages. Although TG2−/− mice are developmentally normal, they develop splenomegaly and immune complex glomerulonephritis beyond 12 months, putatively via dysregulated apoptotic T cell removal. TG2 expression is upregulated in apoptotic cells. Moreover, TG2 promotes not only dysregulated macrophage

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release of TGF-β but also dysregulated expression of interleukin-12 and certain chemokines in vitro and defective autoinhibition of hepatic inflammation induced by lead nitrate and modulated by apoptotic cell uptake in vivo. Therefore, we tested the hypothesis that leukocyte TG2 expression limits atherosclerotic lesion size.

Methods

Chemical reagents were from Sigma-Aldrich unless specified.

Mice Studied and Diet

All animal procedures were done humanely under institutionally approved protocols. Low-density lipoprotein receptor (LDLR)−/− mice on C57BL/6J background were from Jackson Laboratories (Bar Harbor, Me). TG2−/− mice were originally on a hybrid (50:50) C57BL/6J × 129 background. TG2+/+ and TG2−/− mice were created with the same genetic background by backcrossing mice on the hybrid (50:50) C57BL/6J × 129 background 5 times onto a C57BL/6J background before the described studies of their tissues. Mice weaned at 3 to 4 weeks were fed standard chow diet (5015; Harlan Teklad) with autoclaved water, housed 4 per cage in autoclaved filter-top cages, and kept on 12-hour light/dark cycle.

Irradiation, Bone Marrow Transplantation, and Atherosclerosis Induction

In total, 24 6- to 8-week-old male LDLR−/− mice were subjected to 1000 rad total body irradiation, and bone marrow cells were extracted from femurs and tibiae of 8-week-old male TG2−/− and TG2+/+ mice as described. Irradiated LDLR−/− mice received 3×10⁶ marrow cells from TG2−/− or TG2+/+ mice (TG2−/− bone marrow transplantation [BMT], and TG2+/+ BMT mice, respectively) via tail vein injection. Four weeks after BMT, verified as sufficient for bone marrow reconstitution, all animals were fed atherogenic high-fat diet (HFD) (Harlan Teklad diet 94059) for 16 weeks.

Cholesterol and Blood Leukocyte Counts

Blood was obtained by retro-orbital puncture under methoxyflurane-induced anesthesia after a 6-hour fast at weeks designated 0 (pre-BMT), 4 (4 weeks post-BMT; pre-HFD), 8 (4 weeks post-HFD), and 20 (immediately before euthanasia). Plasma cholesterol and triglycerides were measured enzymatically, and neutrophils, monocytes, and lymphocytes per mm³ blood manually counted on Wright-stained smears.

Evaluation of Atherosclerosis

On euthanization, mice were perfused with cold PBS followed by cold 4% paraformaldehyde/5% sucrose and excised hearts embedded in OCT, snap-frozen, and kept at −70°C until sectioning. Serial sections of 10-μm thickness were cut through a 250-μm segment of the aortic valve, and 5 sections, each separated by 40 μm encompassing 200 μm of the valve, were examined from each mouse. Sections were stained with Oil Red O and counterstained with hematoxylin, and lesional Oil Red O–stained areas of each section were quantified using the NIH Image software and compared against the total lesion area. For each section, the percentage of the lesion containing the necrotic core was then determined to be either above or <50% of the total lesion area.

Immunohistochemical Staining of Atherosclerotic Lesions

Serial aortic valve frozen sections with similar lesion morphology were selected for immunohistochemical detection of macrophages using rat anti-mouse MOMA-2 (Serotec). After fixing for 2 minutes in acetone at −20°C, sections were incubated at 22°C for staining of macrophages successively with 5% normal blocking serum for 30 minutes; 1:250 dilution MOMA-2 antibody or nonimmune rat IgG in PBS containing 0.1% BSA and 0.015% Triton X-100 for 2 hours; 5 μg/mL biotinylated goat anti-rat secondary antibodies (Vector Laboratories) for 1 hour; Vectastain (Vector) for 30 minutes and developed with 3-amino-9-ethylcarbazole (AEC; Vector). For immunohistochemical analysis of lesional TG2 and anti-N(γ-glutamyl) lysine cross-links, acetone-fixed frozen sections were blocked and stained using the HistoMouse-kit (Zymed Laboratories). Detection of peroxidase used AEC. Control sections were stained with respective species-specific, isotype-specific IgG in place of primary antibody (each at 1:100 dilution). Primary antibody to TG2 was from Upstate Biotechnology and to N(γ-glutamyl) lysine from Abcam.

Phagocytosis of Apoptotic Leukocytes

Resident mouse peritoneal macrophages were collected by lavage with ice-cold PBS containing 5 mmol/L EDTA and verified as >85% macrophages by manual counting of Wright-Giemsa–stained slides after sedimentation via Cytospin4 cytocentrifuge (Thermo Shandon, Inc.). Cells were washed twice and aliquots of 0.2×10⁶ cells/well plated in 24-well plates (Costar) in 1 mL of RPMI 1640 medium containing 10% FBS, 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 15 ng/mL phorbol myristate acetate (PMA). After 18 hours, nonadherent cells were removed by washing. Murine thymocytes were induced to undergo apoptosis in dexamethasone (1.0 μmol/L) for 12 hours. Thymocytes were consistently >85% apoptotic, as evaluated by annexin V binding. One million apoptotic thymocytes were added to each well containing macrophages, and phagocytosis was allowed to proceed for 2 hours at 37°C. Then, nonphagocytosed cells were removed by washing, and macrophage monolayers fixed with 4% formalin and stained with hematoxylin–eosin. Phagocytosis was enumerated by counting apoptotic cells ingested per 100 macrophages per well. Results are expressed as phagocytic index, representing percentage of macrophages that ingested apoptotic cells × average apoptotic cells taken up per macrophage.

Lipoprotein Preparation and Modification

LDL (density 1.019 to 1.063 g/mL) was isolated from normolipemic human plasma by ultracentrifugation, dialyzed against 0.3 mmol/L EDTA in PBS, and protein concentration determined. Native LDL (OxLDL) was prepared by incubating LDL (100 μg/mL) with 10 μg/mL CuSO₄ for 18 hours at 37°C, with oxidation confirmed by thiobarbituric acid reactive substances assay. Butylated hydroxytoluene (20 μmol/L) and EDTA (0.1 mmol/L) were added to prevent further oxidation. Unlabeled OxLDL was concentrated to ~1 mg/mL, sterile-filtered, and stored at 4°C.

OxLDL Cell Association and Degradation

Mouse peritoneal macrophages, isolated and pretreated with PMA as above, were washed and incubated with 5 μg/mL of ¹²⁵I-OxLDL ± 30-fold excess of cold OxLDL for 5 hours at 37°C. For degradation assays, media were removed and trichloroacetic acid–soluble radioactivity determined. After incubation, cells were washed twice with ice cold PBS containing 0.1% BSA, then twice with PBS and lysed in 0.2 N NaOH. Protein content was determined, and cell-associated radioactivity was measured. Results for cell association were expressed as μg of ¹²⁵I-OxLDL bound/mg cell protein, and for...
degradation assay, μg of 125I-OxLDL degraded/mg cell protein. We determined specific cell association or degradation by subtracting nonspecific values in presence of competitors from totals with competitors absent.

**ABCA1 Induction**

Aliquots of 1.5×10^6 peritoneal macrophages were plated in supplemented RPMI 1640 medium containing PMA, as for phagocytosis studies. For bone marrow macrophage studies, marrows of 8-week-old mice were flushed from femoral and tibial medullary cavities with PBS containing 2% FCS, and washed in RPMI 1640 containing 10% FCS, 2 mmol/L-glutamine, 100 IU/mL penicillin, and 100 μg/mL streptomycin. Aliquots of 1×10^5 adherent bone marrow cells were plated in 6-well plates (Costar) and incubated for 5 days at 37°C in medium containing 40 ng/mL granulocyte/macrophage–colony-stimulating factor (GM-CSF) (Biosource International). Under these conditions, ≥85% of the isolated bone marrow–derived macrophages were confirmed by flow cytometry (using allophycocyanin [activated protein C]–conjugated antibodies [Caltag Laboratory]) as F4/80 positive.24 After 1 day or 5 days incubation at 37°C, 50 ng/mL all-trans retinoic acid (RA) was added for 72 hours and cells lysed by sonication. Aliquots of 30 μg protein were analyzed by Western blotting using polyclonal anti–ABCA1 (Novus Biologicals) and monoclonal anti–α-tubulin (Sigma) (1:1000), with signal detection as described.6

For quantitative polymerase chain reaction (PCR) analyses of ABCA1 expression, macrophages were lysed and RNA extracted with Trizol reagent and reverse transcribed using SuperScriptII and Random Primers (Invitrogen). Real-time PCR for ABCA1 was performed using LightCycler 2.0 (Roche Sciences) and forward primer 5′-ATGAAGTTAATGATGCTATCAAGCAA-3′; reverse primer 5′-CAGCCCTTGTTATTGAACCAC-3′. Thermocycling was performed in 20-μL capillaries at 95°C for 10 minutes, followed by 40 cycles at 63°C. Fluorescence was measured during each amplification step, with mRNA values expressed relative to amplification of GAPDH measured on the same samples.

**Statistical Analyses**

Aortic valve lesion areas were quantified by averaging 5 sections from each mouse heart and then calculating mean lesion area of all mice in each group, which were compared by Mann–Whitney U test. Other in vivo and in vitro results were analyzed by Student t test.

**Results**

**Effects of TG2+/− BMT on Atherosclerosis**

**In Vivo**

We observed marked hypercholesterolemia and hypertriglyceridemia at 8 and 16 weeks of consuming HFD in both TG2+/+ BMT and TG2+/− BMT mice without significant differences in cholesterol elevation but significantly lower triglyceride levels at 4 and 8 weeks after BMT in recipients of TG2+/− bone marrow (Figure 1). Peripheral blood neutrophil, monocyte, and lymphocyte concentrations did not significantly differ between TG2+/+ BMT and TG2+/− BMT between 0 and 16 weeks of the atherosclerosis induction (data not shown). After 16 weeks of HFD, atherosclerosis was assessed by examining Oil Red O–stained frozen serial sections of aortic valves. The mean lesion area demonstrated a 22.5% increase (P<0.05) in the TG2+/− BMT mice (207 275±14 169 μm²) normalized to the size of lesions in TG2+/+ BMT mice (169 085±43 327 μm²; Figure 2). Qualitative differences in plaque morphology also were observed because atherosclerotic lesions in TG2+/− BMT mice appeared to have more expanded necrotic cores compared with the lesions from TG2+/+ BMT mice (Figure 3). The number of atherosclerotic plaques in which the necrotic core occupied >50% of the plaque volume was estimated to be 3 of 12 (25%) for recipients of TG2+/+ BMT and 7 of 11 (64%) studied for recipients of TG2−/− BMT (P=0.071).

**Figure 1.** Hyperlipidemia induced by HFD in lethally irradiated LDLR−/− mice subjected to BMT from TG2+/+ and TG2−/− mouse donors. Male LDLR−/− mice of 6 to 8 weeks of age were subjected to lethal irradiation and engrafted with bone marrow cells from 8-week-old male TG2+/− and TG2+/+ mice, performed as described in the Methods. Four weeks after BMT, all animals were fed an atherogenic HFD for 16 weeks. The figure shows results of total plasma cholesterol (A) and triglycerides (B) at specific time points. n=12 in both the TG2+/+ BMT and TG2−/− BMT groups.

**Figure 2.** Aortic valve atherosclerotic lesion size is increased in LDLR−/− mice repopulated with TG2+/− leukocytes via BMT. The figure shows aortic valve lesion sizes (measured as described in the Methods and shown here in μm²) from the LDLR−/− mice subjected to BMT and after 16 weeks of HFD, as described in Figure 1. n=12 each. *P<0.05.
Immunohistochemical analyses of aortic valve atherosclerotic lesions for TG2 revealed robust and predominantly intimal staining in TG2+/+ BMT mice, and intimal staining was robust for MOMA-2 in TG2+/+ BMT mice (Figure 4). In contrast, TG2 staining was not detected in intima of atherosclerotic lesions in the TG2−/− BMT group, and MOMA-2 staining in TG2−/− BMT mice predominantly was in deep intima, rather than at or near endothelium, as was seen for TG2+/+ BMT mice (Figure 4). Comparable distinctions to those for MOMA-2 staining in TG2+/+ BMT versus TG2−/− BMT mice were observed for localization of immunohistochemical staining for Nα-(γ-glutamyl) lysine cross-links (Figure 4), a product of TG2-catalyzed transamidation.

Effects of TG2 Deficiency on Macrophages In Vitro

We next isolated TG2−/− and TG2+/+ peritoneal macrophages and PMA-treated the cells. We demonstrated decreased phagocytosis of apoptotic leukocytes by TG2−/− macrophages (Figure 5A). In contrast, labeled OxLDL uptake and degradation did not differ significantly between TG2−/− and TG2+/+ peritoneal macrophages (Figure 5B). Because cell activation in response to retinoids including all-trans RA (abbreviated RA) is altered in TG2−/− cells,6,7 we assessed the effects of TG2 deficiency on the capacity of RA to induce ABCA1,25 a pivotal mediator of reverse cholesterol transport26 and potentially other macrophage atherogenic-regulatory activities including apoptotic cell uptake.27–29 Basal ABCA1 expression was robust in both TG2+/+ and TG2−/− peritoneal macrophages, in which there was no further ABCA1 induction by RA (data not shown). Hence, we examined ABCA1 induction in GM-CSF–treated bone marrow–derived macrophages observed to have low basal ABCA1 expression. RA robustly induced ABCA1 protein and mRNA expression in TG2+/+ but not TG2−/− bone marrow macrophages (Figure 6A and 6B).

Discussion

In this study, we observed that LDLR−/− mouse aortic valve atherosclerotic lesions in recipients of TG2−/− bone marrow were significantly larger than in recipients of TG2+/+ bone marrow. TG2 is the most broadly expressed TG isoenzyme10 and had previously been observed to colocalize more with smooth muscle cells and endothelial cells than with macrophages in atherosclerotic lesions.8 But here, immunohistochemical analyses for lesion TG2 expression in bone marrow chimeric mice suggested that leukocytes were the major source of TG2 in atherosclerotic lesions. Significantly, the predominant localization of MOMA-2–positive macrophages in atherosclerotic lesions was intimal in recipients of TG2+/+ BMT but in contrast appeared predominantly deep intimal in recipients of TG2−/− BMT. The capacity of TG2 to regulate macrophage adhesion and migration,2 and possibly differential intimal and necrotic core localization of mediators of macrophage movement, may have acted singly or in concert to produce these qualitative differences in macrophage distribution in atherosclerotic lesions. TG2-dependent distinctions in extracellular matrix organization may also have contributed because Nα-(γ-glutamyl) lysine cross-links, which were concentrated in the superficial intima in recipients of TG2+/+ BMT, appeared contrastingly more diffuse, less intense, and deeper in the intima in recipients of TG2−/− BMT.

TG2 is not the only macrophage-expressed TG. Specifically, FXIIIa, the homodimeric tissue form of the heterotet-
FXIIIA is a ramemic FXIII coagulation factor, is expressed by mononuclear phagocytes, and is also upregulated in atherosclerotic lesions, as confirmed in LDLR−/− murine atherosclerotic lesions (Johnson K et al, unpublished observations, 2005). The notion that FXIIIA genetic variants modulate human atherosclerosis remains highly controversial. FXIIIA is a constitutively latent TG the activation of which is stimulated by thrombin, which may account for the inability of potentially redundant macrophage-expressed TGs other than TG2 to limit atherosclerotic lesion expansion. Unique activities of TG2, including functions as TG2/GTPase dual-enzyme and integrin coreceptor, also could contribute to macrophage TG2 modulation of atherosclerosis.

In atherosclerotic lesions, TGF-β receptor expression is most robust in the fibrous cap. Because TGF-β theoretically promotes plaque stability by stimulating collagen synthesis, decreased in PMA-pretreated TG2−/− peritoneal macrophages. Under these conditions, TG2−/− and TG2+/+ peritoneal macrophages demonstrated no significant differences in OxLDL uptake or OxLDL degradation, indicative of selectively impaired apoptotic leukocyte ingestion in TG2−/− macrophages.

RA-stimulated expression of ABCA1 was attenuated in TG2−/− bone marrow macrophages in vitro. ABCA1 expression is antiatherogenic in vivo, and ABCA1 mediates not only reverse cholesterol transport but also other macrophage inflammatory functions including apoptotic cell uptake. Hence, macrophage TG2 expression could modulate lesion size in atherosclerosis partly by regulating ABCA1 expression.

Figure 4. Immunohistochemical staining for localizations of TG2, MOMA-2, TG-catalyzed Nε-(γ-glutamyl) lysine cross-links in TG2+/+ BMT versus TG2−/− BMT mouse atherosclerotic aortic valve lesions. Frozen sections were stained to show TG2, using mouse anti-TG2, C and D. Staining for MOMA-2, using rat anti-MOMA-2, E and F. Staining for Nε-(γ-glutamyl) lysine, the cross-link generated by the TG-catalyzed transamidation reaction, using murine monoclonal antibody to the cross-link. The arrows illustrate positive staining for each antigen and, where indicated, the asterisks indicate the necrotic core of the atherosclerotic lesion. Noteworthy findings included more robust TG2 staining in lesions after TG2+/+ BMT and apparent differences in intrallesional distributions in TG2−/− BMT versus TG2−/− BMT mice of MOMA-2 and Nε-(γ-glutamyl) lysine. Isotype-specific negative controls for each immunohistochemical staining reaction were negative and are not shown. Magnifications ×160.

Figure 5. Comparisons of phagocytosis of apoptotic leukocytes and OxLDL uptake and degradation by peritoneal macrophages of TG2−/− versus TG2+/+ mice. A, Results are shown for phagocytosis of apoptotic thymocytes by peritoneal macrophages from TG2+/+ and TG2−/− mice, performed as described in the Methods. Data are expressed as phagocytic index, representing the percentage of macrophages that phagocytosed apoptotic cells multiplied by the average number of apoptotic cells taken up per macrophage. B, Results shown for specific uptake and degradation of OxLDL by peritoneal macrophages from TG2+/+ and TG2−/− mice, performed as described in the Methods. Cells were incubated with 125I-labeled OxLDL for 5 hours at 37°C and cell association and degradation of OxLDL determined. Results from A and B represent mean±SEM from 5 separate experiments on distinct donors. *P<0.05.

Limitations of this study included in vivo analyses that were partly qualitative and restricted to a single, relatively severe form of experimental atherosclerosis. Aortic valve atherosclerotic lesions in recipients of TG2−/− bone marrow exhibited a trend (P=0.071) to more expanded lipid cores, a feature characteristic of vulnerable atherosclerotic plaques. In this study, we adapted described approaches to estimate the size of atherosclerotic necrotic cores in histological sections, but without advanced imaging of the lesions, there was no validated method to provide further comparative analyses of necrotic core size. This study did not rule out that effects on adaptive...
immune function\(^{6,37}\) induced by TG2\(\rightarrow\)−/− BMT, including dysregulated apoptotic T cell phagocytosis and autoantibody production, factored into the altered atherosclerosis. However, TG2\(\rightarrow\)−/− mice of breeding age do not exhibit gross abnormalities and breed normally,\(^7\) and immune-mediated organ damage in TG2\(\rightarrow\)−/− mice supervenes well past breeding age.\(^4\) It is possible that BMT induced repopulation of cells other than leukocytes, but effects of TG2 expression on cells other macrophages were not explored here. Last, we did not define the cause of the less pronounced hypertriglyceridemia in TG2\(\rightarrow\)−/− BMT recipients on HFD or potential implications of this finding on atherosclerosis.

TG2 transamidation catalytic effects that promote stabilization and reorganization of connective tissue matrices and promote TGFB activation\(^{10}\) could contribute to modulation of the repair response within atherosclerotic lesions. But TG2 is markedly upregulated in association with downregulation of FXIIIa in monocyte to macrophage maturation.\(^{38}\) TG2 deficiency fundamentally impairs the capacity of macrophages to ingest apoptotic cells and promotes dysregulated macrophage release of TGFB and other cytokines, dysregulated expression of ABCA1 in this study, and defective autoinhibition of inflammation modulated by apoptotic cell uptake in vivo.\(^{4,17}\) Hence, we conclude that macrophage-expressed TG2 appears to function as an endogenous apoptotic cell clearance and anti-inflammatory factor that thereby limits expansion of atherosclerotic plaques.

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