Inhibitory Effect on Arterial Injury-Induced Neointimal Formation by Adoptive B-Cell Transfer in Rag-1 Knockout Mice

Paul Dimayuga, Bojan Cercek, Sumito Oguchi, Gunilla Nordin Fredrikson, Juliana Yano, Prediman K. Shah, Stefan Jovinge, Jan Nilsson

Abstract—We investigated the effect of B-cell reconstitution in immune-deficient Rag-1 knockout (KO) mice subjected to arterial injury. After 21 days, injury induced a 4- to 5-fold increase in neointimal formation in Rag-1 KO mice fed normal chow compared with wild-type (WT) mice (0.020±0.0160 [n=8] versus 0.0049±0.0022 [n=8] mm², respectively; P<0.05) and in western-type diet-fed Rag-1 KO mice compared with WT mice (0.0312±0.0174 [n=7] versus 0.0050±0.0028 [n=6] mm², respectively; P<0.05). To investigate the role of B cells in response to injury, Rag-1 KO mice were reconstituted with B cells derived from the spleens of WT mice, with donors and recipients on the same diet. Reconstitution of Rag-1 KO mice with B cells from WT mice (both fed normal chow) reduced neointimal formation compared with the effect in unreconstituted Rag-1 KO mice (0.0076±0.0039 [n=9] versus 0.020±0.0160 [n=8] mm², respectively; P<0.05). Reconstitution of Rag-1 KO mice with B cells from WT mice (both fed a western diet) reduced neointimal formation compared with the effect in Rag-1 KO mice (0.0087±0.0037 [n=8] versus 0.0312±0.0174 [n=7] mm², respectively; P<0.05). Injured carotid arteries from reconstituted Rag-1 KO mice had detectable IgM and IgG, indicating viable transfer of B cells. The results suggest that B cells modulate the response to arterial injury. (Arterioscler Thromb Vasc Biol. 2002;22:●●●●●●.)

Key Words: neointima ■ B cells ■ arterial injury ■ Rag-1 knockout mice

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Antibody-mediated immunomodulation has been proposed to affect the development of atherosclerosis in various models. In general, immunization with LDL and its various oxidized forms reduces atherogenesis, whereas heat shock protein and β2-glycoprotein I, among several other immunogens tested, accelerate it. Administration of intravenous immunoglobulin to apoE-deficient mice has resulted in reduced atherosclerosis. These findings suggest a role for antibodies in the pathogenesis of the disease. In addition, the presence of B cells has been described in atherosclerotic plaques. It is unclear whether the immunomodulatory effects primarily involve humoral or cellular responses. However, cellular immune responses have been implicated in increased atherogenesis. The T-lymphocyte product interferon-γ has also been shown to contribute to plaque formation.

Immune-mediated modulation of the response to arterial injury has also been postulated to play a role in neointimal formation. Studies on immune modulation of the response to injury have focused primarily on T-lymphocyte effector functions. The main finding of those studies indicates that interferon-γ, as opposed to its proatherogenic role in cholesterol-induced plaque formation, plays an inhibitory role in the response to arterial injury. It is unclear what role B cells may have in arterial injury. Some studies have implicated the humoral immune system, particularly the antibodies, as playing a role in injury and have suggested that there may be antibody-mediated responses to arterial injury.

To test the hypothesis that B cells may modulate neointimal thickening after arterial injury, we used the carotid cuff injury model in immune-deficient Rag-1 knockout (KO) mice, which lack mature B and T cells, and the transfer of B cells from spleens of wild-type (WT) mice into Rag-1 KO mice. Rag KO mice have previously been used to assess B-lymphocyte survival and renewal kinetics after adoptive transfer. The mice were fed either normal chow or a western-type diet to examine the effect of diet on B-cell function after injury. The effects on neointimal formation were then assessed.

Our results show a significant increase in neointimal formation in immune-deficient Rag-1 KO mice compared with WT mice. In addition, we show that reconstitution with B cells reduced neointimal formation in Rag-1 KO mice fed...
Response to Arterial Injury in Rag-1 KO Mice 21 Days After Cuff Placement

<table>
<thead>
<tr>
<th></th>
<th>WT NC</th>
<th>Rag-1 KO NC</th>
<th>Rag-1 KO NC Brec</th>
<th>WT diet</th>
<th>Rag-1 KO diet</th>
<th>Rag-1 KO diet Brec</th>
</tr>
</thead>
<tbody>
<tr>
<td>EEL, mm²</td>
<td>0.0484±0.0152</td>
<td>0.0873±0.0261*</td>
<td>0.0742±0.0344</td>
<td>0.0525±0.0185</td>
<td>0.0913±0.0140†</td>
<td>0.0806±0.0203</td>
</tr>
<tr>
<td>Media, mm²</td>
<td>0.0183±0.0058</td>
<td>0.0277±0.0087</td>
<td>0.0332±0.0064</td>
<td>0.0227±0.0081</td>
<td>0.0327±0.0075†</td>
<td>0.0349±0.0048</td>
</tr>
<tr>
<td>Neointima, mm²</td>
<td>0.0049±0.0022</td>
<td>0.0200±0.0160*</td>
<td>0.0076±0.0039†</td>
<td>0.0050±0.0028</td>
<td>0.0312±0.0174†</td>
<td>0.0087±0.0037§</td>
</tr>
<tr>
<td>Intima/media ratio</td>
<td>0.26±0.08</td>
<td>0.85±0.34</td>
<td>0.22±0.11</td>
<td>0.21±0.08</td>
<td>0.93±0.43†</td>
<td>0.25±0.1§</td>
</tr>
<tr>
<td>Serum cholesterol, mg/dL</td>
<td>122.8±24.4 (n=5)</td>
<td>108.3±42.1 (n=6)</td>
<td>ND</td>
<td>230.6±77.4 (n=5)</td>
<td>186.5±20.5 (n=4)</td>
<td>182.8±31.2 (n=5)</td>
</tr>
</tbody>
</table>

WT NC indicates WT mice fed normal chow, Rag-1 KO NC, Rag-1 KO mice fed normal chow, Rag-1 KO NC Brec, Rag-1 KO mice fed normal chow with B-cell reconstitution; WT diet, WT mice fed western diet; Rag-1 KO diet, Rag-1 KO mice fed western diet; Rag-1 KO diet Brec, Rag-1 KO mice fed western diet with B-cell reconstitution; and ND, not done. Comparisons between groups were made by Mann-Whitney test. Numbers in parentheses for serum cholesterol are the numbers of mice, which differ from those for the other variables.

*P<0.05 vs WT NC; †P<0.05 vs Rag-1 KO NC; ‡P<0.05 vs WT diet; and §P<0.05 vs Rag-1 KO diet.

Methods

Animals

Male mice aged 8 weeks (B6/129S-Rag-1 and B6/129SF2) were purchased from Jackson Laboratories (Bar Harbor, Me). When the mice were euthanized after 21 days, and the carotid artery was perfused with PBS for 10 minutes and frozen at −70°C after it was embedded in OCT (Tissue-Tek, Aligance). Baseline characteristics of the right carotid artery were determined by harvesting tissue from uninjured mice at the same age (28 weeks). Blood was collected by retro-orbital bleed before tissue harvest. Another group of mice was fed a western-type diet (0.15% cholesterol and 21% fat, AnalyCen Nordic) at the age of 10 weeks, and cuff injury was performed accordingly. The experimental protocols used in the present study were approved by the Animal Care and Use Committee.

Splenocyte Reconstitution

Spleen cells from age-matched WT mice fed the same diet were isolated as previously described. Briefly, individual spleens were sliced into 2-mm pieces and pressed through a sterile wire mesh (Cellator, Bellco Glass). Red blood cells were lysed, and the cells were suspended in PBS/1% FBS (GIBCO-BRL). Cells were then incubated overnight, blocked with 1% BSA, and incubated overnight with sera diluted 1:100 in PBS. Wells were washed and incubated with horseradish peroxidase–conjugated IgM and IgG2a detection antibody for 1 hour. The substrate for color development was ABTS, as provided in the kit. Optical density was recorded 20 minutes later on a SpectraMax 190 plate reader (Molecular Devices).

Statistical Analysis

Data are presented as mean±SD. Statistical comparisons were made by the Mann-Whitney test because of the small sample size. Significance was set at a value of P<0.05.

Results

Neointimal Thickening in Rag-1 KO Mice

Cuffing of the carotid artery of the normal chow–fed Rag-1 KO mice, compared with the WT mice, after 21 days resulted in increased neointimal area (P<0.05, Table). Medial area and the intima-to-media ratio were significantly increased in the Rag-1 KO mice (P<0.05, Table). Cholesterol levels were similar (Table). To determine the effect of injury on vessel wall size, the external elastic lamina (EEL) and medial area were compared between age-matched mice without injury and the injured groups. There was no significant difference in the EEL area of the WT mice with and without injury (0.0484±0.0152 [n=8] versus 0.0409±0.0076 [n=7] mm², respectively). However, a significant difference was observed in the injured versus noninjured Rag-1 KO mouse EEL (0.0873±0.0261 [n=8] versus 0.0458±0.0233 [n=7] mm², respectively; P<0.05). There was no difference in the EEL and medial area of the uninjured carotid arteries of WT compared with Rag-1 KO mice.

Injury to the carotid artery of immune-deficient Rag-1 KO mice fed a western diet compared with WT mice also on western diet resulted in significantly increased neointimal formation, medial area, and intima-to-media ratio (P<0.05, Table and Figure 1A and 1B). The neointima of both groups

teral arteries served as an unjured control. A DAB detection kit (Vector) was used for color development.

Serum Cholesterol

Serum cholesterol was determined as previously described. Briefly, serum was collected 21 days after injury during euthanasia. Cholesterol levels were determined by using a commercially available kit (Sigma).

Enzyme-Linked Immunosorbent Assay

Relative serum immunoglobulin levels were determined by using an isotyping ELISA kit from Southern Biotechnology Associates. Briefly, 96-well ELISA plates were coated with the capture antibody overnight, blocked with 1% BSA, and incubated overnight with sera diluted 1:100 in PBS. Wells were washed and incubated with horseradish peroxidase–conjugated IgM and IgG2a detection antibody for 1 hour. The substrate for color development was ABTS, as provided in the kit. Optical density was recorded 20 minutes later on a SpectraMax 190 plate reader (Molecular Devices).

Morphometric Analysis and Immunostaining

Frozen sections 6 to 8 µm thick were collected from the injured carotid arteries as described previously. Briefly, 4 or 5 sections were collected per slide for approximately half of the injured segment. Four slides equally spaced from first to last were then stained with hematoxylin and eosin, and the vessel area was measured by using computer-assisted morphometric analysis (Olympus Micro Image).

For immunostaining, the following antibodies were used: rat anti-mouse MOMA-2 (BMA Biomedicals), FITC-labeled anti–α-actin (Sigma Chemical Co), biotinylated anti-mouse IgM (Vector Laboratories), and biotinylated anti-mouse IgG (Sigma). Biotinylated rabbit anti-rat IgG (Vector) was used for MOMA-2 detection. Nonimmune serum, omission of primary antibody, and isotype IgG/FITC (for α-actin) were used as a negative control. Contralateral

normal and western diets. The possible role of IgM in this response and implications of the findings are also discussed.
consisted predominantly of smooth muscle cells (SMCs), as determined by SMC α-actin staining (Figure 1D and 1E). Serum cholesterol levels were not significantly different between the 2 groups (Table). Monocyte/macrophages as detected by MOMA-2 stain were minimal in WT and Rag-1 arteries 21 days after injury (Figure 1F and 1G).

Compared with WT mice fed normal chow, WT mice fed a western-type diet had significantly increased cholesterol levels but similar neointimal formation (Table). Similarly, compared Rag-1 KO mice fed normal chow, Rag-1 KO mice fed a western diet had increased cholesterol levels but only a slight augmentation of neointimal formation (P<0.23, Table) and no difference in the intima-to-media ratio.

**B-Cell Reconstitution of Rag-1 KO Mice**
The transfer of B cells from WT mice fed normal chow to Rag-1 KO mice resulted in significantly reduced neointimal formation compared with that in Rag-1 KO mice without B-cell transfer (Figure 2A; P<0.05). Reconstitution of western diet–fed Rag-1 KO mice with B cells enriched from WT mice fed a western diet also resulted in significantly reduced neointimal formation after injury (P<0.05, Figure 1C and Figure 2B). The neointima consisted mostly of SMCs (Figure 1F), with minimal staining for monocytes/macrophages (Figure II). The medial area was similar between the 2 groups (Table), but the intima-to-media ratio was decreased in the B-cell–reconstituted mice compared with unreconstituted mice (Table, P<0.05).

**Increased IgM Presence in Injured Arteries**
An increased presence of IgM was observed in the right carotid arteries of WT mice 21 days after injury (Figure 3A). This was observed in the media and the neointima. IgM was not observed in the media of the uninjured contralateral artery (Figure 3B) or in the injured arteries of Rag-1 KO mice.
The presence of IgM was also observed in the injured arteries of reconstituted Rag-1 KO mice (Figure 3D), indicating that the B cells transferred were viable.

**Presence of IgG in Neointima**

The presence of IgG was also detected in the injured carotid arteries of the WT mice 21 days after injury, but unlike IgM, IgG was localized mainly in the neointima and adventitia, with faint patchy stains in the media (Figure 3E). Uninjured contralateral arteries had only faint adventitial stains (Figure 3F), whereas no IgG was detected in the injured arteries of the Rag-1 KO mice (Figure 3G). The arteries of B-cell–reconstituted mice had a staining pattern similar to that of injured WT arteries, with IgG mainly in the neointimal layer (Figure 3H).

**Serum Immunoglobulin**

In contrast to WT mice, Rag-1 KO mice had no detectable serum IgM or IgG2a before injury (Figure 4). Reconstitution of Rag-1 KO mice with B cells slightly increased serum IgM on the day of injury (D0 in Figure 4), but IgG levels remained undetectable. IgM and IgG levels were at or near WT levels 21 days after injury in the Rag-1 KO mice with B-cell reconstitution (Figure 4), whereas Rag-1 KO mice without B-cell transfer had no detectable immunoglobulins. Similar results were observed in the normal chow-fed mice (not shown).

**Discussion**

Injury to the carotid arteries of Rag-1 KO mice (which lack mature B and T cells) compared with WT mice resulted in a 4- to 5-fold increase in neointimal formation. This is a clear indication of the modulatory role of the immune system in the response to injury. Studies on the role of the immune system in response to arterial injury have focused primarily on...

(Figure 3C). The presence of IgM was also observed in the injured arteries of reconstituted Rag-1 KO mice (Figure 3D), indicating that the B cells transferred were viable.

(Figure 4).

Figure 3. Presence of IgM was detected in WT cuffed carotid arteries (A) but not in the contralateral left carotid artery (B). Rag-1 KO mice had no detectable IgM in the cuffed artery (C). Reconstitution of B cells into Rag-1 KO mice resulted in increased presence of IgM in the cuffed artery (D). Mice were fed a western-type diet. IgG was localized mainly in the neointimal layer of the cuffed artery of WT mice, with only faint staining in the media (E) and minimal presence in the uninjured contralateral left carotid artery (F). Rag-1 KO mice had no detectable IgG in the cuffed artery (G), but transfer of B cells resulted in staining of the neointima (H) in a pattern similar to that of the WT artery (E).

Figure 4. Serum immunoglobulins of the IgM and IgG2a isotypes were detectable in the WT mice before injury (WT D0). Rag-1 KO mice had no detectable immunoglobulins at the same time point (Rag D0), but they had low levels of IgM 48 hours after B-cell transfer (Rag Brec D0). Twenty-one days after B-cell reconstitution, Rag-1 KO mice had detectable serum immunoglobulins of IgM and IgG2a isotypes (Rag Brec D21, n=4). Unreconstituted Rag-1 KO mice had no immunoglobulins at the 21-day time point (n=2, not shown). Sera were from western diet-fed mice.
T-cell–mediated effector functions.\textsuperscript{11,14} Infusion with antibodies against T-cell surface markers has resulted in increased neointimal formation after injury.\textsuperscript{11,12} Those studies were instrumental in demonstrating the role of the immune response, specifically of the T helper response to arterial injury. The present study further shows that the combined deficiency in cellular and humoral immunity also results in increased neointimal formation. The Rag-1 KO model also provided the opportunity to study neointimal formation after injury, with B-cell immune response in the absence of T-cell mediation. Reconstitution of Rag-1 KO mice with B cells would only inherently suggest that the effect was not due to T helper–mediated immune mechanisms because of the lack of mature B cells and T cells in Rag-1 KO mice. Few studies have implicated B cells in the response to injury. B lymphocytes have been shown to act as a limiting factor in the healing response to bone marrow ablation.\textsuperscript{22} Another study has implicated B lymphocytes in dermal wound healing in humans.\textsuperscript{23}

The use of a western diet did not alter the outcome in the WT mice, but there was a slight augmentation of neointimal formation in Rag-1 KO mice. This finding suggests that the diet may play a role in the response of the arterial wall to injury when the immune system is compromised. The difference in the medial area coupled with an increase in the EEL area in injured Rag-1 KO arteries suggests increased tissue remodeling. This was observed only after injury, because the age-matched uninjured arteries of WT and Rag-1 KO groups had similar areas. It is interesting that immune deficiency, but not the western diet, seems to have had an effect on this process in the present study. A similar increase in medial area was observed in injured arteries of apoE KO mice fed a western diet, suggesting that cholesterol levels must be profoundly increased to have any effect on injury-induced neointimal formation.\textsuperscript{18} Our results show that the B-cell response in experimental injury was not significantly affected by the diet.

The presence of IgM in the injured arteries of WT mice suggests that cuff injury increases tissue permeability or the localization of antibodies to neoantigens expressed in the arterial wall after injury. Tissue permeability has been described to be a possible source of medial SMC activation in the rabbit cuff injury model.\textsuperscript{24} However, the present results also suggest that tissue permeability may lead to increased IgM in the injured tissue and may modulate the response to injury. It is also possible that the cuff placement disrupts lymphatic drainage from the affected area. However, the localization of IgG mainly in the neointima would not support this notion. The presence of IgG mainly in the neointimal layer is in agreement with a previous study by Hansson et al\textsuperscript{25} in a rabbit injury model. It is not clear whether the localization of IgG in the neointima has any functional significance. IgM and IgG in the vascular wall have been previously described in plaques of LDL receptor–deficient mice,\textsuperscript{26} suggesting a role for localized antibodies in atherogenesis. In our previous study, immunization with oxidized LDL reduced injury-induced neointimal thickening in hypercholesterolemic rabbits.\textsuperscript{15} Although arterial tissue was not examined for the presence of antibodies in that study, antibody titers against oxidized LDL of the IgG isotype were similar between immunized and nonimmunized rabbits. This observation is not in agreement with other immunization studies on the antibody response,\textsuperscript{1–3} although immunization is seemingly beneficial in both situations. The Rag-1 KO mice used in the present study have no mature B cells and T cells; therefore, reconstitution with B cells alone would suggest lack of T helper cell–mediated antibody maturation from the IgM class after injury. However, the presence of IgG in the reconstituted mice 21 days after injury suggests the presence of memory B cells. Which of the 2 isotypes is important in the response to injury is not clear. The beneficial effects of intravenous immunoglobulin that have been reported in atherosclerosis\textsuperscript{6} and injury\textsuperscript{27} in mice were found to be primarily through the modulation of T helper cell function. In the present study, the presence of antibodies in the B-cell–reconstituted Rag-1 KO mice, which lack mature T cells, suggests a mechanism not involving the modulation of T helper cell functions.

Although the mechanism is currently unclear, one way for IgM to play a role in arterial injury could be by the enhancement of phagocytosis by macrophages. However, the low presence of macrophages in the injured arteries in the present study would argue against this. IgM may also be involved in complement activation, as has been reported in early atherosclerotic lesions (Torzewski et al\textsuperscript{28}). The tissue presence of IgM, but not IgG, has been demonstrated in ischemia/reperfusion injury, which has been shown to be mediated by IgM and complement. The role of IgM and complement in mediating injury in the study of Torzewski et al was further supported by an apparent lack of injury in Rag KO mice.\textsuperscript{29,30} Although the present study and the others cited identify a role for IgM in tissue injury, we propose that IgM may have an inhibitory effect on neointimal formation in arterial injury.

IgM has been proposed to be a first line of defense for the organism, as supported by a study in which B-cell reconstitution of Rag-2 KO mice resulted in a rapid normalization of serum IgM levels despite low numbers of B cells.\textsuperscript{17} In the present study, serum levels of IgM, but not IgG, were detectable at very low amounts in the reconstituted Rag-1 KO mice 48 hours after adoptive cell transfer. Our results suggest that natural antibodies may have effector functions not clearly defined that may yet explain our findings.

In summary, the present study identified a possible role for the humoral immune response in arterial injury and suggests that B cells may be important in this response. This was confirmed in normal diet– and western diet–fed mice. Antibodies of the IgM and IgG isotypes were detected in different areas of the injured artery, suggesting isotype specificity in the modulation of neointimal formation after injury.

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References


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