Transforming Growth Factor Activity Is a Key Determinant for the Effect of Estradiol on Fatty Streak Deposit in Hypercholesterolemic Mice

Pierre Gourdy, Alexia Schambourg, Cédric Filipe, Victoire Douin-Echinard, Barbara Garmy-Susini, Bertrand Calippe, François Tercé, Francis Bayard, Jean-François Arnal

Objective—Whereas estradiol prevents fatty streak deposit in immunocompetent apoE−/− or LDLr−/− mice, it is totally ineffective in immunodeficient mice, underlining the key role of immunoinflammation in this effect. In the present work, the role of several major pro- and antiinflammatory cytokines involved in the atheromatous process was evaluated in the effect of estradiol on fatty streak constitution.

Methods and Results—The preventive effect of estradiol was fully maintained in LDLr−/− mice grafted with bone marrow from either IFN-γ or interleukin (IL)-12–deficient mice, showing that this beneficial effect was not mediated through a specific decrease in the production of these 2 proinflammatory cytokines. Furthermore, IL-10−/− apoE−/− mice remained protected by estradiol, excluding a significant contribution of this antiinflammatory cytokine. In contrast, the protective effect of estradiol was (1) associated with enhanced aortic expression of TGF-β1 in apoE−/− mice during early steps of atherogenesis; (2) abolished and even reversed in apoE−/− mice administered with a neutralizing anti–TGF-β antibody; (3) abolished in LDLr−/− mice grafted with bone marrow from Smad3-deficient mice.

Conclusions—The status of the TGF-β pathway crucially determines the antiatherogenic effect of estradiol in hypercholesterolemic mice, whereas neither IFN-γ, IL-12, nor IL-10 are specifically involved in this protection. (Arterioscler Thromb Vasc Biol. 2007;27:2214-2221.)

Key Words: atherosclerosis ■ inflammation ■ cytokine ■ TGF-β ■ estrogen

Determining the mechanisms of the vascular effects of estrogens is now identified as a research priority, because epidemiological surveys, experimental observations, and clinical intervention trials have led to conflicting results.1 Indeed, whereas epidemiological studies suggested that both endogenous and exogenous estrogens protect women against cardiovascular diseases,2 2 recent controlled prospective and randomized studies did not demonstrate a beneficial effect of hormone replacement therapy (HRT) neither in secondary3 nor in primary prevention.4 Although evaluating late steps of atherothrombosis, these clinical trials strikingly contrast with the strong preventive effect of estrogens in all animal models of atherosclerosis, from mice to monkey.5,6

During the last decade, these hypercholesterolemic mouse models allowed to establish the major contribution of the immune system in the development of early lesions of atherosclerosis, and to highlight the influence of several cytokines in this process.7 Indeed, proinflammatory cytokines, namely IL-12, IL-18, and IFN-γ, have been demonstrated to promote fatty streak constitution7 and probably plaque instability,8 whereas the prototypic antiinflammatory cytokines TGF-β and IL-10 appear clearly protective.7,9 TGF-β activity was shown to prevent vascular inflammation and to promote plaque stabilization in mice models of atherosclerosis, including apolipoprotein E–deficient (apoE−/−) and low-density lipoprotein receptor–deficient (LDLr−/−) mice.10-14

We previously reported that, whereas estradiol-17β (E2) strongly prevents fatty streak deposit in immunocompetent hypercholesterolemic mice, it is totally ineffective in both apoE−/− and LDLr−/− mice also deficient in recombination activating gene 2 (RAG-2), demonstrating that lymphocytes are absolutely required for the protective effect of the hormone.15,16 As lymphocytes are both source and target of cytokines, we hypothesized that the protective effect of E2 could be mediated through a favorable effect on the pro- versus antiinflammatory cytokine balance, because estrogens are known to alter the secretion of several pro- and antiinflammatory cytokines by immune cells in various pathophysiological models.17-19

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From the Institut National de la Santé et de la Recherche Médicale, INSERM U858 (P.G., A.S., C.F., V.D.E., B.G.S., B.C., F.B., J.F.A.), Institut de Médecine Moléculaire de Rangueil, Toulouse, France; Service de Diabétologie (P.G.), Pôle Cardio-Vasculaire et Métabolique, CHU Toulouse Rangueil, France; and INSERM U563 (F.T.), Département Lipoprotéines et Médiateurs Lipidiques, CHU Toulouse Purpan, France.

Correspondence to Pierre Gourdy, INSERM U858, Institut de Médecine Moléculaire de Rangueil, BP 84225, 31432 Toulouse Cedex 4, France. E-mail gourdyp@toulouse.insERM.fr

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2214
In the present work, we separately evaluated the role of major pro- and antiinflammatory cytokines involved in the atheromatous process in the effect of E2 on fatty streak constitution, with an approach consisting in suppressing their expression or activity in hypercholesterolemic mouse models. Whereas neither IFN-γ, IL-12, nor IL-10 deficiency altered the protective effect of E2, we demonstrate that the integrity of the TGF-β pathway appears to be crucial for this beneficial effect of estrogens.

Materials and Methods
Mice and Experimental Procedures
Female apoE−/− mice, apoE and IL-10 double deficient mice (apoE−/− IL-10−/−), provided by G.K. Hansson, Karolinska Institute, Stockholm, Sweden, were ovariectomized at 4 weeks of age, then administered with either 60-day time-release placebo or 0.1 mg estradiol-17β pellets (Innovative Research of America), and maintained on a chow diet. In some experiments, apoE−/− mice were injected intraperitoneally with either an anti–TGF-β1/β2/β3 monoclonal antibody (clone 2G7, provided by D. Fradelizi, INSERM U477, Paris, France) or an isotype matched (IgG2b) control mAb, devoid of neutralizing activity, once a week for 11 weeks, as previously reported.11

Six-week-old ovariectomized LDLr−/− female mice were lethally irradiated, then were intravenously reconstituted with bone marrow cells from either wild-type C57Bl/6J, IFN-γ–deficient (IFN-γ−/−), or IL-12p40–deficient (IL-12−/−) mice, all purchased from Charles River (L’Arbresle, France), or from Smad3-deficient mice (Smad3−/−, provided by C. Deng, National Institute of Health, Bethesda, Md).22 Four weeks later, transplanted mice received placebo or E2 pellets, and were switched to a high fat atherogenic diet containing 1.25% cholesterol (TD96335, Harlan Teklad).23 All mice were euthanized after a 12-week period of E2 or placebo treatment, and total and HDL plasma cholesterol concentrations were determined. In some experiments, spleen cells were isolated and stimulated with coated anti-CD3 mAb (1 μg/mL, BD Pharmingen) for 48 hours. Then, IFN-γ, IL-10, and IL-4 concentrations were determined in supernatants by ELISA as previously described.24 (For detailed materials and methods, please see supplemental materials available online at http://atvb.ahajournals.org).

Analyses of Fatty Streak Lesion and TGF-β1 Expression
Fatty streak lesion size was estimated at the aortic sinus, as previously described.20 Collagen content was assessed by Sirius red staining. For immunohistochemical staining, a goat polyclonal anti-TGF-β1/2 (Santa Cruz Biotechnology) was used. TGF-β1 mRNA expression was determined by RT-PCR in the aortic arch and the descending aorta from ovariectomized apoE−/− female mice administered with either placebo or E2 pellets for 5 weeks (For detailed materials and methods, please see supplemental materials).

Statistical Analyses
Results were expressed as means±SEM. A 2-way ANOVA was performed to test for the respective effects of E2 treatment, cytokine deficiency, or anti–TGF-β mAb treatment on various parameters. When allowed, paired comparisons were performed with the Fisher PLSD procedure. P<0.05 was considered as statistically significant.

Results
IFN-γ or IL-12 Deficiency Does Not Alter the Effect of E2 on Fatty Streak Constitution in LDLr−/− Mice
To determine whether the preventive effect of E2 on fatty streak constitution could be mediated by a specific modula-

![Figure 1](http://atvb.ahajournals.org/)

Figure 1. Influence of IFN-γ, IL-12, or IL-10 deficiency on the effect of estradiol on fatty streak constitution in hypercholesterolemic mice. Six-week-old ovariectomized LDLr−/− female mice were lethally irradiated and reconstituted with bone marrow cells from wild-type, IFN-γ−/− (A), or IL-12−/− (B) mice, then were administered with placebo or estradiol (E2) and switched to atherogenic diet from the age of 10 to 22 weeks. Six-week-old ovariectomized apoE−/− IL-10−/− female mice were maintained on a chow diet and treated with placebo or E2 for 12 weeks (C). Mean lesion sizes at the aortic sinus are shown (at least 7 mice per group). Results are expressed as means±SEM. Fisher PLSD test: *P<0.05, ***P<0.001.
Effect of E2 Treatment on Fatty Streak Development in ApoE\(^{-/-}\) Female Mice

Table 1. Effect of E2 Treatment and Anti–TGF-\(\beta\) mAb Administration in ApoE\(^{-/-}\) Female Mice

<table>
<thead>
<tr>
<th></th>
<th>Control Ab-Treated ApoE(^{-/-})</th>
<th>Anti–TGF-(\beta) Ab-Treated ApoE(^{-/-})</th>
<th>(P), Two-Factor ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Placebo</td>
<td>E2</td>
<td>Placebo</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>n=9</td>
<td>n=9</td>
<td>n=9</td>
</tr>
<tr>
<td>Uterine weight, mg</td>
<td>&lt;10</td>
<td>101±16</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Total Chol., mg/dL</td>
<td>536±65</td>
<td>483±44</td>
<td>584±61</td>
</tr>
<tr>
<td>HDL Chol., mg/dL</td>
<td>45±7</td>
<td>34±7</td>
<td>56±15</td>
</tr>
<tr>
<td>Lesions, (\times10^3) (\mu)m(^2)</td>
<td>98.2±10.2</td>
<td>47.4±8.7*</td>
<td>48.2±5.0†</td>
</tr>
</tbody>
</table>

Results are expressed as means±SEM. *\(P<0.001\) vs respective placebo group; †\(P<0.001\) vs control Ab-treated apoE\(^{-/-}\) placebo group. ‡\(P<0.05\) vs control Ab-treated apoE\(^{-/-}\) E2 group. §\(P<0.05\) vs anti–TGF-\(\beta\) Ab-treated apoE\(^{-/-}\) placebo group.

Effect of TGF-\(\beta\) Activity Neutralization on Fatty Streak Constitution in Placebo- or E2-Treated ApoE\(^{-/-}\) Female Mice

To explore the contribution of TGF-\(\beta\) to the protective effect of estrogens, placebo or E2 were administered to ovariolectomized apoE\(^{-/-}\) female mice simultaneously treated with an anti–TGF-\(\beta\) mAb, according to the procedure reported to neutralize both systemic and local TGF-\(\beta\) activity.\(^{11}\) Anti–TGF-\(\beta\) mAb treatment did not affect body weight and lipid parameters (Table 1). However, body weight was reduced by E2 in apoE\(^{-/-}\) mice treated or not with the anti–TGF-\(\beta\) mAb and, plasma total and HDL cholesterol tended to decrease under E2 treatment in both groups, although not significantly (Table 1). Compared with control mice, administration of either anti–TGF-\(\beta\) mAb or E2 alone resulted in a strong reduction of fatty streak area (Figure 2A). In contrast, the combination of E2 and anti–TGF-\(\beta\) mAb treatment was no longer protective, inducing a significant increase in lesion size compared with mice given anti–TGF-\(\beta\) mAb alone (+68%, \(P<0.05\)) or E2 alone (+71%, \(P<0.05\)) (Figure 2A). Indeed, the 2-factor ANOVA showed a strong interaction (\(P<0.0001\)) between E2 and anti–TGF-\(\beta\) mAb (Table 1).

Influence of TGF-\(\beta\) Activity Neutralization on Lesion Composition and Cytokine Production

As previously reported, neutralizing TGF-\(\beta\) activity influenced lesion composition, especially the balance between collagen and inflammatory cell content. Indeed, anti–TGF-\(\beta\) mAb treatment induced a 2-fold decrease in collagen content and a 2.3-fold increase in lymphocyte infiltration in placebo-treated mice. A significant increase in T lymphocyte density was also observed in the adventitia and adjacent myocardial area (data not shown). A similar effect was observed in E2-treated mice (Figure 2B and 2C).

To further assess the inflammatory status of mice from the 4 experimental groups, we analyzed the cytokine production profile by anti–CD3-activated splenocytes. As shown in Table 2, no change was observed in IL-10 production, and IL-4 concentrations were not detectable. In contrast, anti–TGF-\(\beta\) mAb or E2 treatment alone increased IFN-\(\gamma\) production by splenic T lymphocytes. Furthermore, their combination appeared additive, without interaction between the 2 factors, leading to a further increase in the production of this proinflammatory cytokine.

tion of IFN-\(\gamma\) or IL-12 production, LDLr\(^{-/-}\) female mice received a lethal whole body irradiation and were subsequently reconstituted with bone marrow (BM) from either IFN-\(\gamma\)\(^{-/-}\), IL-12\(^{-/-}\) or wild-type female mice. In mice grafted with IFN-\(\gamma\)\(^{-/-}\) or IL-12\(^{-/-}\) bone marrow cells, respectively, the abolition of IFN-\(\gamma\) production by anti–CD3-activated splenocytes and the abolition of IL-12 synthesis by lipopolysaccharide (LPS)-activated peritoneal macrophages were systematically controlled (data not shown). In both cases, cytokine deficiency did not affect total and HDL plasma cholesterol (supplemental Tables I and II). At the level of the aortic sinus, fatty streak lesion area was significantly reduced in placebo-treated IFN-\(\gamma\)-deficient mice (Figure 1A), whereas no significant change (\(P=0.18\)) was observed in IL-12-deficient mice (Figure 1B).

E2 treatment significantly decreased body weight and tended to decrease total plasma cholesterol in E2-treated mice irrespective of IFN-\(\gamma\) or IL-12 deficiency (supplemental Tables I and II), whereas no change in HDL-cholesterol was observed. Finally, E2 treatment decreased fatty streak development in IFN-\(\gamma\)\(^{-/-}\)(BM)/LDL-r\(^{-/-}\) mice (-60.6%, \(P<0.001\), Figure 1A) and in IL-12\(^{-/-}\)(BM)/LDL-r\(^{-/-}\) mice (-53.9%, \(P<0.01\), Figure 1B) as it did in WT(BM)/LDL-r\(^{-/-}\) mice, without any interaction between E2 and the respective cytokine deficiency (Two-factor ANOVA). These data clearly indicate that the atheroprotective effect of E2 is independent of the proinflammatory cytokines IFN-\(\gamma\) and IL-12.

Effect of TGF-\(\beta\) Activity Neutralization on Fatty Streak Constitutions in Placebo- or E2-Treated ApoE\(^{-/-}\) Female Mice

Table 2, no change was observed in IL-10 production, and IL-4 concentrations were not detectable. In contrast, anti–TGF-\(\beta\) mAb or E2 treatment alone increased IFN-\(\gamma\) production by splenic T lymphocytes. Furthermore, their combination appeared additive, without interaction between the 2 factors, leading to a further increase in the production of this proinflammatory cytokine.
Figure 2. Effect of estradiol on atherosclerotic lesion size and composition in 18-week-old ovariectomized apoE−/− female mice treated with either anti–TGF-β mAb or control mAb for 12 weeks. A, Oil-red-O staining (original magnification ×40) and quantification of total lesion surface at the aortic sinus. B, Sirius-red staining (×125) and quantification of lesion collagen content (sirius-red–stained surface/total lesion surface). C, CD3 staining (×200) and quantitative analysis of CD3-positive cells (T lymphocytes, number of positive cells per mm² lesion area). Representative photomicrographs from at least 9 mice per group were analyzed. Results are expressed as means±SEM. Fisher’s PLSD test: *P<0.05, **P<0.01, ***P<0.001.
Disruption of TGF-β Signaling in Bone Marrow–Derived Cells Abolishes the Protective Effect of E2 on Fatty Streak

To determine whether TGF-β contributes to the atheroprotective effect of E2 through its regulating actions on immune cells, we then grafted irradiated LDLr<sup>−/−</sup> mice with bone marrow cells from Smad3<sup>−/−</sup> or wild-type mice. Indeed, Smad3, one of the intracellular mediators that transduce signals from TGF-β and activin receptors, was previously shown to mediate the antiinflammatory effects of TGF-β1 on immune cells, especially T lymphocytes and monocytes/macrophages. The deletion of Smad3 gene was systematically controlled by PCR in splenocytes and peritoneal macrophages from Smad3<sup>−/−</sup>(BM)/LDL-r<sup>−/−</sup> mice (data not shown). The 2-factor ANOVA indicates an interaction between E2 and Smad3 genotype on lesion size (P<0.05; Table 3). Indeed, whereas the protective effect of E2 was observed as expected in WT(BM)/LDL-r<sup>−/−</sup> mice (–61.5% in lesion area), it was no longer significant in Smad3<sup>−/−</sup>(BM)/LDL-r<sup>−/−</sup> mice (–16.9%). As observed above under TGF-β activity neutralization, lesion from Smad3<sup>−/−</sup>(BM)/LDL-r<sup>−/−</sup> mice were characterized by an increase in T lymphocyte density and a decrease in collagen content (data not shown).

**E2 Treatment Enhances TGF-β1 Expression in the Aorta From apoE<sup>−/−</sup> Mice**

The demonstration that TGF-β activity strongly influences the vascular expression of estrogens led us to finally determine whether E2 could modulate the production of TGF-β in the vascular wall during the atheromatous process. As shown in Figure 3A, TGF-β1 mRNA expression was enhanced by a 6-week E2 treatment in both aortic arch (2-fold) and descending aorta (3-fold) from ovariectomized apoE<sup>−/−</sup> female mice, whereas TGF-β2, TGF-β3, and TGF-βR2 mRNA levels were not affected (data not shown). Furthermore, the expression of TGF-β1/2 was increased in fatty streak lesions from E2-treated ovariectomized apoE<sup>−/−</sup> mice when compared with lesions from placebo-treated mice, irrespective of fatty streak size (Figure 3B).

**Discussion**

The abolition of the protective effect of E2 against fatty streak constitution in immunodeficient mice first underlined the crucial contribution of the immune system to the atheroprotective effect of estrogens. According to the key role played by lymphocyte-derived cytokines in atherosclerosis, we logically hypothesized that estrogens prevent atherosclerosis constitution by a direct influence on the intimal production of cytokines, more precisely by decreasing proinflammatory cytokines or increasing the vascular expression of antiinflammatory cytokines. To test this assumption, we decided to assess the functional role of several major cytokines known to influence atheroma development. This experimental strategy allowed us to conclude that the integrity of the TGF-β pathway is absolutely required for the expression of the beneficial effect of E2 on the atheromatous process.

By contrast, our experiments tend to exclude a significant role of IL-10 in this preventive effect of E2, although the expression of this antiinflammatory cytokine is clearly atheroprotective and was reported elsewhere to be influenced by estrogens. Similarly, the atheroprotective effect of E2 in hypercholesterolemic mice does not appear to be mediated by a specific decrease in the expression of one of the proinflammatory cytokines IFN-γ or IL-12, although their clear-cut proatherogenic effects have been demonstrated.

<table>
<thead>
<tr>
<th>Table 2. Effect of E2 Treatment and Anti–TGF-β mAb Administration on Cytokine Production by Anti–CD3–Activated Spleen Cells From ApoE&lt;sup&gt;−/−&lt;/sup&gt; Female Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Ab-Treated ApoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>IFN-γ, ng/ml</td>
</tr>
<tr>
<td>IL-10, pg/ml</td>
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<td>IL-4, pg/ml</td>
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</tbody>
</table>

Results are expressed as means±SEM. *<i>P</i>&lt;0.001 vs control Ab-treated apoE<sup>−/−</sup> placebo group. †<i>P</i>&lt;0.05 vs respective placebo group. ‡<i>P</i>&lt;0.01 vs control Ab-treated apoE<sup>−/−</sup> E2 group.

<table>
<thead>
<tr>
<th>Table 3. Effect of E2 Treatment in Irradiated LDL-r&lt;sup&gt;−/−&lt;/sup&gt; Female Mice Reconstituted With Bone Marrow From Either Wild-Type (WT) or Smad3&lt;sup&gt;−/−&lt;/sup&gt; Mice</th>
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<tr>
<td>WT → LDL-r&lt;sup&gt;−/−&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Body weight, g</td>
</tr>
<tr>
<td>Uterine weight, mg</td>
</tr>
<tr>
<td>Total Chol., mg/dL</td>
</tr>
<tr>
<td>HDL Chol., mg/dL</td>
</tr>
<tr>
<td>Lesions, ×10&lt;sup&gt;2&lt;/sup&gt;/μm&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Results are expressed as means±SEM. *<i>P</i>&lt;0.001 vs WT → LDL-r<sup>−/−</sup> placebo group.
Moreover, we previously showed that the protective effect of E2 was also fully maintained in apoE−/−/IL-6−/−/IL-18−/− female mice (Elhage et al., unpublished data, 2002). As apoE deletion influences immune responses, we cannot exclude that IL-10, IL-6 or IL-18 play some role in the protective effect of E2 in LDLr−/− mice. It is of importance to emphasize that the crucial role of the TGF-β pathway in the protective effect of E2 was demonstrated here in the 2 models of atherosclerosis: apoE−/− mice using a neutralizing anti–TGF-β mAb and LDLr−/− mice using disruption of TGF-β signaling in hematopoietic cells.

Noteworthy, we found here that the administration of the anti–TGF-β mAb in placebo-treated ovariectomized apoE−/− mice led to a substantial decrease in lesion size. This observation differs from the original report by Mallat et al., despite the use of the same anti–TGF-β mAb, but is in perfect agreement with the data reported by Lutgens et al. using a recombinant soluble dominant negative form of the TGF-β receptor II. Similarly, Smad3 deficiency in hematopoietic cells did not significantly influence fatty streak area in our graft experiments, contrasting with the strong acceleration of lesion constitution reported by Robertson et al. in T cells carrying a dominant negative TGF-β receptor 2 in T cells. More than the strategy of TGF-β activity inhibition used, the comparison between these series of experimental procedures suggests that the specific conditions of animal care, pathogen-free (in Lutgens’ and the present studies) versus conventional (in Mallat’s and Robertson’s studies), could crucially determine the effect of TGF-β blockade on fatty streak deposit, as previously demonstrated for IL-10 deficiency. Overall, our data totally agree with previous observations in mouse models when considering the increased inflammatory components and the decreased collagen content in atheromatous lesions, which reflects the imbalance between inflammation and fibrosis under TGF-β blockade.

The ability of estrogens to increase the expression of TGF-β isoforms has been previously demonstrated in extra-reproductive tissues, especially in the reproductive tract and in bone. We report here, for the first time, that chronic E2 administration enhances the expression of TGF-β1 in the aortic wall during the early steps of atherosclerosis in hypercholesterolemic mice, especially in fatty streak lesions. Interestingly, tamoxifen, a selective estrogen receptor modulator, was previously reported to prevent fatty streak constitution in C57Bl/6 mice fed a high-fat diet, with a concomitant increase of both circulating and aortic concentrations of active and latent TGF-β. Noteworthy, almost all the cell types involved in the atheromatous process, namely smooth muscle cells, macrophages, platelets, and lymphocytes including regulatory T cells can be source of TGF-β and target for estrogens. However, neither immunohistochemistry analysis nor mRNA quantification allowed us to determine the specific cell type(s) responsible for the E2-induced increase in TGF-β1 production within the atherosclerotic lesions. Interestingly, natural CD4+ CD25+ regulatory T cells...
were recently reported to prevent the constitution of fatty streak in apoE−/− mice through the modulation of CD4+ T lymphocyte activity by TGF-β.35 Because estrogens were shown to expand the CD4+ CD25+ regulatory T cell compartment in mice,16 it is tempting to speculate that these cells contribute, at least in part, to the enhanced expression of TGF-β observed in E2-treated mice, and thus to the hormonal atheroprotective effect.

The mechanisms responsible for the TGF-β antiatherogenic effects, especially reduction of the intimal inflammatory process and promotion of plaque stabilization, were recently further analyzed.17,37 Thus, a major part of this protective effect of TGF-β is mediated through its inhibitory action on T cells, as the selective disruption of TGF-β signaling in T cells promotes atherosclerosis.13,14 Because chronic in vivo E2 administration was recently reported to enhance IFN-γ production by CD4+ T helper lymphocytes as well as Natural Killer T lymphocytes, it remains possible and even likely that TGF-β contributes to the atheroprotective effect of estrogens by inhibiting the secretion of proinflammatory cytokines in the vessel wall.18,38 According to this hypothesis, we observed that: (1) the combination of E2 administration and TGF-β blockade in apoE−/− mice resulted in a significant increase in IFN-γ production by activated splenocytes; (2) the disruption of TGF-β signaling in bone marrow–derived cells abolishes the preventive effect of E2 on fatty streak constitution. Interestingly, E2 was recently reported to prevent bone loss through the silencing of IFN-γ receptor signaling by TGF-β,19 suggesting some similarity of interaction between E2, IFN-γ, and TGF-β in vessel and bone.

Finally, the complex interactions between cytokines, as well as the heterogeneity of the atheromatous tissue, greatly limit the information provided by the assessment of their level. For these reasons, we favored a functional approach consisting in studying the atheroprotective effect of E2 in a context of suppression of cytokine expression or activity. Although most of the cytokines are expressed during the atheromatous process, their precise role is probably not constant at the different steps of atherothrombosis (for instance in asymptomatic fatty streaks and during plaque rupture or erosion leading to clinical events). Whereas several proinflammatory cytokines, which level can be enhanced by E2 in several contexts, does not appear to alter the effect of E2 in asymptomatic fatty streaks (present experimental models), they could contribute to plaque rupture in unstable plaque at the initiation of the hormonal replacement therapy (HERS and WHI trials). This deleterious effect could be largely favored in women with a defect in the TGF-β pathway.11–14 However, despite numerous studies have suggested that lower levels of TGF-β1 activity predispose to unstable atherosclerotic disease (correlation with serum concentration of active TGF-β1 and association with polymorphisms of the TGF-β1 gene), the precise role of the TGF-β pathway in human atherosclerosis remains to be elucidated.9

In conclusion, the present work clearly demonstrates the importance of TGF-β status in the vascular effects of estrogens. Thus, it will be of interest to consider the TGF-β pathway in past and future clinical trials concerning menopausal women. More precisely, serum active TGF-β concentrations both at baseline and after initiation of HRT, along with the TGF-β and TGF-β receptors gene polymorphisms, should be assessed in such trials and could represent one of the keys of the individual occurrence of cardiovascular events after initiation of HRT.

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Disclosures

None.

References


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On-line Supplemental Materials and Methods.

Mice.

C57Bl/6J, LDLr<sup>/−</sup>, IFN-γ-deficient (IFN-γ<sup>/−</sup>) and IL-12p40-deficient (IL-12<sup>/−</sup>) mice were purchased from Charles River (L’arbresle, France). ApoE<sup>/−</sup> mice were bred in-house as previously described<sup>20</sup>. ApoE and IL-10 double deficient mice (apoE<sup>/−</sup> IL-10<sup>/−</sup>) were kindly provided by G.K. Hansson (Karolinska Intitute, Stockholm, Sweden)<sup>21</sup>. Smad3 deficient mice (Smad3<sup>/−</sup>) were kindly provided by C. Deng (National Institute of Health, Bethesda), bred in our animal facilities in heterozygotic state, and screened by Polymerase Chain Reaction (PCR) genotyping as previously described<sup>22</sup>. All mice had been backcrossed into a C57Bl/6 background for more than 6 generations and were maintained under specific pathogen-free conditions.

Female mice were systematically ovariectomized at 4 weeks of age, then administered with either 60-day time-release placebo or 0.1mg estradiol-17β pellets (Innovative Research of America, Sarasota, FL), implanted s.c. in the back of the animals at 6 weeks of age (excepted in bone marrow transplantation experiments: 10 weeks of age). New pellets were reimplemented 7 weeks later. The dose of 0.1mg E2, releasing 80 µg/kg/d, had previously been defined as adequate for a maximal effect on fatty streak constitution in female apoE<sup>/−</sup> mice<sup>20</sup>. Female mice on apoE<sup>/−</sup> genetic background were maintained on a chow diet (4.3% fat and 0.02% cholesterol) throughout the entire experiments, while LDLr<sup>/−</sup> mice were switched to a high-fat diet (1.25% cholesterol, 6% fat, no cholate, TD96335, Harlan Teklad, Wisconsin) when placebo or E2-treatment was started, as previously described<sup>23</sup>. After 12 weeks of E2 or placebo treatment, blood was collected from the retro-orbital venous plexus of anesthetized mice after a 16h fast. Mice were sacrificed, then, the heart with the ascending aorta, the spleen and the uterus were carefully removed. In all experiments, the efficacy of E2 treatment was systematically assessed by the induction of uterus hypertrophy contrasting with the total atrophy observed in placebo-treated ovariectomized female mice. All procedures were performed in accordance with the guidelines established by the National Institute of Medical Research.
Bone marrow transplantation.

Six week-old ovariectomized LDLr⁻/⁻ female mice were subjected to 9.5-Gy lethal total body γ-irradiation to eliminate endogenous bone marrow stem cells and bone marrow-derived cells. Twelve hours later, they were intravenously reconstituted with bone marrow cells (4x10⁶) extracted from the femur and tibia of either IFN-γ⁻/⁻, IL-12⁻/⁻, Smad3⁻/⁻, or wild type C57Bl/6J female mice. Following irradiation and bone marrow transplantation, mice were allowed to recover for 4 weeks under a chow diet. Then, all transplanted mice were implanted subcutaneously with placebo or E2 pellets and were switched to the high fat atherogenic diet to induce atherosclerotic lesion formation. Mice were sacrificed 12 weeks later (at 22 weeks of age) and tissues prepared as described above. One week before and four weeks following bone marrow transplantation, Bactrim (sulfamethoxazole 200 mg/ml, trimethoprim 48 mg/ml) was added to drinking water.

In vivo treatment of animals with anti-TGF-β antibody.

Mice were injected intraperitoneally in a volume of 240 µl with either an anti-TGF-β1/β2/β3 monoclonal antibody (clone 2G7; ascitic fluid at 1:10 dilution) or an isotype matched (IgG2b) anti-human cytotrophoblast JEG13 control mAb (ascitic fluid at 1:10 dilution), devoid of neutralizing activity, as previously shown 11. Both mAbs were kindly provided by D. Fradelizi (INSERM U477, Paris, France). Injections were started at 6 weeks of age and repeated once a week for 11 weeks, during the placebo- or E2-treatment period. The determination of 2G7 mAb concentration was based on in vitro preliminary tests, as previously described 11.

Determination of serum lipids.

Lipids determination was performed by the technical platform for lipid mediators analysis of INSERM IFR30, Toulouse Purpan, France. Total plasma cholesterol was assayed using the CHOD-PAP kit (Randox Laboratories, Montpellier, France). The high density lipoprotein (HDL) fraction was isolated from 10 µl of serum by precipitation of the apoB-containing particles with 20µl of the phosphotungstate/MgCl2 kit (Randox Laboratories), and HDL cholesterol was assayed on 10 µl of the supernatant using the CHOD-PAP kit.
**Morphometric and immunohistochemical analyses.**

Fatty streak lesion size was estimated at the aortic sinus, as previously described\(^{20}\). Other sections were stored at -80°C and dedicated to lesion composition analysis, including collagen detection and immunohistochemical staining. Collagen fibers were stained with Sirius red. The lesion collagen content was determined by measuring the relative area/density in 12 contiguous fields in each Sirius red-stained section. For immunohistochemical staining, a goat polyclonal anti-CD3 and a rabbit polyclonal anti-TGF-β1/2 (Santa Cruz Biotechnology, Santa Cruz, CA) were used. Immunostains were visualized after incubation with an anti-goat or anti-rabbit preadsorbed secondary biotinylated antibody (Vector laboratories, Burlingame, CA) and the use of an avidin DH-biotinylated peroxidase complex (Vectorstain ABC kit, Vector Laboratories) and AEC peroxidase substrate kit (Vector Laboratories). Countercoloration was carried out using Mayer’s hemalum. At least four sections per animal were analyzed for each immunostaining.

**Analysis of cytokine production by spleen cells.**

Spleen cells were prepared using a homogenizer, and red blood cells were lysed in a hemolysis buffer (NH\(_4\)Cl, KHCO\(_3\), EDTA). Splenocytes were resuspended in RPMI 1640 complete medium (Cambrex, Verviers, Belgium) containing 10% FCS, seeded into 96-well flat-bottom cultured plates (5x10\(^5\) cells/well), and stimulated with coated anti-CD3 mAb (1 µg/ml, BD Pharmingen, San Diego, CA) for 48h. Then, supernatants were harvested and stored at -80°C until cytokine assays. IFN-γ, IL-10 and IL-4 were quantified by two-site sandwich ELISA as previously described\(^{24}\). For each assay, detection limits was 15 pg/ml.

**Analysis of TGF-β1 mRNA in aorta by RT-PCR.**

Six-week-old ovariectomized apoE\(^{-/-}\) female mice were administered with either placebo or E2 pellets and were sacrificed 5 weeks later. The aortic arch and the descending aorta were carefully removed and immediately frozen for RNA extraction. A DNase I treatment was realized (AMP-D1 kit, Sigma), then a reverse transcription was performed using 1 µg of total RNA, according to the manufacturer’s instructions (Invitrogen, superscriptII First-Strand kit). Quantitative PCR was performed using SYBR green JumpStart™ Taq ReadyMix (Sigma). As an internal control, the quantification of ribosomal 18S RNA was used (5’- AGCCTGCGGCTAATTTGAC-
3' and 3'- CAACTAAGAACGCCATGCA-5'), and the amplification of TG-β1 was performed with specific probes (5'-ACTGGAGTTGTACGGCAGTGG-3' and 3'- GCAGTGAGCGCTGAATCGA-5'). The amplification conditions for Gene Amp 7900 (Applied Biosystem) consisted of an initial step of 2 min at 50°C, 10 min 95°C, followed by 40 cycles: 15 s 95°C, 1 min 60°C.

Statistical analyses.

Statistical analyses were performed using the Statview® software (Abacus Concepts, Inc., Berkeley, CA). Results were expressed as means ± S.E.M. A two-way ANOVA was performed to test for the respective effects of E2 treatment, cytokine deficiency or anti-TGF-β mAb treatment on various parameters. When the F-test allowed rejection of the null hypothesis of no difference between groups, paired comparisons were performed with the Fisher’s PLSD procedure. \( P<0.05 \) was considered as statistically significant.
**On-line Supplemental tables.**

Table I. Effect of E2 treatment in irradiated LDL-r⁻/⁻ female mice reconstituted with bone marrow from either wild type (WT) or IFN-γ⁻/⁻ mice.

<table>
<thead>
<tr>
<th></th>
<th>WT → LDL-r⁻/⁻</th>
<th></th>
<th>IFN-γ⁻/⁻ → LDL-r⁻/⁻</th>
<th></th>
<th>P, two-factor ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Placebo n= 8</td>
<td>E2 n= 8</td>
<td>Placebo n= 9</td>
<td>E2 n= 9</td>
<td>Genotype effect</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>27.3 ± 0.5</td>
<td>24.3 ± 0.5</td>
<td>23.8 ± 0.9</td>
<td>22.4 ± 0.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Uterine weight (mg)</td>
<td>&lt; 10</td>
<td>95 ± 9</td>
<td>&lt; 10</td>
<td>62 ± 4</td>
<td>-</td>
</tr>
<tr>
<td>Total Chol. (mg/dL)</td>
<td>1105 ± 63</td>
<td>986 ± 118</td>
<td>1198 ± 98</td>
<td>1084 ± 100</td>
<td>NS</td>
</tr>
<tr>
<td>HDL Chol. (mg/dL)</td>
<td>102 ± 7</td>
<td>104 ± 7</td>
<td>92 ± 6</td>
<td>97 ± 4</td>
<td>NS</td>
</tr>
<tr>
<td>Lesions (x 10³ µm²)</td>
<td>106.0 ± 17.2</td>
<td>25.9 ± 5.6</td>
<td>68.1 ± 6.3</td>
<td>26.8 ± 3.4</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Results are expressed as means ± SEM.
Table II. Effect of E2 treatment in irradiated LDL-r⁻/⁻ female mice reconstituted with bone marrow from either wild type (WT) or IL-12⁻/⁻ mice.

<table>
<thead>
<tr>
<th></th>
<th>WT → LDL-r⁻/⁻</th>
<th>IL-12⁻/⁻ → LDL-r⁻/⁻</th>
<th>P, two-factor ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Placebo n=7</td>
<td>E2 n=7</td>
<td>Placebo n=8</td>
</tr>
<tr>
<td><strong>Body weight (g)</strong></td>
<td>27.1 ± 0.6</td>
<td>24.0 ± 0.5</td>
<td>25.8 ± 0.8</td>
</tr>
<tr>
<td><strong>Uterine weight (mg)</strong></td>
<td>&lt; 10</td>
<td>93 ± 4</td>
<td>&lt; 10</td>
</tr>
<tr>
<td><strong>Total Chol. (mg/dL)</strong></td>
<td>1133 ± 79</td>
<td>975 ± 107</td>
<td>1219 ± 67</td>
</tr>
<tr>
<td><strong>HDL Chol. (mg/dL)</strong></td>
<td>98 ± 4</td>
<td>101 ± 4</td>
<td>99 ± 9</td>
</tr>
<tr>
<td><strong>Lesions (x 10³ µm²)</strong></td>
<td>118.4 ± 8.7</td>
<td>42.8 ± 6.6</td>
<td>145.5 ± 17.9</td>
</tr>
</tbody>
</table>

Results are expressed as means ± SEM.
Table III. Effect of E2 treatment in apoE−/− and apoE−/− IL-10−/− female mice.

<table>
<thead>
<tr>
<th></th>
<th>apoE−/−</th>
<th>apoE−/− IL-10−/−</th>
<th>P, two-factor ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Placebo n= 7</td>
<td>E2 n= 7</td>
<td>Placebo n= 6</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>26.8 ± 1.1</td>
<td>23.7 ± 0.9</td>
<td>21.9 ± 1.3</td>
</tr>
<tr>
<td>Uterine weight (mg)</td>
<td>&lt; 10</td>
<td>166 ± 12</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>Total Chol. (mg/dL)</td>
<td>517 ± 18</td>
<td>388 ± 15</td>
<td>399 ± 11</td>
</tr>
<tr>
<td>HDL Chol. (mg/dL)</td>
<td>41 ± 4</td>
<td>34 ± 3</td>
<td>36 ± 4</td>
</tr>
<tr>
<td>Lesions (x 10³ µm²)</td>
<td>111.4 ± 10.6</td>
<td>54.8 ± 7.3</td>
<td>150.3 ± 14.3</td>
</tr>
</tbody>
</table>

Results are expressed as means ± SEM.