Suppression of Atherosclerotic Development in Watanabe Heritable Hyperlipidemic Rabbits Treated With an Oral Antiallergic Drug, Tranilast

Toshiyuki Matsumura, MD; Kiyotaka Kugiyama, MD; Seigo Sugiyama, MD; Yasutaka Ota, MD; Hideki Doi, MD; Nobuhiko Ogata, MD; Hideki Oka, MD; Hirofumi Yasue, MD

Background—Inflammatory and immunological responses of vascular cells have been shown to play a significant role in the progression of atheromatous formation. Tranilast [N-(3,4-dimethoxycinnamoyl) anthranilic acid] inhibits release of cytokines and chemical mediators from various cells, including macrophages, leading to suppression of inflammatory and immunological responses. This study tested whether tranilast may suppress atheromatous formation in Watanabe heritable hyperlipidemic (WHHL) rabbits.

Methods and Results—WHHL rabbits (2 months old) were given either 300 mg · kg⁻¹ · d⁻¹ of tranilast (Tranilast, n=12) or vehicle (Control, n=13) PO for 6 months. Tranilast treatment was found to suppress the aortic area covered with plaque. Immunohistochemical analysis showed that there was no difference in the percentage of the RAM11-positive macrophage area and the frequency of CD5-positive cells (T cells) in intimal plaques between Tranilast and Control. Major histocompatibility complex (MHC) class II expression in macrophages and interleukin-2 (IL-2) receptor expression in T cells, as markers of the immunological activation in these cells, was suppressed in atheromatous plaque by tranilast treatment. Flow cytometry analysis of isolated human and rabbit peripheral blood mononuclear cells showed that an increase in expression both of MHC class II antigen on monocytes by incubation with interferon-γ and of IL-2 receptor on T cells by IL-2 was suppressed by the combined incubation with tranilast.

Conclusions—The results indicate that tranilast suppresses atherosclerotic development partly through direct inhibition of immunological activation of monocytes/macrophages and T cells in the atheromatous plaque. (Circulation. 1999;99:919-924.)

Key Words: atherosclerosis ■ cells ■ lymphocytes ■ immune system

It has been shown that inflammatory and immunological responses may play at least a partial role in the pathogenesis of atherosclerosis.1–3 In the past, a number of anti-inflammatory or immunosuppressive drugs were examined in vivo animal experiments as to whether the drugs might have suppressive effects on the development and progression of atherosclerosis.4,5 However, the results were inconsistent among experiments in different laboratories, and the effect of immunodepression on atherogenesis remains inconclusive.4–6 Furthermore, most of these drugs induce nonspecific immunosuppression, thereby having serious side effects for long-term clinical use.4,5 Tranilast, N-(3,4-dimethoxycinnamoyl) anthranilic acid, has been used clinically as an oral antiallergic drug for nearly a decade in Japan. The antiallergic effects of tranilast are thought to be mediated by the inhibition of the release of chemical mediators from mast cells and basophils.7 Very recently, a clinical trial showed that oral administration of tranilast for 3 months significantly prevented coronary restenosis with minimal side effects after directional coronary atherectomy in humans.8 To the best of our knowledge, tranilast is the first anti-immunological drug to show inhibitory effects on atherosclerotic development without significant side effects during long-term use in vivo in humans. Mechanisms responsible for the inhibition of restenosis by tranilast remain undetermined. It has been shown that tranilast inhibits release of cytokines from activated human macrophages and T cells in vitro.9 It is known that chronically activated macrophages and T cells within the lesion participate in local immune responses during atherogenesis and that various cytokines released from these cells affect growth, differentiation, and lipid metabolism in vascular cells and could thereby play a role in the development and progression of atherosclerosis.1–3 Thus, we hypothesized that tranilast may suppress atherosclerosis, possibly through inhibitory effects on the functions of macrophages and T cells. We tested this hypothesis in Watanabe heritable hyperlipidemic (WHHL) rabbits and focused on the effects of tranilast...
treatment on the expression of the major histocompatibility complex (MHC) class II on macrophages and of interleukin-2 (IL-2) receptor on T cells in the atheromatous plaque and on isolated cells. MHC class II on macrophages and IL-2 receptor on T cells are known to contribute to the immunological responses and have been recognized as a marker of the local immunological activation in the atherosclerotic arterial walls.2,10,11

Methods

Animal Experiments

Twenty-five WHHL rabbits (3 months old) weighing between 2.0 and 2.3 kg were housed individually in wire-bottomed cages in an air-conditioned room at 20°C and 50% humidity with 12 hours light/12 hours dark cycles. After an adaptation period of 1 week, the rabbits were randomly separated into 2 groups: 13 rabbits in the control group (standard chow containing placebo) and 12 rabbits in the tranilast-treated group (standard chow containing tranilast). Tranilast powder (Kissei Pharmaceutical Co) was mixed with the pelleted diet at an oral dose of 300 mg·kg body wt−1·d−1. This dose was selected because its oral administration maintained plasma concentrations of tranilast to ~10 to 100 μmol/L, which have an anti-immunological action in in vitro experiments.12 Standard chows were purchased from Cler Japan Inc. The amount of daily diet for each animal was restricted to 120 g during the study period. Water was provided ad libitum.

At 6 months into the dietary period, arterial blood pressure and heart rate in conscious rabbits were measured by the central ear technique11 in the early morning 2 hours after rabbits were provided access to the diet. Blood samples from the central ear artery after a 24-hour fast were collected into tubes containing EDTA-Na2 (1 mg/mL blood) for the lipid assay. Then, rabbits were killed under pentobarbital anesthesia (30 mg/kg IV). The entire aortas from the origin of the aortic valve to the iliac bifurcation were excised and placed immediately into ice-cold PBS. The isolated aortas were cleaned of perivascular tissues. The aortas from the origin of the first intercostal arteries to the origin of the third intercostal arteries were used for assays of tissue lipids and for the immunohistological examination. The remaining aortas from the origin of the aortic valve to the first intercostal arteries and from the third intercostal arteries to the iliac bifurcation were used for morphometric assessment of atheromatous plaque area.13 All experiments were performed in accordance with the guidelines on experimental animals issued by Kumamoto University School of Medicine and were approved by the Center for Laboratory Animals.

Atheromatous Plaque Area

The aortas from the origin of the aortic valve to the first intercostal arteries and from the third intercostal arteries to the iliac bifurcations were opened longitudinally to expose the intimal surface and were fixed in 10% buffered formalin overnight. The preparations were then stained with Sudan IV to reveal sudanophilic plaques and subsequently photographed. Then, the photographs were copied onto graph paper at ×2 magnification, and the outlines of the aorta and the Sudan-positive area were scanned and estimated by computerized planimetry, as reported previously.13 The atheromatous plaque areas within each aorta were summed, and the extent of atheromatous plaques was expressed as a percentage of surface area of the aorta.

Lipid Analysis of EDTA Plasma and Aortic Samples

Parts of the aortas from the first to the third intercostal arteries were blotted, kept in an incubator for 48 hours at 60°C, and weighed. Measurements of lipids and lipoproteins in plasma and aortic tissues were performed with commercial kits (Wako Pure Chemical) as previously described.13

LDL Isolation and Oxidation

At 6 months into the dietary period, LDL (d=1.021 to 1.063) was isolated from both groups of WHHL rabbits by sequential ultracentrifugation. The oxidative susceptibility of LDL isolated from each rabbit was determined by measurement of diene formation as described previously.14

Immunohistochemistry

Parts of the aortas at the level between the first and the third intercostal arteries were rinsed and embedded in O.C.T. compound (Miles Inc), quickly frozen, and stored at –80°C. Frozen tissue specimens were cut into 8-μm-thick sections and stained either by a single indirect immunoperoxidase method or by a sequential double-labeling method11 using the primary monoclonal antibodies listed in Table 1.

Adjacent tissue sections from atherosclerotic lesions were examined to determine lesion size and cellularity in the atherosclerotic lesions. The area density was calculated by use of the cross-sectional area of the lesion measured by computerized image analysis. The percentages of RAM11-positive cell area in intimal plaque areas were calculated by dividing the RAM11-positive area by the area of intimal plaques in the adjacent sections. The percentages of MHC class II antigen–positive cell area in RAM11-positive cell area were calculated by dividing the MHC class II antigen–positive cell area by the RAM11-positive cell area in the adjacent sections. The percentages of CD25-positive (IL-2 receptor–expressing) T cells in CD5-positive cells (all T cells) in intimal plaques were calculated by

<table>
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<tr>
<th>Antibody</th>
<th>Antigen</th>
<th>Specificity</th>
<th>Clone</th>
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<tr>
<td>RAM 11†</td>
<td>Rabbit</td>
<td>Macrophages</td>
<td>RAM 11</td>
</tr>
<tr>
<td>Class II RLA-DQ†</td>
<td>Rabbit</td>
<td>MHC class II determinant</td>
<td>MCA 811</td>
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<tr>
<td>CD5†</td>
<td>Rabbit</td>
<td>Pan-T cells</td>
<td>KEN-5</td>
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<tr>
<td>CD25‡</td>
<td>Rabbit</td>
<td>IL-2 receptor on activated T cells</td>
<td>Kei-α1</td>
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| Flow cytometry | | | |
|----------------|-----------------|-------------------|
| CD 14 PE* | Human | Monocytes/macrophages | TÜK4 |
| HLA-DP, DQ, DR FITC* | Human | MHC class II determinant | CR3/43 |
| CD3 PE‡ | Human | Pan-T cells | HIT3a |
| CD25 FITC‡ | Human | IL-2 receptor on activated T cells | M-A251 |
| Class II RLA-DQ† | Rabbit | MHC class II determinant | MCA 811 |

*DAKO A/S; †Serotec Ltd; ‡Pharmingen.
dividing the number of CD25-positive cells by the number of CD5-positive cells in the adjacent sections.

**Immunofluorescence Staining and Fluorescence-Activated Cell Sorter Analysis**

Antibodies used for flow cytometry were also listed in Table 1. Peripheral blood mononuclear cells (PBMCs) were purified from citrate-anticoagulated venous blood samples from healthy volunteers by use of Ficoll-Hypaque gradients.16 The isolated PBMCs (10⁶ cells/mL) were incubated with or without recombinant human IL-2 (200 U/mL; Genzyme) in the presence or absence of tranilast at the indicated concentrations (3 to 300 µmol/L) at 37°C for 24 hours in 5% CO₂ and 98% humidity. The concentrations, ranging from 3 to 300 µmol/L, tranilast, used in this in vitro experiment are comparable to the plasma levels in rabbits that had oral doses of 300 mg·kg⁻¹·d⁻¹ of tranilast (plasma concentration reached 450 µmol/L 30 minutes after a single administration of 300 mg/kg of tranilast and decreased to 3 µmol/L 24 hours after the single administration). After the incubation, expression of IL-2 receptor (CD25) on T cells (CD3-positive) was evaluated by flow cytometry with the direct method as described previously.16,17 CD25 (IL-2 receptor) expression on the treated T cells was expressed as the mean channel of fluorescence intensity among the CD3-positive cells (all T cells).

In the other set of experiments, human PBMCs (10⁶ cells/mL) and rabbit monocytes isolated from peripheral blood were incubated with or without recombinant human interferon-γ (IFN-γ; 100 ng/mL; Pharmingen) in the presence or absence of tranilast 3 to 300 µmol/L at 37°C for 24 hours. Then, HLA-DP, DQ, DR (MHC class II) expression on human monocytes and class II RLA-DQ (MHC class II) expression on rabbit monocytes were also evaluated by flow cytometry with the direct and indirect methods,19 respectively.

**Measurements of Cytosolic Free Calcium in Monocytes**

The concentration of cytosolic free calcium ([Ca²⁺]) in the isolated monocytes was measured by use of the intracellular calcium indicator fura 2, as described previously.16 [Ca²⁺], in single fura 2-loaded monocytes was monitored continuously with dual-excitation microfluorimetry equipment (ARGUS 50/CA System) coupled to an image acquisition system (Nikon inverted microscope). Monocytes adhering to plastic culture dishes were stimulated by For-Met-Leu-Phe (FMLP; 100 nmol/L; Peptide Institute) in the presence or absence of tranilast 3 to 300 µmol/L under continuous monitoring of [Ca²⁺], at 37°C. More than 95% of the adherent cells were CD14-positive cells.

**Reagents**

All reagents for cell culture were from Gibco. Other chemicals were from Sigma Chemical Co.

**Statistical Analysis**

All values were expressed as mean±SEM. Statistical analysis of the data was performed with Student’s t test for paired or unpaired observations. When >2 groups were compared, ANOVA was used. Values were considered to be statistically different at P<0.05.

**Results**

**Body Weight and Hemodynamics**

There was no significant difference in body weight (Control, 3.0±0.2 kg versus Tranilast, 3.2±0.2 kg, P=NS), mean blood pressure (Control, 99±6 mm Hg versus Tranilast, 98±4 mm Hg, P=NS), or heart rate (Control, 232±14 bpm versus Tranilast, 224±18 bpm, P=NS) at 6 months (on the day of death) of the dietary period between the 2 groups of rabbits.

**Lipids in Plasma and in Aortic Tissues**

There was no significant difference in the plasma levels of total cholesterol (Control, 472±50 mg/dL, versus Tranilast, 548±31 mg/dL, P=NS), triglycerides (Control, 398±45 mg/dL versus Tranilast, 444±54 mg/dL, P=NS), or HDL cholesterol (Control, 13±2 mg/dL versus Tranilast, 11±1 mg/dL, P=NS) at 6 months (on the day of death) of the dietary period between the 2 groups. However, tissue concentrations of both total cholesterol and cholesteryl esters in the aortas were significantly lower in the tranilast-treated group than in the control group (total cholesterol, 25±4 versus 62±12 µg/mg protein, respectively, P<0.01; cholesteryl esters, 19±3 versus 48±9 µg/mg protein, respectively, P<0.01).

**Oxidative Susceptibility of LDL**

Susceptibility of isolated rabbit LDL (300 µg protein/mL) to oxidative modification with Cu²⁺ 5 µmol/L was not different between the control and tranilast-treated groups (lag time of diene formation: Control, 63±3 minutes versus Tranilast, 61±2 minutes, n=6 in each experiment, P=NS). Also, the addition of tranilast to the incubation mixture containing LDL isolated from the control rabbits did not affect the lag time of conjugated diene formation (diene lag time: 61±2 minutes without tranilast versus 60±3 minutes with tranilast 300 µmol/L, n=6 in each experiment, P=NS).

**Histological Findings**

There was no significant difference in the percentage of RAM11-positive macrophage area in the intimal plaque area between the placebo- and tranilast-treated groups, as shown in Table 2. Most of the MHC class II antigen–positive cells were identical with macrophages (Figure 2), a result in agreement with previous reports.5,6 Table 2 shows that the ratio of class II MHC antigen–positive cells to RAM11-positive macrophages was significantly lower in the tranilast-treated group than in the control group.

As shown in Figure 3A, most T cells (CD5-positive) were localized in the subendothelium. CD25-positive cells (IL-2 receptor–positive cells) were found to be identical with T
TABLE 2. Percentage of RAM11-Positive Macrophage Area in Intimal Plaque Area and Percentage of MHC Class II Antigen–Positive Area in RAM11-Positive Macrophage Area

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Tranilast</th>
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<tr>
<td>% RAM11-positive area in intimal plaques</td>
<td>64±9</td>
<td>59±8</td>
<td>NS</td>
</tr>
<tr>
<td>% MHC class II antigen–positive area in RAM11 positive area</td>
<td>58±12</td>
<td>18±7</td>
<td>&lt;0.01</td>
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RAM11-positive area and MHC class II antigen–positive area were identified in the adjacent sections. The percentage of RAM11-positive area in intimal plaque area was calculated by dividing the RAM11-positive area by the area of intimal plaques in the adjacent sections. The percentage of MHC class II antigen–positive area in RAM11-positive area was calculated by dividing the MHC class II antigen–positive area by the RAM11-positive area in the adjacent sections. Data are mean±SEM. Control indicates rabbits fed standard chow (n=13); Tranilast, rabbits fed chow containing tranilast (n=12).

Effect of Tranilast on Cell Surface Antigen Expression on Isolated PBMCs

Figure 4 shows that incubation of human T cells with IL-2 upregulated expression of CD25 (IL-2 receptor) on the T cells and that combined incubation with tranilast suppressed the upregulation of CD25 (IL-2 receptor) expression on T cells (mean channel of fluorescence intensity: no treatment, 8.4±0.1; IL-2 200 U/mL alone, 15.9±0.3*; IL-2 plus tranilast 300 μmol/L, 9.7±0.3*; n=5 or 6; *P<0.01 versus no treatment, †P<0.01 versus IL-2 alone).

Figure 5 shows that incubation of human PBMCs with IFN-γ upregulated expression of MHC class II antigen in monocytes and that combined incubation with tranilast suppressed the upregulation of MHC class II expression in monocytes. Tranilast 300 μmol/L also suppressed the upregulation of class II RLA-DQ (MHC class II) expression on the rabbit monocytes treated with IFN-γ 100 ng/mL (mean channel of fluorescence intensity: no treatment, 1315±16; IFN-γ alone, 2024±26*; IFN-γ plus tranilast, 1641±36*; n=6 in each experiment; *P<0.01 versus no treatment, †P<0.01 versus IFN-γ alone).

Effect of Tranilast on FMLP-Induced Transients of [Ca^{2+}] in Monocytes

FMLP 100 mmol/L induced a rapid and transient rise in [Ca^{2+}], in all monocytes examined (Figure 6). Tranilast reduced the transients in [Ca^{2+}], in response to FMLP (Figure 6B and 6C, peak [Ca^{2+}], [μmol/L]: control, 465±19; tranilast 30 μmol/L, 174±15*; tranilast 300 μmol/L, 124±9*; n=6 to 9 in each experiment; *P<0.01 versus control).

Discussion

The present study showed that tranilast, an antiallergic drug, suppressed atheromatous formation and decreased contents of cholesterol and cholesteryl esters in the aortas of WHHL rabbits. These inhibitory effects of tranilast were associated with the suppression of MHC class II expression on monocytes/macrophages and of IL-2 receptor (CD25) expression on T cells, both of which are known to contribute to the immunological responses and have been recognized as a marker of the local immunological activation in the atherosclerotic arterial walls.2,10,11 Tranilast is unlikely to exert...
nonspecific cytotoxic effects on monocytes and T cells, because it did not affect the extent of areas with RAM11 (macrophage) and the frequency of CD5 (all T cell)–positive cells in the intimal plaque area. Furthermore, tranilast did not influence plasma lipid profiles in the WHHL rabbits, and it did not affect the susceptibility of LDL oxidation. The results indicate that tranilast may inhibit the immunological responses of macrophages and T cells in the arterial wall, leading to suppression of atheromatous formation. A number of cytokines, including IFN-γ and IL-2, secreted from the activated T cells can stimulate macrophages and regulate their lipoprotein uptake, leading to foam cell formation.1–3,19 The activated macrophages, in turn, can secrete a variety of potentially atherogenic mediators, including monocyte/macrophage-derived growth factors, lipoprotein lipase, cytotoxic substances, plasminogen activators, and substances that regulate LDL metabolism.1–3,10 Thus, the present study suggests that tranilast may suppress the activation of T cells and macrophages in atherosclerotic plaques, possibly leading to the inhibition of production/secretion of cytokines promoting atherosclerosis from these cells. This may explain the suppressive effects of tranilast on the atheromatous formation in WHHL rabbits.

It has been shown that IFN-γ secreted from the activated T cells in turn stimulates MHC class II expression in macrophages,18,20 and IL-2 from the T cells stimulates IL-2 receptor expression in T cells themselves in an autocrine manner.2,3,20 In this context, the present study further showed that tranilast suppressed both IFN-γ–induced expression of MHC class II in the isolated macrophages and IL-2–induced expression of IL-2 receptor in the isolated T cells in in vitro experiments, using concentrations of tranilast comparable to the plasma level in WHHL rabbits that were treated with the oral dose of 300 mg · kg body wt⁻¹ · d⁻¹ used in the present study. These results indicate that the inhibitory effects of tranilast on the actions of IFN-γ and IL-2 may contribute to the lower frequencies of macrophages with MHC class II antigen and of T cells with IL-2 receptor in atheromatous plaques,21 and IL-2 receptor is also expressed in certain macrophages and B cells.11 However, MHC class II antigen in smooth muscle cells and IL-2 receptor in macrophages were rarely shown in the intimal plaque area in the present study.

Various mechanisms of these suppressions with tranilast can be considered. A variety of cellular responses need...
elevation of $[\text{Ca}^{2+}]$, in the isolated macrophage, and a rise in $[\text{Ca}^{2+}]$, has been implicated to have a regulatory role in various functions in macrophages.\textsuperscript{22} Considering that tranilast suppressed the elevation of $[\text{Ca}^{2+}]$, in response to FMLP in macrophages, as shown in the present study, tranilast may suppress MHC class II expression in macrophages partially through the mechanism mediated by inhibition of intracellular calcium mobilization.

Most RAM11-positive macrophages and CD5-positive T cells in the atherosclerotic intima are derived from circulating monocytes and T cells. The recruitment of these cells has been shown to involve redox-sensitive mechanisms.\textsuperscript{23} In this sense, lack of antioxidant effect in tranilast may possibly cause failure to inhibit the recruitment of these cells into the arterial intima on the basis of the present finding of comparable numbers of these cells in the plaques between tranilast- and placebo-treated rabbits. Thus, tranilast is unlikely to exert nonspecific inhibition of all functions of macrophages and T cells, but it might inhibit their interactions and the subsequent activation of these cells only after their recruitment into the arterial intima. From this point of view, the present study strongly suggests that immune activation of monocytes/macrophages and T cells after their recruitment into the arterial intima may play an important role in foam cell formation.

In conclusion, tranilast merits further investigation as an agent that may be therapeutically useful for suppression of atherosclerotic development.

Acknowledgments

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References

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