Expression of G2A, a Receptor for Lysophosphatidylcholine, by Macrophages in Murine, Rabbit, and Human Atherosclerotic Plaques

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Objective—Lysophosphatidylcholine (LPC), a major phospholipid component of oxidized low density lipoprotein, has been demonstrated to induce multiple functional alterations of vasculature that are potentially involved in atherosclerosis. Recently, an orphan G-protein–coupled receptor, G2A, has been identified as a high-affinity receptor for LPC. Although it has been demonstrated that G2A is expressed predominantly in lymphoid tissues and lymphocytes, there are no reports to determine whether G2A is expressed in atherosclerotic lesions and cardiovascular cells.

Methods and Results—Immunohistochemistry with an anti-G2A antibody revealed that G2A was expressed predominantly by macrophages within atherosclerotic lesions at the aortic root of apolipoprotein E–deficient mice and the thoracic aortas of Watanabe heritable hyperlipidemic rabbits. In atherosclerotic plaques of human coronary arterial specimens, G2A was expressed by macrophages within the lipid-rich plaques, whereas no immunoreactivity of G2A was observed in fibrous plaques where macrophages did not exist. Reverse transcription–polymerase chain reaction analysis demonstrated that G2A mRNA was highly expressed in human and murine monocytes/macrophages. The expression of G2A protein was detected in human and murine monocytes/macrophages by immunoblotting.

Conclusions—These findings demonstrate that monocytes/macrophages abundantly express G2A and suggest that G2A may play a role in the formation and progression of atherosclerotic lesions. (Arterioscler Thromb Vasc Biol. 2002;22: 2049-2053.)

Key Words: atherosclerosis ▪ lipids ▪ plaque ▪ receptors

Oxidized LDL has been implicated in vascular diseases, including atherosclerosis.1 Lysophosphatidylcholine (LPC), a phospholipid component of oxidized LDL, has been demonstrated to induce multiple functional alterations of the vasculature that are potentially involved in atherosclerosis. We and other groups have reported that LPC inhibits endothelium-dependent vasorelaxation, upregulates genes encoding vasoactive substances, growth factors, and adhesion molecules, and regulates cell migration and proliferation.2–6 LPC is known to increase intracellular calcium concentration and activate the divergent intracellular signal cascades.7–9 In contrast to the accumulating evidence regarding multiple receptors for oxidized LDL in the cardiovascular system, little is known about receptors for LPC. Although some actions of LPC are suggested to be mediated through G-protein–coupled receptors (GPCRs), a GPCR specific for LPC has not yet been identified.

Recently, G2A, an orphan GPCR, has been identified as a high-affinity receptor for LPC.10 Previous studies revealed that G2A was expressed predominantly in lymphoid tissues and cells.11 Little is known about the expression of G2A in vascular cells, including monocytes/macrophages, smooth muscle cells, and endothelial cells, and no studies on the expression of G2A in atherosclerotic lesions have been reported. Resolving these issues is important for elucidating the, as yet, completely unknown function of G2A.

In the present study, we performed immunohistochemistry by using an anti-G2A antibody to investigate whether or not G2A was expressed in atherosclerotic lesions of apoE-deficient mice and Watanabe heritable hyperlipidemic (WHHL) rabbits. In both animal models, infiltrating macrophages within atherosclerotic lesions abundantly expressed G2A. Interestingly, in human coronary arteries, G2A staining was observed in lipid-rich plaques, whereas none of the fibrous plaques showed G2A immunoreactivity. We also examined whether or not the cardiovascular cells express G2A and demonstrated that mRNA and protein of G2A are highly expressed in human and murine monocytes/macrophages. These results suggest the possible involvement of G2A in the formation and progression of atherosclerotic lesions.
Methods

Immunohistochemistry

Aortic roots and thoracic aortas were isolated from apoE-deficient mice (n = 5) fed a western-type diet for 8 weeks and homozygous WHHL rabbits (n = 2) aged 3 months, respectively. All procedures were conducted according to the Guidelines for Animal Experimentation at Kobe University School of Medicine. Human coronary arteries were obtained from 19 autopsy cases (aged 29 to 92 years) after informed consent was given by their families. Segments were fixed in 10% neutral buffered formalin and then frozen in OCT compound (Tissue-Tek). Serial cryostat sections (6 μm thick) were preincubated with 0.5% hydrogen peroxide in methanol. The sections were blocked with carrier protein (DAKO) and subsequently incubated with goat polyclonal anti-G2A antibody (Santa Cruz, 1:50 dilution), rat monoclonal anti-mouse macrophage antibody (MOMA2, Biosource; 1:20 dilution), mouse monoclonal anti-rabbit macrophage antibody (RAM11, DAKO; 1:30 dilution), mouse monoclonal anti-human CD68 antibody (KP-1, DAKO; 1:100 dilution), or mouse monoclonal anti-human smooth muscle α-actin antibody (1A4, Sigma Chemical Co; 1:400 dilution). After they were washed with PBS, the sections were incubated with biotinylated antibody (1A4, Sigma Chemical Co; 1:400 dilution). After they were preincubated with 0.5% hydrogen peroxide in methanol. The sections were blocked with carrier protein (DAKO) and subsequently incubated with goat polyclonal anti-G2A antibody (Santa Cruz, 1:50 dilution), rat monoclonal anti-mouse macrophage antibody (MOMA2, Biosource; 1:20 dilution), mouse monoclonal anti-rabbit macrophage antibody (RAM11, DAKO; 1:30 dilution). After color development with 0.03% diaminobenzidine, the sections were counterstained with hematoxylin.

Double-Labeling Immunofluorescence

The sections of human coronary arteries were incubated with anti-G2A antibody and anti-human CD68 antibody, followed by incubation with Texas red–conjugated swine anti-goat IgG (EY Laboratories) and FITC-conjugated sheep anti-mouse immunoglobulins (Amersham). The samples were examined by a laser scanning confocal imaging system (MRC-1024, Bio-Rad Laboratories).

Cell Culture

Human monocyte–derived macrophages were prepared as previously described.13 Peripheral blood mononuclear cells were isolated from whole blood of normal human subjects by Ficoll-Paque density gradient centrifugation. After they were washed with PBS, the cells were plated in 35-mm dishes and allowed to adhere for 2 hours in RPMI 1640 medium (GIBCO-BRL) supplemented with 10% heat-inactivated human serum. After nonadherent cells were discarded, premature macrophages were further incubated for 7 days in RPMI 1640 medium containing 10% human serum to differentiate into mature macrophages. The medium was replaced every 3 days. Mouse peritoneal macrophages were collected by peritoneal lavage with PBS from mice given an intraperitoneal injection of 4% thiglycollate in PBS. The cells were cultured in RPMI 1640 medium containing 10% FBS. A human monocytic cell line (THP-1), a human T-cell line (Jurkat T cells), and a murine monocyte/macrophage cell line (J774.A.1) were obtained from the American Type Culture Collection and cultured in RPMI 1640 medium containing 10% FBS. Human coronary artery smooth muscle cells and human umbilical vein endothelial cells were purchased from Clonetics.

Reverse Transcription–Polymerase Chain Reaction

G2A mRNA expression was analyzed by using reverse transcription (RT)–polymerase chain reaction (PCR) as described previously.9 In brief, the first-strand cDNAs were synthesized from total mRNA isolated from cultured cells with the use of the manufacturer’s kit (RETRoscript, Ambron). Aliquots of cDNA were amplified with Ex-Taq DNA polymerase (Takara) with the use of human G2A-specific primers, 5′-CAG TGG TTG TCA TCT TCC TA-3′ (forward) and 5′-TTA GCC GGC GCC GCT CAG CAG GAC TCC TCA ATC AG-3′ (reverse), or mouse G2A-specific primers, 5′-TAG CGG TCG CAG GAA ATG CAG-3′ (forward) and 5′-CAG GAC TGG CTT GGG GCT TTA TT-3′ (reverse).11 Primers for the amplification of GAPDH, 5′-ACC ACA GTC CAT GCC ATC AC-3′ (forward) and 5′-TCC ACC CTG TCA TT-3′ (reverse), were used as an internal control. PCR products were analyzed on 1.5% agarose gel containing ethidium bromide and photographed.

Immunoblotting

Cells were lysed in a buffer (50 mmol/L Tris/HCl [pH 7.4], 50 mmol/L NaCl, 5 mmol/L EDTA, 0.05% Triton X-100, 0.5% NP-40, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 10 mmol/L NaF, and 1 mmol/L phenylmethylsulfonyl fluoride). Immunoblotting was performed as described by using an anti-G2A antibody (1:500 dilution).

Results

Expression of G2A in Atherosclerotic Lesions of ApoE-Deficient Mice and WHHL Rabbits

Immunohistochemistry with a specific anti-G2A antibody was used to examine the G2A expression in atherosclerotic lesions. Accumulating lipids within atherosclerotic plaques in
the aortic root of apoE-deficient mice were stained with Sudan III (Figure 1A). Foam cell lesions within atherosclerotic plaques were positive for G2A (Figure 1B). When the primary antibody was replaced with a nonimmune goat IgG, no reactivity was detected (Figure 1C). Immunohistochemistry using cell-specific antibodies revealed that the majority of the G2A-expressing cells were positive for anti-mouse macrophage antibody (Figure 1D) but not for anti-human smooth muscle α-actin antibody (Figure 1E). Consistently, macrophages, which infiltrated into the subendothelial space, expressed G2A in atherosclerotic lesions at the aortas of WHHL rabbits (Figure 2A through 2C). Immunoreactivity of G2A was not detected in nonatherosclerotic lesions (D). Sections shown are representative of atherosclerotic lesions from WHHL rabbits (n=2). Original magnification ×200.

Expression of G2A in Human Coronary Atherosclerotic Plaques

We next examined the G2A expression in human coronary arteries. In lipid-rich plaques, G2A expression was observed around the lipid core and at the shoulder region (Figure 3A and 3E), where it colocalized with macrophage immunoreactivity (Figure 3C and 3G), but the expression pattern of G2A did not overlap the expression of smooth muscle α-actin antibody (Figure 1D) but not for anti-human smooth muscle α-actin antibody (Figure 1E). Consistently, macrophages, which infiltrated into the subendothelial space, expressed G2A in atherosclerotic lesions at the aortas of WHHL rabbits (Figure 2A through 2C). Immunoreactivity of G2A was not detected in nonatherosclerotic lesions (D). Sections shown are representative of atherosclerotic lesions from WHHL rabbits (n=2). Original magnification ×200.

Expression of G2A in Cardiovascular Cells

Human monocytic THP-1 and monocyte-derived macrophages express G2A mRNA expression (please see online Figure 1A, available at http://atvb.ahajournals.org). Weak mRNA expression of G2A was observed in Jurkat T cells, human coronary artery smooth muscle cells, and human umbilical vein endothelial cells. Murine macrophage J774A.1 as well as thioglycollate-elicited peritoneal macrophages abundantly expressed G2A mRNA, whereas no significant expression was detected in murine endothelial cells (PY4.1) and fibroblasts (NIH 3T3) or in rat aortic smooth muscle cells (please see online Figure 1B). Although rat G2A has not yet been cloned, the present RT-PCR analysis using murine primers was likely able to detect rat G2A cDNA, because a single band with the same molecular size was detected in the RT-PCR product of rat spleen cells.

A high level of the G2A protein was detected in human and murine monocytes/macrophages, whereas no significant staining was detectable in other tested cells, indicating the higher expression level of G2A protein in monocytes and macrophages (please see online Figure II, available at http://atvb.ahajournals.org). These results clearly demonstrated that G2A is abundantly expressed in monocytes/macrophages.

Discussion

Although it has been reported that G2A is expressed predominantly in T and B lymphocytes,11 little is known about the expression of G2A in cardiovascular cells. In the present study, we demonstrated that G2A was highly expressed in cultured human and murine monocytic cell lines and macrophages. The potential physiological role of G2A in these cells remains unclear. G2A was originally identified as a Bcl-Ab1-inducible gene.11 Ectopic expression of G2A antagonizes the transformation of Rat-1 fibroblasts by Bcl-Ab1 and results in the accumulation of cells within G2/M. By contrast, another group independently isolated G2A and showed that ectopic expression of G2A induces oncogenic transformation of NIH 3T3 fibroblasts.14 Thus, whether G2A may promote or antagonize cell growth may depend on the presence of other signals and/or cell-type differences. Indeed, it has been
reported that LPC has biphasic effects on cell growth, i.e., a stimulatory effect for macrophages and vascular smooth muscle cells and an inhibitory effect for endothelial cells, but it remains to be determined whether G2A may exhibit a growth-promoting or growth-antagonizing function in the vascular cells.

As reported previously, binding of LPC with G2A can induce calcium mobilization and mitogen-activated protein kinase activation via a pertussis toxin-sensitive pathway. Oxidized LDL is known to increase intracellular calcium concentration and activate mitogen-activated protein kinase. It was interesting to discover that these effects of oxidized LDL are mediated by LPC via G2A. However, it remains unknown whether G2A can recognize LPC associated with oxidized LDL. Besides monocytes/macrophages, vascular endothelial and smooth muscle cells play an important role in atherogenesis. However, the expression of G2A was not detected in these cells. Another GPCR, GPR4, has been also identified as a receptor for LPC. It is possible that these GPR4 and/or other unidentified receptors may be expressed by vascular endothelial and smooth muscle cells. Further investigations are necessary to solve these issues.

Immunohistochemistry revealed that G2A is highly expressed in infiltrating macrophages within atherosclerotic lesions. The pathological role of G2A in atherosclerotic lesions remains unknown. It is suggested that G2A may play a role as a sensor of LPC levels at sites of inflammation, thus limiting the expansion of tissue-infiltrating cells and the progression to overt autoimmune disease, inasmuch as it has been reported that T lymphocytes from G2A-deficient mice exhibit hyperproliferative responses to antigen receptors that are cross-linked with stimulation. As for monocytes/macrophages, it has been demonstrated that LPC is chemotactic for monocytes, stimulates monocyte production of heparin-binding epidermal growth factor-like growth factor, a potent mitogen for smooth muscle cells, and increases DNA synthesis in macrophages. Therefore, we speculate that LPC might induce the recruitment of monocytes and stimulate the proliferation of macrophages and smooth muscle cells through G2A in atherosclerotic lesions, resulting in the progression of atherosclerotic lesion formation. Thus, to specifically interrupt G2A activation by LPC might develop into a novel therapeutic strategy to alter the development of atherosclerotic lesions.
References


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Figure 1. Expression of G2A mRNA in cultured cardiovascular cells.

Expression of human (A) or murine (B) G2A mRNA was analyzed by RT-PCR analysis. cDNAs were synthesized from total mRNA isolated from Jurkat T cells, THP-1, monocyte-derived macrophages, human coronary artery smooth muscle cells (HCASMC), and human umbilical vein endothelial cells (HUVEC), J774A.1, thioglycollate-elicited peritoneal macrophages, PY4.1, NIH 3T3, rat aortic smooth muscle cells (RASMC), and rat spleen, and then amplified using specific primers for human or murine G2A, or GAPDH. RT: reverse transcription. The results are representative of at least three experiments and identical results were obtained.
Figure II. Expression of G2A protein in cultured cardiovascular cells.

Expression of G2A protein was analyzed by immunoblot analysis using a specific G2A antibody. Whole cell lysates from cultured cells in which G2A mRNA expression was determined were subjected. The results are representative of at least three experiments and identical results were obtained.