A product of growth arrest-specific gene 6 modulates scavenger receptor expression in human vascular smooth muscle cells

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Abstract Although Gas6 is identified as a growth factor for vascular smooth muscle cells (VSMCs), its roles in these cells have not been clearly elucidated. To examine the role of Gas6 in atherosclerosis, we examined the effects of Gas6 on scavenger receptor family expression in VSMCs. Scavenger receptor class A, one of the scavenger receptor family members, was upregulated in VSMCs by Gas6. Furthermore, the atherogenic lipoprotein, oxidized LDL, induced Gas6 production in these cells. These results indicate that Gas6 plays an important role in foam cell formation in human VSMCs.

Key words: Growth arrest-specific gene 6; Scavenger receptor; Vascular smooth muscle cell

1. Introduction

Gas6, the product of growth arrest-specific gene 6, potentiates the proliferation and prevents the cell death of vascular smooth muscle cells (VSMCs) [1,2]. Gas6 was originally cloned as one of the growth arrest-specific genes isolated by subtractive hybridization on the basis of preferential expression in the G_0 phase of the cell cycle [3]. It has been demonstrated that Gas6 is a ligand of receptor tyrosine kinases, Axl [4]. Recent reports indicated that Gas6 promotes Axl-mediated adhesion, suggesting adhesion as one function for the Gas6-Axl interaction [5], and Gas6 can act as a chemoattractant for migration in both rat and human primary VSMCs [6]. However, Gas6's biological activity is still poorly understood.

During atherogenesis, VSMCs migrate from the media to the intima of the arterial wall, where they proliferate and accumulate lipid, becoming foam cells. Although the factors contributing to the regulation of smooth muscle cell scavenger receptor activity in vivo are unknown, several cytokines are reported to regulate the activity of scavenger receptor in VSMCs [7,8]. Thus, these cytokines are presumed to play important roles in the phenotypic change of VSMCs acting in an autocrine or paracrine manner [9].

It has been proposed that modified LDL is the atherogenic ligand for scavenger receptor on macrophages and smooth muscle cells that causes the massive accumulation of lipid and foam cell formation [10]. The uptake of oxidized low density lipoprotein (OxLDL) by macrophages is a key event implicated in the initiation and development of atherosclerotic lesions. Several macrophage surface receptors, CD36 (a class B scavenger receptor) [11], CD68 [12] and the macrophage scavenger receptor (a class A scavenger receptor; SRA) [13], have been identified as the major receptors that bind and internalize OxLDL. Scavenger receptor is detected in VSMCs in the intima but not in normal VSMCs, demonstrating that smooth muscle cell scavenger receptor activity is upregulated in atherosclerotic lesions [7,8].

In this study, the regulation of scavenger receptor expression in VSMCs was investigated using an established human vascular smooth muscle cell line.

2. Materials and methods

2.1. Materials

Interferon-γ (IFN-γ) and interleukin-1β (IL-1β) were obtained from Research Biochemicals International (Natick, MA, USA). Phorbol 12-myristate 13-acetate (PMA) was purchased from Sigma (St. Louis, MO, USA). OxLDL was prepared as described previously [12]. All other reagents were of analytical grade.

2.2. Cell culture

A human vascular smooth muscle cell line, ISS10, was established as described previously [14]. ISS10 cells were plated in DMEM medium (Gibco BRL, Osaka, Japan) supplemented with 10% fetal calf serum, 100 μg/ml streptomycin and 100 U/ml penicillin in a humidified atmosphere containing 5% CO_2.

2.3. Preparation of recombinant human Gas6 and anti-human Gas6 antibody

CHO cells were transfected with human Gas6 expression plasmid. Confluent cells were cultured in protein-free culture medium, PM-1000 (Eiken, Tokyo, Japan), in the presence of 4 μM vitamin K2. Recombinant human Gas6 was purified from the culture medium as described elsewhere [1]. Rabbits were immunized with recombinant human Gas6. Anti-human Gas6 IgG was purified using the Ampure PA kit (Amersham, Arlington Heights, IL, USA).

2.4. RNA isolation and RT-PCR analysis

Total RNA was isolated from ISS10 cells by single-step acid guanidinium thiocyanate-phenol-chloroform extraction [18]. Axl and SRA expression was determined by PCR analysis of the reverse transcribed RNA as described previously [15]. A primer pair was designed as matching the published sequence of Axl [16] (sense: 5′-GGTGGCTGTGAAGACGATGA-3′ and antisense: 5′-CTCAGATA-

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Abbreviations: FBS, fetal bovine serum; RT-PCR, reverse transcription polymerase chain reaction; VSMCs, vascular smooth muscle cells; SRA, class A scavenger receptor; OxLDL, oxidized low density lipoprotein; Gas6, product of growth arrest-specific gene 6; DiI-AcLDL, 1,1′-dioctadecyl-1-13,3,3′3′-tetramethyl-indo-carbocyanine perchlorate-labeled acetyl-LDL; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PMA, phorbol 12-myristate 13-acetate

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CTCCATGCCACT-3′) and SR-A [13]; (5′ common type I and type II sense primer: 5′-GGGAACATTCTCAGACCTTGAG-3′, 3′ type I-specific antisense primer: 5′-TGTCCATAGTGAGCTGCCTTGT-3′, and 3′ type II-specific primer: 5′-TGCCCTAATATGATCAGT-GAGTTG-3′). As a control, β-actin was amplified and analyzed under identical conditions using an appropriate set of primers.

2.5. Proliferation assay

ISS10 cells (10^5) were cultured in 96-well plates in 200 μl of DMEM complete medium. Four hours before harvesting, cells were pulsed with 1 μCi/well [^{3}H]TdR (specific activity = 6.7Ci/mmol, DuPont Company, Wilmington, DE, USA) and then harvested onto glass fiber filters using an automated cell harvester and counted in a Packard liquid scintillation counter (Hewlett-Packard Co., Palo Alto, CA, USA). The data are presented as the mean cpm of triplicate cultures ± S.E.M.

2.6. Western blot analysis

ISS10 cells were washed and scraped in phosphate-buffered saline (PBS) and lysed as described previously [17]. The proteins were fractionated by size on 7.5% SDS-polyacrylamide gels, and transferred to polyvinylidene difluoride membranes for immunoblotting. The membranes were incubated with 0.1% Tween 20 in PBS (PBS-T) containing anti-SRA antibody (diluted 1/3000 from whole antiserum), anti-cyclophilin A, or anti-Gas6 antibody. The membranes were then washed with PBS-T, and incubated for 1 h at room temperature in PBS-T containing horseradish peroxidase-linked second antibodies. The antibody binding was visualized by chemiluminescence detection (ECL, Amersham Corp., Arlington Heights, IL, USA).

2.7. Uptake of AcLDL in ISS10 cells

1,1′-Dioctadecyl-1-13,3,3′3′-tetramethyl-indo-carbocyanine perchlorate-labeled acetyl-LDL (DiI-AcLDL; Biomedical Technologies Inc., Stoughton, MA, USA) was added to the culture medium at a final concentration of 10 μg/ml. The cells were incubated for 4 h at 37°C, washed with PBS, and then fixed and analyzed using a fluorescence microscope.

2.8. Statistical analysis

Statistical comparisons were made by one-way analysis of variance and Student's *t*-test, with $P < 0.05$ considered significant.

3. Results

3.1. Gene expression of the receptor for Gas6 and effects of Gas6 on smooth muscle cell line growth

Gas6 is a common ligand for Axl, one of the members of the Axl/Sky receptor family [4]. To examine Axl gene expression in a vascular smooth muscle cell line, we employed the reverse-transcribed PCR analysis with mRNA from ISS10 cells. Although a previous study reported that Axl was upregulated in a human leukemia cell line, K562 treated with PMA [16], Axl mRNA was detected in ISS10 cells at the same level found in K562 treated with PMA (Fig. 1A). When ISS10 cells were stimulated for 24 h in the presence of Gas6 at given concentrations, their proliferative response to Gas6 was increased in a dose-dependent manner (Fig. 1B).

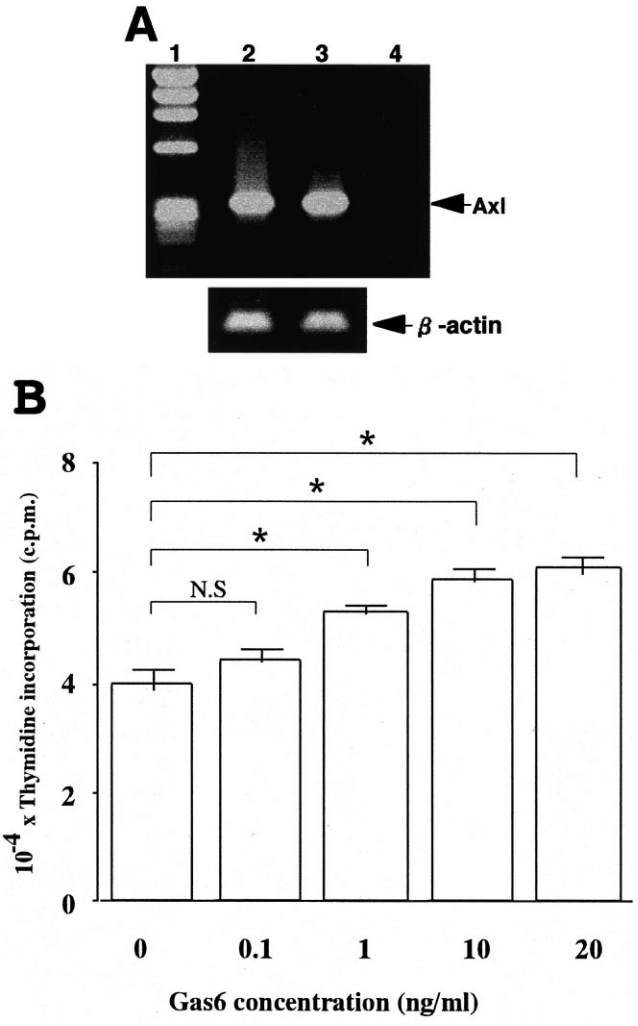

Fig. 1. A: Axl gene expression in ISS10 cells. Total RNA was isolated from ISS10 cells and K562 treated with PMA, then RT-PCR was performed as described in Section 2. β-Actin was amplified as an internal control. Lane 1, DNA size marker (ΦX174); lane 2, ISS10 cells; lane 3, K562 treated with PMA. An identical experiment independently performed gave similar results. B: Gas6-induced DNA synthesis in ISS10 cells. In 24-well plates, confluent ISS10 cells were rendered quiescent after incubation in DMEM for 24 h. ISS10 cells were stimulated with the indicated amount of Gas6 for 24 h, and then the cells were pulsed with [^{3}H]thymidine for 4 h. The data are presented as the mean cpm of triplicate cultures ± S.E.M.

3.2. Effects of Gas6 on scavenger receptor expression in human smooth muscle cells

We analyzed the effects of Gas6 on scavenger receptor expression in ISS10 cells with a specific antibody against human SRA. Fig. 2A shows that Gas6 stimulated SRA expression in ISS10. Positive controls (IFN-γ) also increased SR-A expression in ISS10 (Fig. 2). To determine whether enhanced SRA expression by Gas6 depends on increased levels of SRA mRNA, RT-PCR analyses were performed. ISS10 cells were incubated with 10 ng/ml of Gas6 for 24 h, and total RNA was extracted and subjected to RT-PCR analyses. As shown in Fig. 2B, Gas6 treatment at 10 ng/ml for 24 h increased the SRA mRNA level. In contrast, Gas6 had no effect on the expression of other scavenger receptors including CD36 and CD68 in ISS10 cells (data not shown).

3.3. Effects of Gas6 on uptake of AcLDL

To determine whether the upregulated expression of SRA by Gas6 is correlated with an enhanced uptake of AcLDL, amounts of DiI-AcLDL internalized into ISS10 were measured. After treatment with Gas6 for 24 h, confluent monolayers of ISS10 were incubated with DiI-AcLDL. Fluorescence microscopy was carried out to evaluate the cellular uptake of DiI-AcLDL. In Gas6-treated ISS10, significant amounts of DiI-labeled AcLDL were internalized (Fig. 3).

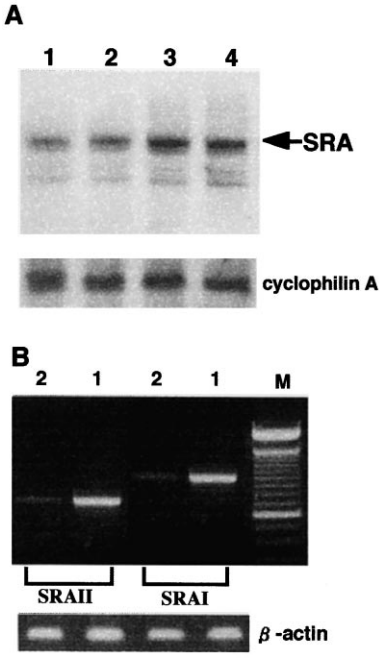

Fig. 2. Effect of Gas6 on SRA expression in ISS10 cells. A: Western blot analysis. Total cell protein extracted with 1% NP40 from untreated ISS10 cells (lanes 1 and 2) and ISS10 cells treated for 24 h with IL-1β, IFN-γ, or Gas6 was separated by SDS-PAGE, transferred to a nitrocellulose membrane and blotted with anti-SRA antibody. Each lane contained 10 μg of protein. Lane 1, control; lane 2, IL-1β; lane 3; INF-γ; lane 4, Gas6. An identical experiment independently performed gave similar results. B: RT-PCR analysis of SRA mRNA expression. Total RNA was isolated from ISS10 cells with or without Gas6 and RT-PCR was performed as described in Section 2. β-Actin was amplified as an internal control. Lane M, DNA size marker (ΦX174); lane 1, Gas6-treated ISS10; lane 2, untreated ISS10. An identical experiment independently performed gave similar results.

These results demonstrate that increased SRA expression by Gas6 was associated with enhanced AcLDL uptake in ISS10.

3.4. OxLDL induced Gas6 expression

A previous report indicated that human VSMCs express Gas6 [1], and we have confirmed that ISS10 also expressed Gas6 by Western blot analysis. It was recently reported that modified LDL enhances SRA expression [18]. To examine the effects of the modified form of LDL on Gas6 expression in ISS10 cells, we incubated ISS10 cells with various concentrations of OxLDL. As shown in Fig. 4A, OxLDL stimulated Gas6 expression in a dose-dependent manner in ISS10 cells. In contrast, cyclophilin A expression was not affected by OxLDL treatment in ISS10 cells. Gas6 was maximally induced by OxLDL after 12 h incubation with OxLDL. On the other hand, OxLDL induced SRA expression in ISS10 cells after 24 h incubation (Fig. 4B).

4. Discussion

In the current study, we have demonstrated an increased expression of SRA, a receptor for OxLDL, in a human smooth muscle cell line after Gas6 treatment. We also revealed that the increase in type I and II scavenger receptor mRNA expression is correlated with an increase in scavenger receptor activity in human smooth muscle cells by Gas6 treatment. Furthermore, we have shown that the expression of Gas6 was increased in the presence of OxLDL in ISS10. Our results suggest important interactions between Gas6-Axl and SRA, which is the receptor for OxLDL. Thus, the future characterization of Gas6-Axl signaling should yield important discoveries regarding the mechanisms underlying the foam cell formation of human VSMCs.

Whereas the scavenger receptor activity in differentiated macrophages can be regulated in a narrow range by various growth factors and cytokines, that in VSMCs is regulated over a wide range [9]. The regulation of scavenger receptor activity in VSMCs and macrophages, however, is fundamentally different. In macrophages, scavenger receptor expression is stimulated constitutively, and there is a high level of receptor activity. In smooth muscle cells, scavenger receptor expression is weak in the absence of stimulation [7,8].

Li et al. [8] demonstrated that after balloon injury, the aorta smooth muscle cells in the neointima of hypercholesterolemic rabbits express scavenger receptors, whereas smooth muscle cells in the media do not. These results clearly demonstrate that scavenger receptor expression is upregulated in smooth muscle cells in atherosclerotic lesions. The factors leading to scavenger receptor regulation in VSMCs in vivo are unknown. However, Melaragno et al. reported that the expression of Axl is increased in neointima VSMCs of rat carotid arteries after balloon injury and that Axl expression is selectively regulated by G protein-coupled receptor agonists in vitro [19]. Wu et al. [20] have shown that scavenger receptor expression in human monocyte-derived macrophages is regulated via a signal transduction pathway involving ras, AP1 (c-jun and junB), and ets domain proteins. In the present

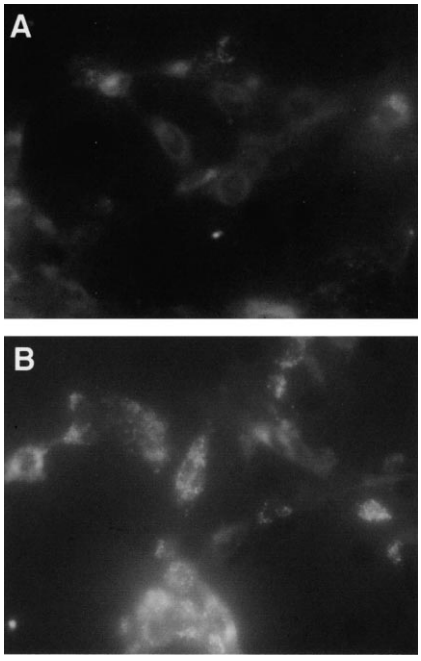

Fig. 3. Effect of Gas6 on uptake of DiI-AcLDL in ISS10 cells. On day 1, ISS10 cells were plated in complete medium. On day 2, the monolayers were incubated without (A) or with 10 ng/ml Gas6 (B) for 24 h at 37°C. On day 3, the subconfluent cells were re-fed with the medium containing DiI-AcLDL (1 μg of protein/ml) and incubated for 4 h at 37°C. The cells were photographed with a fluorescence microscope with a rhodamine filter package. An identical experiment independently performed gave similar results.

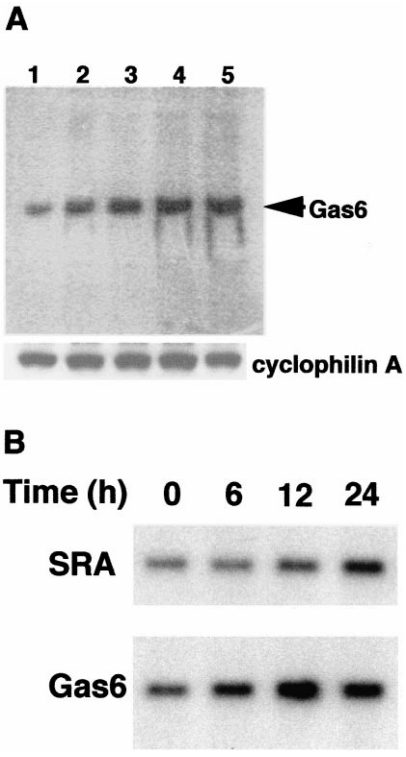

Fig. 4. Effect of OxLDL on Gas6 expression in ISS10 cells. ISS10 cell monolayers were exposed to OxLDL at different concentrations or for different periods of time and cell lysates were analyzed by SDS-PAGE and Western blotting with anti Gas6, anti-SRA or anti-cyclophilin A antibody. A: Dose response of OxLDL on Gas6 expression. Cyclophilin A was used as an internal control. Lane 1, control; lane 2, OxLDL 5 μg/ml; lane 3, OxLDL 10 μg/ml; lane 4, OxLDL 20 μg/ml; lane 5, OxLDL 50 μg/ml. B: Western blot analysis was performed to determine the time course of SRA and Gas6 expression in ISS10 cells after 50 μg/ml OxLDL treatment. An identical experiment independently performed gave similar results.

study, we showed that Gas6 stimulated the expression of scavenger receptor in an Axl-expressing human smooth muscle cell line. Thus, Gas6 may play an important role in foam cell formation in atherosclerotic lesions.

Many studies also demonstrated that VSMCs express type I and II scavenger receptors that bind, internalize, and degrade modified LDL, suggesting that the scavenger receptor-mediated uptake of modified LDL by VSMCs also leads to lipid accumulation and foam cell formation in vivo as proposed in macrophages [7–9]. Growth factors secreted by cells in developing atherosclerotic lesions could stimulate scavenger receptor activity in VSMCs and contribute to lipid accumulation and foam cell formation [7]. OxLDL, one of the atherogenic lipoproteins, induces the expression of several genes including scavenger receptors [21]. The present study indicated that Gas6 induced SRA expression and the time-course study (Fig. 4B) showed that OxLDL stimulated Gas6 expression before the induction of SRA expression. Taken together, the results suggest that Gas6 induced by OxLDL stimulated SRA expression to take up OxLDL. Further investigation is needed to test this hypothesis.

These results, showing Gas6 upregulation of scavenger receptor expression in a human smooth muscle cell line, suggest that Gas6 may play an important role in foam cell formation in the progress of atherosclerosis.

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