Release of a product of growth arrest-specific gene 6 from rat platelets

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Abstract A product of growth arrest-specific gene 6 (Gas6) is known to be synthesized by growth-arrested cells. In this study, we found that several rat tissues including platelets contain Gas6 and activation of the platelets with thrombin provoked the release of Gas6. ADP and collagen, which as well as thrombin stimulated release of ATP from platelets, also enhanced the release of Gas6, suggesting that the mechanism of its release was similar to that of ATP release. This study provides the first evidence of growth arrest-independent secretion of Gas6 and suggests the involvement of Gas6 in vascular diseases as well as hemostasis.

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Key words: Growth arrest-specific gene 6; Platelet; Secretion; Thrombin; ADP; Collagen

1. Introduction

Growth arrest-specific gene 6 (*gas6*) was initially identified as a gene whose expression in fibroblasts increased during the growth-arrested state [1]. Thereafter, some biological activities of the product of *gas6* (Gas6) have been reported. One is stimulation of cell proliferation. We purified Gas6 as a protein which potentiated cell proliferation mediated by Ca^{2+}-mobilizing receptors [2]. Gas6 was also reported to stimulate proliferation of fibroblasts [3]. Another Gas6 activity is the prevention of cell death induced by serum deprivation [3,4]. These activities of Gas6 were similar to those of growth factors activating receptor tyrosine kinases. Indeed, it was clarified that Gas6 became bound to and stimulated receptor tyrosine kinases, Axl, Sky and Mer [5–8]. Therefore, the above biological activities are very likely to be mediated by activation of tyrosine kinases.

Gas6 has a homology to protein S, a negative regulator of blood coagulation, and is composed of three structurally distinct domains, the Gla domain, epidermal growth factor-like repeat and C-terminal domain [1]. The Gla domain is rich in γ-carboxyglutamic acid (Gla) residues and found in some blood coagulation factors. The function of the Gla domain in the coagulation factors is thought to be the mediation of their Ca^{2+}-dependent binding to negatively charged phospholipids. In a previous paper, we demonstrated that Gas6 is bound to a negatively charged phospholipid, phosphatidylserine (PS), and that Gas6 links receptor-expressing cells to PS-exposing surfaces [9]. Thus, Gas6 may function as a cell adhesion molecule in addition to a growth factor.

As described above, the production and secretion of Gas6 have been reported to be upregulated when cell proliferation is prohibited. In this study, however, we found that rat platelets, which do not proliferate, possess Gas6 and that activation of the platelets with thrombin, ADP or collagen can stimulate the release of Gas6 from them. This finding suggests that Gas6 is involved in vascular diseases such as atherosclerosis as well as hemostasis.

2. Materials and methods

2.1. Preparation of Gas6 and anti-rat Gas6 IgG

Recombinant rat Gas6 and anti-rat Gas6 were prepared as described previously [10].

2.2. Preparation of rat tissue samples and immunoblotting

Tissues of male Sprague–Dawley rats were homogenized in a sample buffer of sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (SDS–PAGE). Insoluble substances were removed by centrifugation and protein concentrations in the supernatants were measured. The proteins extracted from each tissue (10 μg/lane) were separated with SDS–PAGE and transferred onto polyvinylidene difluoride membranes. Gas6 on the membranes was visualized using anti-rat Gas6 IgG.

2.3. Preparation of rat platelets

Blood was drawn from the abdominal aorta of anesthetized male Sprague–Dawley rats into a syringe containing 3.8% trisodium citrate (1:9, v/v). The blood was centrifuged at $160\times g$ for 10 min at room temperature to obtain platelet-rich plasma (PRP). The PRP was overlaid on 0.5 ml of 40% bovine serum albumin (BSA) in a tube, and centrifuged at $1200\times g$ for 25 min at room temperature. The platelets were resuspended in the suspension buffer (140 mM NaCl, 2.7 mM KCl, 12 mM $NaHCO_3$, 0.4 mM NaH_2PO_4, 0.8 mM $MgCl_2$, 5 mM glucose, 10 mM HEPES, 1 mM $CaCl_2$, 0.1% BSA, pH 7.4) and purified with a column of Sepharose 2B. The platelets were suspended at 1×10^9/ml.

2.4. Stimulation of platelets

Thrombin (Sigma), ADP (Sigma), collagen (Sigma) or calcium ionophore A23187 (Sigma) was added to 400 μl of platelets, which were then incubated at 37°C. Next, PGE_1 (2.5 μg/ml) was added to stop the reactions. The samples were centrifuged at $1000\times g$ for 0.5 min at room temperature and Gas6 in the supernatant was analyzed by immunoblotting or enzyme-linked immunosorbent assay (ELISA).

2.5. Quantification of Gas6

ELISA plates (Corning) were coated with 100 μl/well of anti-rat Gas6 IgG dissolved in Tris-buffered saline (TBS) (10 mM Tris–HCl, pH 7.4, 150 mM NaCl). The plates were incubated for 18 h at 4°C and then blocked with 3% BSA in TBS for 2 h at room temperature. The wells were washed with TBS containing 0.05% Tween-20 and incubated with samples or Gas6. Gas6 bound to the wells was determined using biotinylated anti-rat Gas6 IgG and peroxidase-conjugated streptavidin (Zymed).

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Abbreviations: Gas6, growth arrest-specific gene 6; PS, phosphatidylserine; PAGE, polyacrylamide gel electrophoresis; PRP, platelet-rich plasma; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; TBS, Tris-buffered saline

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2.6. ATP measurement

Platelets and luminescence reagent (20 μl ATP Assay Mix; Sigma) were placed in a cuvette, and luminescence and platelet aggregation were measured simultaneously with an aggregometer (Whole Blood Aggregometer, Chrono-Log). ATP released from the platelets was quantified by addition of 2 nmol of ATP to the samples as an internal control at the end of the experiment.

3. Results and discussion

In order to investigate the tissue distribution of Gas6, we analyzed several rat tissues for Gas6 by immunoblotting using anti-rat Gas6 IgG. As shown in Fig. 1, Gas6 existed in several but not all rat tissues. Skin, lung, aorta, thymus, spleen, bone marrow and platelet contained significant amounts of Gas6. Gas6 has been reported to be synthesized and released when cell proliferation is inhibited. However, platelets do not possess the ability to proliferate. We were thus interested in whether the Gas6 in the platelets would be released on adequate stimulation.

Platelets release several substances such as ATP, platelet factors and platelet-derived growth factor upon stimulation with agonists like thrombin, ADP or collagen [11]. Thus, we examined the release of Gas6 from rat platelets stimulated with thrombin. As shown in Fig. 2, thrombin stimulation of the rat platelets increased the levels of Gas6 in the supernatant.

Fig. 3 shows the results of quantification of Gas6 released from rat platelets using ELISA. Fig. 3A demonstrates that Gas6 release was dependent on the concentration of thrombin, with half-maximal release occurring at approximately 0.8 U/ml thrombin. The time course of the release of Gas6 from the thrombin-stimulated rat platelets showed that the release was completed in 0.5 min (Fig. 3B).

As shown in Fig. 4A, other platelet agonists, ADP and collagen also induced secretion of Gas6 from rat platelets while the released Gas6 was much less than that released by thrombin stimulation. Fig. 4B shows the release of ATP from the platelets. The proportions of Gas6 release and ATP release were very similar, suggesting that both are mediated by common intracellular mechanisms. This was also supported by the finding that both Gas6 release and ATP release were similarly induced by calcium ionophore A23187, which activates platelets by increasing intracellular Ca^{2+} concentrations (Fig. 4A,B).

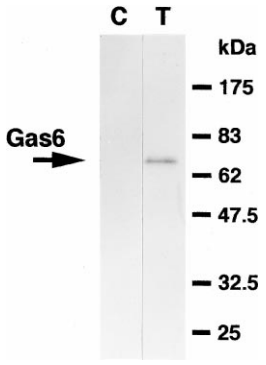

Fig. 2. Secretion of Gas6 from platelets stimulated by thrombin. Rat platelets, 400 μl, were incubated for 5 min at 37°C in the presence (T) or absence (C) of 5 U/ml thrombin. After the incubation, PGE_1 was added to stop the activation, then the sample was centrifuged. The supernatants were separated with SDS–PAGE and transferred onto polyvinylidene difluoride membranes. Gas6 on the membranes was visualized using anti-rat Gas6 IgG.

The production and secretion of Gas6 from cells has been reported to be enhanced when cell proliferation is arrested [1]. Although Gas6 may be produced by growth-arrested cells in order to inhibit growth cell death induced by the growth arrest [4,5], the true purpose of growth arrest-specific production of Gas6 has not been proved.

In this study, we first demonstrated that Gas6 is present in and is released from non-proliferation cells, i.e. platelets. It is known that the stimulation of platelets with many agonists induces release of several substances from the platelets, involving ATP, platelet factors or platelet-derived growth factor, which further enhance platelet aggregation, blood coagulation or proliferation of cells in the vessel walls [11]. The release of growth factors from the platelets is thought to be related to the progress of vascular diseases such as atherosclerosis and restenosis [12]. Since Gas6 stimulates cell proliferation of vascular smooth muscle cells in vitro [2], the Gas6 released from the stimulated platelets may also be involved in these diseases. Melaragno et al. reported the increased expression of Axl after vascular injury [13].

On the other hand, our previous study suggested that Gas6

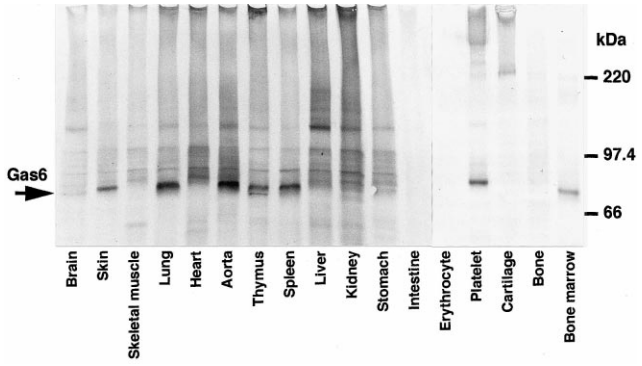

Fig. 1. Tissue distribution of Gas6. The proteins extracted from each rat tissues (10 μg/lane) were separated with SDS–PAGE and transferred onto polyvinylidene difluoride membranes. Gas6 on the membranes was visualized using anti-rat Gas6 IgG. The bands except Gas6 are non-specific signals which were observed even in the absence of first antibody.

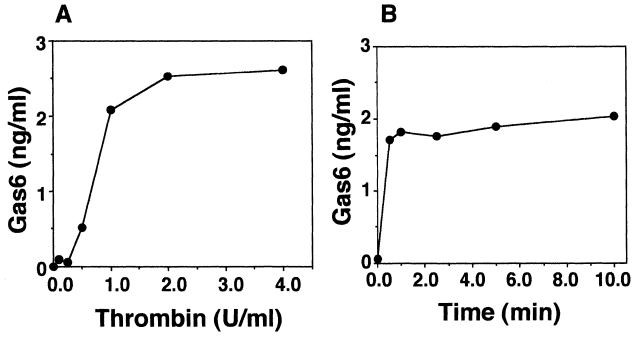

Fig. 3. Dose-dependency and time course of Gas6 secretion from platelets stimulated by thrombin. (A) The platelets were stimulated with various concentrations of thrombin for 5 min. (B) The platelets were stimulated with 5 U/ml thrombin for various periods. The Gas6 concentration in the supernatants of platelets stimulated by thrombin was quantified by ELISA. Data were means ± S.D. ($n = 3$).

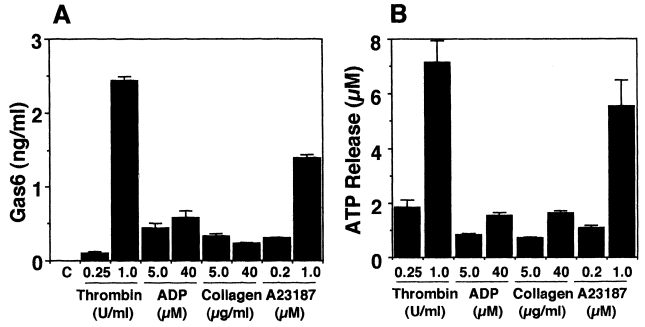

Fig. 4. Secretion of Gas6 (A) and ATP (B) from platelets stimulated by various stimulants. (A) The platelets were incubated for 5 min with thrombin, ADP, collagen, A23187 or none (C). The Gas6 concentration in the supernatants was quantified by ELISA. Data were means ± S.D. ($n = 3$). (B) Platelets and luminescence reagent (20 μl) were placed in a cuvette, and luminescence and platelet aggregation were measured simultaneously in an aggregometer. Maximum concentration of ATP released from the platelets was quantified by addition of 2 nmol of ATP to the samples as an internal control at the end of the experiment. Data were means ± S.D. ($n = 3$).

may function as an adhesion molecule which links the cells expressing Gas6 receptor and the cells expressing PS on the surfaces [9], while it has also been reported that Gas6 inhibited the adhesion of neutrophils to endothelial cells [14]. PS is mainly present only in the inner leaflet of the cell membranes [15]. However, PS emerges to the outer surface of the cell membranes upon platelet activation as well as apoptosis [16,17]. Therefore, a part of the Gas6 released from the platelets may bind to PS exposed on activated platelets, from which it is released. Gas6 bound to platelet surfaces may enhance coagulation by stimulating adhesion of the activated platelets to endothelial cells. Another possible role of platelet surface-associated Gas6 may be a marker which is recognized by macrophages that take up PS-expressing cells and inhibit excessive coagulation.

In conclusion, the findings described in this study suggest the involvement of Gas6 released from the platelets in vascular diseases such as atherosclerosis and intimal thickening, as well as in the regulation of hemostasis.

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