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Prevention of growth arrest-induced cell death of vascular smooth muscle cells by a product of growth arrest-specific gene, *gas6*

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Abstract We have purified Gas6 as a growth-potentiating factor for vascular smooth muscle cells (VSMCs) [Nakano, T. et al. (1995) J. Biol. Chem. 270, 5702–5705]. However, specific production of Gas6 in growth-arrested cells raises an intriguing question as to the physiological function of Gas6. In this study, we found that serum-starved VSMCs secreted some survival factors and depletion of the factors induced cell death of VSMCs. Finally, we demonstrated that cell death was prevented by the addition of Gas6, suggesting that one of the major biological activity of Gas6 is protection of growth-arrested VSMCs from death.

Key words: Vascular smooth muscle cell; Cell death; Gas6; Vitamin K; Growth factor; Survival factor

1. Introduction

In a previous paper, we demonstrated the isolation of a novel vascular smooth muscle cell (VSMC)-derived growth-potentiating factor from the culture medium of VSMCs [1]. The factor specifically potentiates proliferation of VSMCs stimulated with G-protein-coupled substances, such as thrombin, angiotensin II or lysophosphatidic acid, and has a specific, high affinity binding site on VSMC membranes [1]. Thus, it is suggested that the factor plays an important role in the proliferation of VSMCs induced by these growth-stimulating substances. Structural analysis of the growth-potentiating factor revealed that the factor was a γ-carboxyglutamic acid (Gla)-containing protein encoded by a growth arrest-specific gene, *gas6* [1,2]. Therefore, the growth-potentiating factor is referred to as Gas6. The *gas6* gene was 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]. Expression of *gas6* is up-regulated when cells are growth-arrested by serum deprivation and down-regulated upon mitogenic stimulation [2,3]. Recently, it has been reported that Gas6 is a ligand for a receptor tyrosine kinase Axl and its relative Sky [4-6]. Their genes were cloned on the basis of transforming activity or homology with other tyrosine kinases [7-10]. From these findings, it is expected that stimulation of cells with gas6 may activate Axl or Sky, resulting in phosphorylation of tyrosine residues of Axl and Sky, or other substrates, which may enhance the mitogenic response of cells.

However, there is an inconsistency in understanding the major biological function of Gas6 to be the growth-potentation of cells, since the production of Gas6 by cells is specifically induced during the growth-arrested state, when cell proliferation is not necessary [2,3]. In this paper, we report that Gas6 prevents growth arrest-induced cell death of VSMCs, demonstrating that prevention of cell death may be one of the important biological roles of Gas6.

2. Materials and methods

2.1. Materials

Rat Gas6 was purified from the culture medium of CHO cells transfected with rat Gas6 expression plasmid as described elsewhere [1]. Platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF) were purchased from Boehringer Mannheim, and heparin-binding EGF-like growth factor (HB-EGF) were from R&D Systems, Inc. Vitamin K2 was purchased from Sigma.

2.2. Culture of VSMCs

VSMCs isolated from thoracic aortas of male adult rats were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum. Cells at passage 6–10 were used for experiments.

2.3. Induction of cell death

Culture medium of confluent VSMCs in 24-well plates was replaced with DMEM containing 0.05% bovine serum albumin (BSA) and the cells were cultured for 2 days. To induce cell death, the culture medium was replaced again with fresh DMEM containing 0.05% BSA.

2.4. Monitoring of cell death

Cell survival was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, which measures mitochondrial dehydrogenase activity [11]. VSMCs were cultured for 4 h with 0.5 mg/ml MTT. Viable cells with active mitochondria produce dark blue formazan reaction product, whereas dead cells remain uncolored. The formazan product was dissolved with 10% SDS containing 5 mM HCl and quantified by measuring the absorbance at 570 nm. Lactate dehydrogenase (LDH) released into the culture medium was measured using an LDH assay kit (Kyokuto, Japan). Survival is expressed as the value at the second medium change to be 100%. LDH release was expressed as LDH released from intact VSMCs with 0.1% Triton X-100 to be 100%.

3. Results and discussion

Many types of cells in culture have been shown to die by deprivation of essential growth factors [12]. These include vascular endothelial cells deprived of FGF [13], mouse embryo cells after removal of epidermal growth factor [14], rat pheochromocytoma PC12 cell line and sympathetic neurons deprived of nerve growth factor [15], hormone-dependent cells of the breast or prostate deprived of steroids [16,17] and glial

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Abbreviations: VSMC, vascular smooth muscle cell; Gla, γ-carboxyglutamic acid; PDGF, platelet-derived growth factor; bFGF, basic fibroblast growth factor; EGF, epidermal growth factor; HB-EGF, heparin-binding EGF-like growth factor; DMEM, Dulbecco's modified Eagle's medium; BSA, bovine serum albumin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; LDH, lactate dehydrogenase

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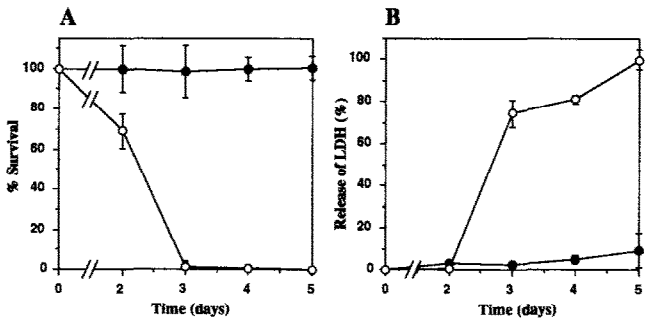

Fig. 1. Growth arrest-induced cell death of VSMCs. Confluent VSMCs were cultured for 2 days in DMEM containing 0.05% BSA, and at day 0, the medium was replaced again with fresh DMEM containing 0.05% BSA (open circles) or not replaced (closed circles). Survival of VSMCs (A) and release of LDH (B) were assayed as described in section 2. Error bars represent S.D. (n=3).

cells deprived of PDGF [18]. Therefore, serum-free culture medium without growth factors appears to be a cell death-favoring factor. However, as reported by Leszczynski et al. [19], normal VSMCs did not die for 5 days in serum-free medium (Fig. 1A). We then postulated that some autocrine survival factors might be released from VSMCs and prevent cell death. To confirm this hypothesis, we replaced the culture medium again with serum-free medium 2 days after the first medium change. In this condition, the cells started to die 2 days after the second medium change when monitored with the MTT assay (Fig. 1A). In parallel with the decrease of cell survival monitored with the MTT assay, release of LDH, one of the indicators of cell damage, was increased (Fig. 1B). These results demonstrated that some survival factors released from VSMCs during the first 2 days of serum starvation prevent cell death of VSMCs induced by serum-free, growth-arrested conditions.

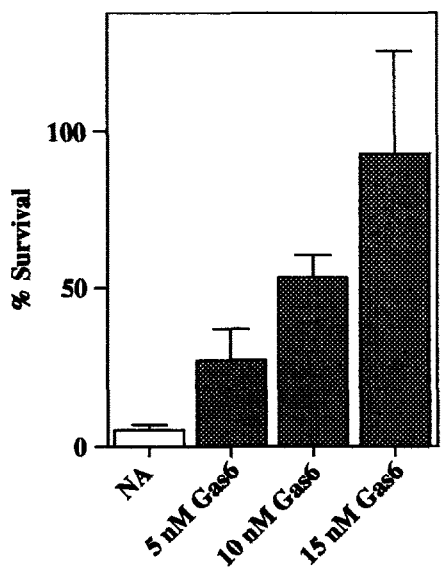

Fig. 2. Prevention of cell death of VSMCs by Gas6. Confluent VSMCs were cultured for 2 days in DMEM containing 0.05% BSA. Then, the medium was replaced again with fresh DMEM containing 0.05% BSA, and the cells were cultured in the presence or absence (NA) of various concentrations of Gas6 for a further 3 days. Survival of VSMCs was assayed as described in section 2. Error bars represent S.D. (n=3).

It was reported that in serum-free, growth-arrested conditions, production of Gas6 was dramatically enhanced [2,3]. We consequently hypothesized that Gas6 might be one of the survival factors and examined the effect of Gas6 on the growth arrest-induced cell death of VSMCs. As shown in Fig. 2, Gas6 dose dependently prevented growth arrest-induced cell death when added to VSMCs after the second medium change. This result indicates that Gas6 exerts an activity of preventing cell death of VSMCs.

We have reported that VSMCs produce active, Gla-containing Gas6 during the first 2 days of serum-starvation [1]. However, after the second medium change with serum-free medium, VSMCs mainly produce inactive Gas6 without Gla residues due to the depletion of vitamin K in the medium (Nakano et al., unpublished results). We then added vitamin K to the medium after the second medium change in order to examine the effect of vitamin K on the cell death of VSMCs. As a result, the addition of vitamin K also prevented cell death of VSMCs (Fig. 3).

The results described so far strongly suggest that Gas6 is a survival factor for growth arrest-induced cell death of VSMCs and vitamin K-dependent γ-carboxylation of Gas6 is essentially indispensable for its activity. However, it has been reported that VSMC releases other growth factors such as PDGF and HB-EGF [20], which are also candidates for the survival factors of VSMC. Indeed, as shown in Fig. 3, PDGF-BB, HB-EGF, and bFGF also prevented growth arrest-induced cell death of VSMCs. However, expression of these growth factors is up-regulated when cells are stimulated with mitogenic substances, and their expression is scarcely detected when cells are growth-arrested [20]. Therefore, these growth factors do not appear to be candidates for the survival factor of growth arrest-induced cell death. Moreover, these

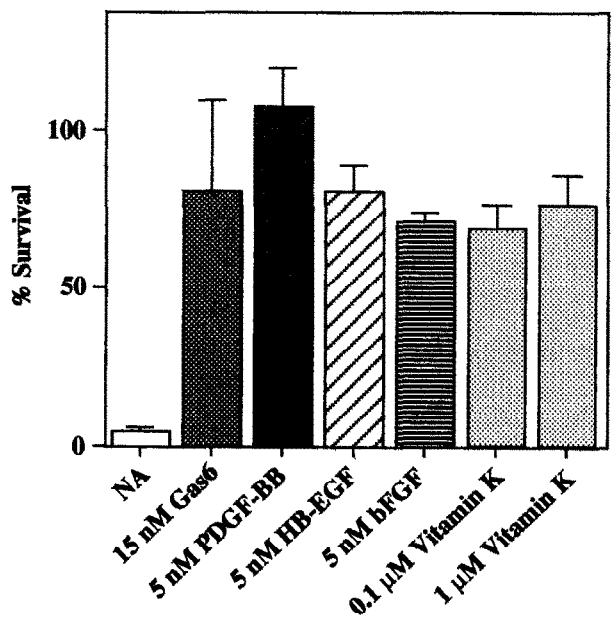

Fig. 3. Prevention of cell death of VSMC by vitamin K and other growth factors. Confluent VSMCs were cultured for 2 days in DMEM containing 0.05% BSA. The medium was then replaced again with fresh DMEM containing 0.05% BSA, and the cells were cultured in the presence or absence (NA) of vitamin K, Gas6, PDGF-BB, HB-EGF or bFGF for a further 3 days. Survival of VSMCs was assayed as described in section 2. Error bars represent S.D. (n=3).

growth factors not only prevent cell death but also induce proliferation of VSMC [21,22], whereas Gas6 does not induce cell proliferation by itself [1]. This specific characteristic of Gas6 may be preferable to maintaining viability of quiescent cells.

Thus, we concluded that one of the major biological activities of Gas6 is to protect VSMC from growth arrest-induced cell death. It is quite reasonable that a role of a product of growth arrest-specific gene is the prevention of growth arrest-induced cell death. However, Gas6 alone may not be a survival factor for the cell death of VSMC, since inhibition of Gas6 activity by anti-Gas6 antibody or inhibition of vitamin K-dependent γ-carboxylation of Gas6 by warfarin, an inhibitor of vitamin K, failed to induce cell death of VSMC when the antibody and warfarin were added instead of the second medium change (data not shown). Furthermore, the concentration of Gas6 in the culture medium of growth-arrested VSMCs was less than 2 nM (data not shown), which is not enough to prevent cell death (Fig. 2). Therefore, VSMCs may also secrete other survival factors to prevent cell death.

Potentiation of VSMC proliferation [1] and prevention of cell death of VSMCs described in this study are the biological activities of Gas6 demonstrated so far. It is interesting to note that both Gas6 and its receptor Axl are widely expressed in various tissues [1,7,8], whereas another type, Sky, is restricted to the brain [9,10]. Thus, Gas6 may have some distinctive physiological roles in respective tissues. We are now conducting further studies to clarify this problem.

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