Regular Article

S-Allyl-L-cysteine Promotes Cell Proliferation by Stimulating Growth Hormone Receptor/Janus Kinase 2/Phospholipase C Pathways and Promoting Insulin-Like Growth Factor Type-I Secretion in Primary Cultures of Adult Rat Hepatocytes

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The mechanism of insulin-like growth factor type-I (IGF-I) secretion stimulated by *S*-allyl-L-cysteine (SAC) was investigated as part of a study of SAC-induced DNA synthesis and cell proliferation in primary cultures of adult rat hepatocytes. When 10^{-6} M SAC was added to the culture, the amount of IGF-I in the medium was significantly increased at 10 min. The peak IGF-I level (140 pg/mL) was observed 20 min after SAC stimulation. The SAC-induced IGF-I secretion was completely suppressed by a selective Janus kinase 2 (JAK2) inhibitor (TG101209), a selective phospholipase C (PLC) inhibitor (U-73122), an intracellular Ca^{2+} chelating agent (BAPTA-AM), and a granule secretion inhibitor (somatostatin). On the other hand, 10^{-6} M SAC-stimulated hepatocytes showed increased intracellular Ca^{2+} concentration in a time-dependent manner from 0 to 10 min. Phosphorylation of SAC-induced JAK2 and IGF-I receptor tyrosine kinase (RTK) was completely suppressed by TG101209. In addition, U-73122, BAPTA-AM, and somatostatin did not suppress SAC-induced JAK2 phosphorylation, but significantly suppressed SAC-induced IGF-I RTK phosphorylation. Furthermore, binding of the monoclonal antibody against growth hormone (GH) to GH receptor was dose-dependently suppressed by SAC on immunofluorescence. These results showed that SAC promotes cell proliferation by stimulating GH receptor/JAK2/phospholipase C pathways and promoting autocrine secretion of IGF-I in primary cultures of adult rat hepatocytes.

Key words *S*-allyl-L-cysteine (SAC); hepatocyte; insulin-like growth factor type-I (IGF-I); secretion; Janus kinase 2 (JAK2)

INTRODUCTION

Insulin-like growth factor type-I (IGF-I) is structurally highly homologous to insulin, and most of it is synthesized in the liver.[1,2] IGF-I, which results in cell proliferation and apoptosis suppression, transmits signals intracellularly like hormones or autocrine/paracrine factors.[1,3] IGF-I biosynthesis is controlled by growth hormone (GH), and binding of ligands to GH receptors promotes IGF-I gene expression through activation of the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathways.[4]

Liver regeneration is systematically regulated by the effects of many growth factors, cytokines, and hormones (*e.g.*, epidermal growth factor, interleukin 6, and thyroid hormone).[5,6] IGF-I and GH are also endogenous liver-regeneration-promoting factors that contribute greatly to cell proliferation.[7,8] In past studies, we demonstrated that IGF-I rapidly promotes cell proliferation *via* the IGF-I receptor tyrosine kinase (RTK)/phosphoinositide 3-kinase (PI3K)/extracellular signal-regulated kinase 2 (ERK2)/mammalian target of rapamycin (mTOR) pathway in primary cultures of adult rat hepatocytes.[9] In addition, GH was found not only to promote IGF-I production *via* the JAK/STAT pathway, but also to secrete pre-stored IGF-I in hepatocytes *via* the JAK2/phospholipase C (PLC)/Ca^{2+} pathway in cultured hepatocytes.[10]

S-Allyl-L-cysteine (SAC) is a water-soluble amino acid in which an allyl group is bonded to the sulfur atom of cysteine. SAC is contained in garlic, and its content is further increased by grating or through the process of aging.[11,12] The pharmacological effect of SAC is cell protection by antioxidant action, and it has been shown to be effective against nervous system diseases such as Alzheimer's disease by preventing oxidative damage.[11,13] On the other hand, a small number of papers have shown that SAC promotes cell proliferation.

We previously showed that SAC promoted cell proliferation by GH receptor/JAK2/PLC pathway stimulation followed by activation of the IGF-I RTK/PI3K/ERK2/mTOR pathway in primary cultures of adult rat hepatocytes. Then, we hypothesized that SAC promotes IGF-I secretion through the GH receptor/JAK2/PLC pathway in cultured hepatocytes. The aim of this study was to further examine the mechanism of SAC-induced IGF-I secretion in primary cultures of adult rat hepatocytes. Thus, the IGF-I secretory effect of SAC on hepatocytes was evaluated by enzyme-linked immunosorbent assay (ELISA) and immunofluorescence. The mechanism of IGF-I secretion by SAC was also investigated using specific signaling factor inhibitors of the JAK2/PLC and JAK/STAT pathways. In addition, the relationship between SAC and the GH receptor that phosphorylates JAK2 was investigated using immunofluorescence techniques.

MATERIALS AND METHODS

Isolation and Culture of Hepatocytes Hepatocytes were isolated from male Wistar rats (Labo Service Corp., Tokyo, Japan) by two-step *in situ* collagenase perfusion.[14] Briefly,

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rats anesthetized with pentobarbital 45 mg/kg intraperitoneally were cannulated in the portal and hepatic veins, and Ca^{2+}-free Hanks solution was refluxed for 10 min. Then, Hanks' solution containing 60 U/mL collagenase type 2 (Worthington Biochemical Corp., Freehold, NJ, U.S.A.) and 50 mM $CaCl_2$ was refluxed for 11 min to digest the connective tissue. Isolated hepatocytes with a survival rate of 96% or higher, which was examined by the trypan blue exclusion test, were seeded in a collagen type 1-coated plastic dish (AGC TECHNO GLASS Co., Ltd., Shizuoka, Japan) at a cell density of 3.3×10^4 cells/cm^2. They were then cultured in newborn bovine serum (Thermo Fisher Scientific, Inc., Waltham, MA, U.S.A.) and 10^{-10} M dexamethasone-containing Williams' medium E (Sigma-Aldrich Co., St. Louis, MO, U.S.A.) for 3 h. After cell attachment and replacement with serum-free medium, hepatocytes were cultured in the presence of SAC with or without specific signal transduction factor inhibitors related to the JAK2/PLC/Ca^{2+} pathway. The reagents added to hepatocytes were SAC, *S*-methyl-L-cysteine (SMC) (Tokyo Chemical Industry Co., Tokyo, Japan), and growth-related signal-transducing inhibitors or monoclonal antibodies. Inhibitors used were TG101209,[15] SH-4-54,[16] U-73122,[17] BAPTA-AM,[18] GF109203X,[19] somatostatin,[20] and AG538,[21] which were obtained from Sigma-Aldrich Co. The antibodies used were monoclonal antibodies against IGF-I (anti-IGF-I mAb) and transforming growth factor (TGF)-α (anti-TGF-α mAb) (Santa Cruz Biotechnology, Dallas, TX, U.S.A.).

This study was approved by the Institutional Animal Care and Use Committee of Josai University (Nos. JU 20035 and JU 21035), and the handling of animals was in accordance with the regulations of the Guidelines for the Care and Use of Laboratory Animals of Josai University.

Counting Nuclei and/or Cells The cell proliferation effect of SAC was evaluated by measuring the number of hepatocyte nuclei with a slight modification to the method of Nakamura *et al.*[22] Briefly, 0.3% trypan blue (Sigma-Aldrich) was used to stain hepatocyte nuclei isolated with 0.1% Triton X-100 containing 0.1 M citric acid; a hemocytometer was then used to count the number of nuclei.

The numbers of total hepatocytes and of mononuclear and binuclear hepatocytes per 0.01 cm^2 (namely, $\times 10^2$ cells/cm^2) were counted in a phase-contrast microscope image. The values were measured from cells in microscopic images taken at any three locations, and the average was taken as one example.

Detection of the S-Phase in the Cell Cycle DNA synthesis was determined by detecting the S-phase of the cell cycle. The cell cycle was measured with a Muse™ cell analyzer with a slight modification to the manufacturer's instructions (Merck Millipore, Darmstadt, Germany).[23] The Muse™ cell analyzer is a cell cycle analysis apparatus based on a flow cytometer. Briefly, hepatocyte nuclei obtained from the above section (**Counting nuclei and/or cells**) were incubated with 5% propidium iodide (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) for 15 min in the dark. Next, the S-phase of hepatocytes was measured using a cell analyzer.

Measurement of IGF-I Secretion into the Medium The amount of IGF-I secreted from hepatocytes was measured using an ELISA kit (Biosensis Pty. Ltd., Thebarton, Australia). Hepatocytes attached to the culture dish were washed with phosphate-buffered saline (PBS) and then incubated with SAC or inhibitor in an incubation medium (PBS containing 1.0 mM $CaCl_2$, 5.5 mM glucose, and 0.1 μg/mL aprotinin (pH 7.4)) for 0 to 30 min. The IGF-I concentration in incubation medium (50 μL) was quantified according to the manufacturer's assay procedure.

Immunolocalization of IGF-I IGF-I expressed in hepatocytes was examined using immunofluorescence techniques.[10,24,25] Hepatocytes (3.3×10^4 cells/cm^2), seeded on the 20×20 mm chamber slide (AGC TECHNO GLASS Co., Ltd.), were attached to the cells as described in the above section (**Isolation and culture of hepatocytes**), and then in serum-free medium containing SAC or an inhibitor for 0 to 30 min. Next, the cultured hepatocytes were incubated in the order of 3.7% buffered formalin for 20 min, 0.1% Triton X-100 for 10 min, and 1% bovine serum albumin (BSA) for 60 min. Then 0.4 μg/mL mouse anti-IGF-I monoclonal antibody (Santa Cruz Biotechnology) was incubated for 3 h. Subsequently, 1 μg/mL Goat anti-mouse immunoglobulin G (IgG) Cross-Adsorbed secondary antibody, Alexa Fluor 488 (Thermo Fisher Scientific, Inc.) was incubated for 45 min, and then 1 μg/mL propidium iodide (Sigma-Aldrich Co.) was incubated for 30 min to label the nuclei of hepatocytes (red signals). Labeled IGF-I was observed under a fluorescence microscope (green signals). Fluorescence intensity per cell was quantified using ImageJ version 1.53.[26]

Measurement of the Binding Ability of SAC to the GH Receptor The binding ability of SAC to the GH receptor was investigated using immunofluorescence techniques. Hepatocytes attached to the chamber slide were incubated with formalin, Triton X-100, and BSA in that order as in the steps above (**Immunolocalization of IGF-I**). Next, 0.01 μg/mL mouse anti-GH receptor monoclonal antibody (Santa Cruz Biotechnology) was incubated with SAC, SMC, or GH for 1.5 h. Subsequently, secondary antibody and propidium iodide treatments were performed in the same manner as in the above section. Labeled GH receptor was observed under a fluorescence microscope (green signals). Fluorescence intensity per cell was quantified using ImageJ version 1.53.[26]

Measurement of the Intracellular Ca^{2+} Concentration The intracellular Ca^{2+} concentration was measured using a Ca^{2+} fluorescent probe (Fluo 4-AM; Dojindo Laboratories, Co., Ltd., Kumamoto, Japan).[27,28] Hepatocytes attached to the culture dish were incubated in a recording buffer (20 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES), 115 mM NaCl, 5.4 mM KCl, 0.8 mM $MgCl_2$, 1.8 mM $CaCl_2$, and 13.8 mM glucose) containing 3 μM Fluo 4-AM for 40 min at 37 °C. Hepatocytes were washed with PBS and then incubated in a recording buffer containing SAC for 0 to 20 min. The intracellular Ca^{2+} at each incubation time was observed under a fluorescence microscope (green signals). Fluorescence intensity per cell was quantified using ImageJ version 1.53.[26]

Measurement of Phosphorylated p125 kDa JAK2 and p95 kDa RTK Phosphorylated p125 kDa JAK2 and p95 kDa RTK were measured by Western blotting analysis.[29,30] Briefly, the protein in hepatocytes was obtained by dissolving in Cell Lysis buffer (Cell Signaling Technology, Inc., Danvers, MA, U.S.A.). The protein in the sample was quantified by the Lowry method. The sample was mixed with Laemmli sample buffer (Bio-Rad Laboratories, Inc., Hercules, CA, U.S.A.), and then 30 μg of protein per lane were run on a polyacrylamide gel. The separated sample was transferred to a Polyscreen polyvinylidene difluoride (PVDF) transfer membrane (Perki-

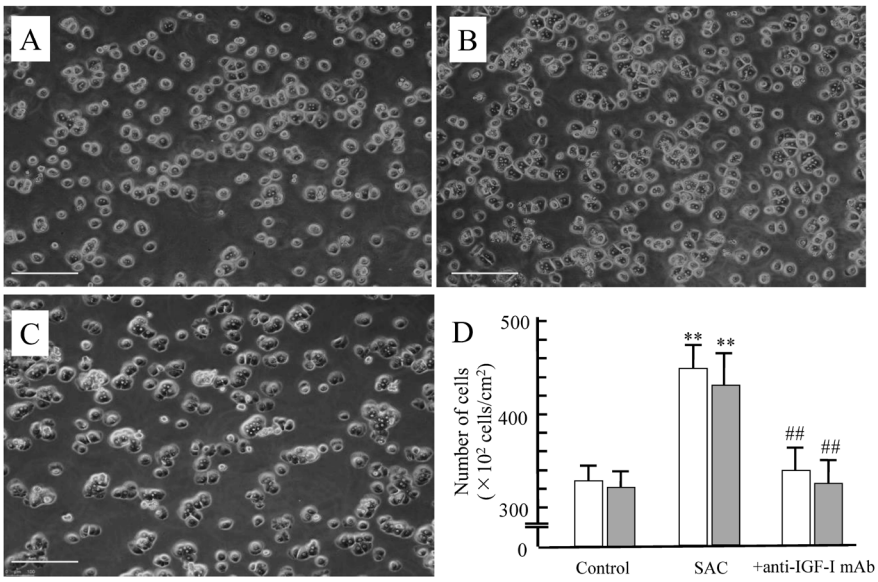

Fig. 1. Phase-Contrast Micrographs of Hepatocytes Cultured with SAC for 5 h

As described in Materials and Methods, after a 3-h attachment period, hepatocytes (3.3×10^4 cells/cm^2) were cultured in serum-free medium containing SAC for 5 h. Photos are (A), control (medium alone); (B), SAC (10^{-6} M); (C), SAC (10^{-6} M)-treated hepatocytes with presence of anti-IGF-I mAb (100 ng/mL). Scale bar, 200 μm. (D), Number of hepatocytes per field of view (per 0.01 cm^2[$\times 10^2$ cells/ cm^2]) under the microscope. White column: number of total hepatocytes, gray column: number of mononuclear and binuclear hepatocytes. Values are shown as means ± S.E.M. *($p < 0.05$), **($p < 0.01$) shows comparison with control. $^{\#}$($p < 0.05$), $^{\#\#}$($p < 0.01$) shows comparison with SAC.

nElmer, Inc. Waltham, MA, U.S.A.). Next, the membrane was incubated in the order of monoclonal antibodies against phosphorylated p125 kDa JAK2 or p95 kDa RTK (Cell Signaling Technology) and the HRP-labeled secondary antibody (Cell Signaling Technology), which was chemiluminescent with the Western Lightning Plus-ECL (PerkinElmer, Inc.). Emission intensity was quantified by ChemiDoc Touch MP (Bio-Rad Laboratories, Inc.). Phosphorylated p125 kDa JAK2 or p95 kDa RTK activity ratios were calculated by dividing the respective expression levels (activity in the total-p125 kDa JAK2 or p95 kDa RTK antibodies; Cell Signaling Technology) by total activity.

Statistical Analysis The results are presented as means ± standard error of the mean (S.E.M.) of three separate experiments. For comparison between the control group and the treatment group, the p value was calculated by multiple comparisons using Dunnett's method, and significance was set at less than 5%.

RESULTS

Effects on SAC-Induced Hepatocyte Proliferation of Monoclonal Antibodies against IGF-I or TGF-α To investigate whether SAC secretes IGF-I or TGF-α and stimulates cell proliferation, the effect on SAC cell proliferation (as the number of nuclei or cells) in the presence of anti-IGF-I mAb or anti-TGF-α mAb was measured. Figure 1 shows a phase-contrast microscopic image of hepatocytes cultured for 5 h. It was confirmed that the number of SAC-stimulated hepatocytes was significantly greater than in control (medium alone) (Figs. 1A, B). In contrast, no increase in the number of hepatocytes was observed with combined treatment by SAC and anti-IGF-I mAb (Fig. 1C). As shown in Fig. 1D, the number of total hepatocytes per field of view (per 0.01 cm^2 [$\times 10^2$ cells/cm^2]) under the microscope in the control treatment (medium only) was 332 ± 22. In contrast, the number of total hepatocytes stimulated with 10^{-6} M SAC was 448 ± 23, which was a significant increase of approximately 1.34-fold compared to the control (Fig. 1D, white column). In addition, the number of total hepatocytes treated with SAC and anti-IGF-I mAb was significantly reduced to control levels. Similar results were observed for the effects of SAC on mononuclear and binuclear hepatocytes (Fig. 1D, gray column).

Figure 2 shows the time-dependent changes in the number of nuclei by SAC. As shown in Fig. 2, SAC showed an increase in hepatic cell nuclei depending on the culture time, whereas anti-IGF-I mAb significantly suppressed the increase in hepatic cell nuclei induced by SAC (Fig. 2A). In addition, the hepatocyte proliferation effect of SAC was significantly suppressed depending on the dose of anti-IGF-I mAb (Fig. 2B). In contrast, anti-TGF-α mAb did not affect the number of nuclei increased by SAC (Figs. 2A, B).

Effects of SAC on Hepatocyte Proliferation in the Presence of Specific Signal Transduction Factor Inhibitors Related to the JAK2/PLC/Ca^{2+} Pathway The effects of JAK/PLC pathway-related inhibitors on SAC-induced cell proliferation and DNA synthesis (as progression of the S-phase in the cell cycle) were examined. The culture time was fixed at 5 h after SAC stimulation. In Fig. 3, the hepatocyte proliferation and DNA synthesis-promoting effect of SAC was completely inhibited to control level by TG101209, U-73122, BAPTA-AM, somatostatin, and AG538. On the other hand, SH-4-54 and GF109203X did not inhibit SAC-induced hepatocyte proliferation and DNA synthesis (Figs. 3A, B).

Effects of SAC on IGF-I Secretion by Cultured Hepatocytes To investigate the effects of SAC on IGF-I secretion by hepatocytes, intracellular IGF-I treated with SAC was observed by fluorescence IGF-I imaging techniques. After an attachment period of 3 h, hepatocytes exchanged for serum-free medium were cultured with SAC (10^{-6} M) or with the co-presence of selective JAK2 inhibitor TG101209 (10^{-6} M) for 20 min. As shown in Fig. 4, it can be confirmed that the

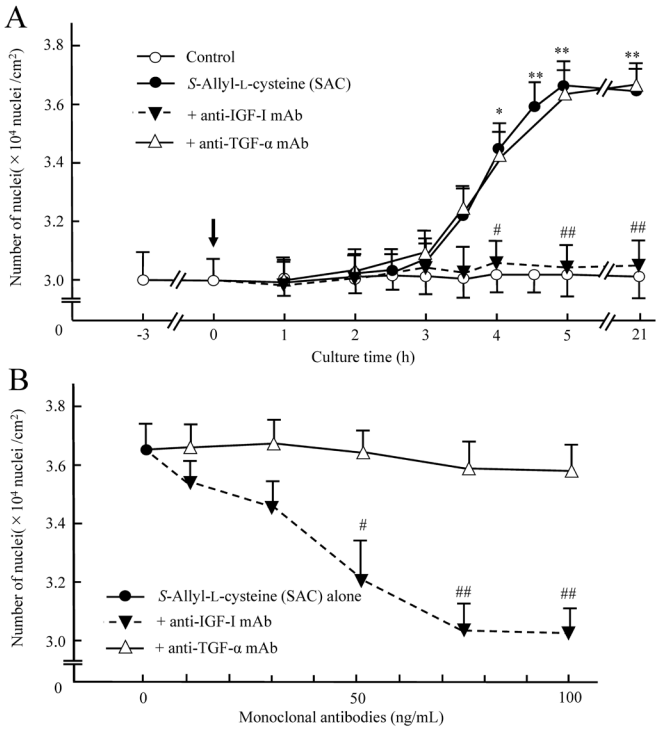

Fig. 2. Effect of Anti-IGF-I mAb or Anti-TGF-α mAb on SAC-Induced Proliferation in Hepatocytes

After cell attachment and replacement with serum-free medium as described in Materials and Methods, hepatocytes were cultured in the presence of SAC (10^{-6}M) with or without monoclonal antibodies against IGF-I or TGF-α. (A) Effects of anti-IGF-I mAb (100 ng/mL) or anti-TGF-α mAb (100 ng/mL). The arrow shows the addition of the SAC (10^{-6}M) with or without antibodies. (B) Dose-dependent effects of anti-IGF-I mAb or anti-TGF-α mAb on SAC (10^{-6}M)-induced proliferation for 5 h. Values are shown as means ± S.E.M. *($p<0.05$), **($p<0.01$) shows comparison with respective control. #($p<0.05$), ##($p<0.01$) shows comparison with respective SAC.

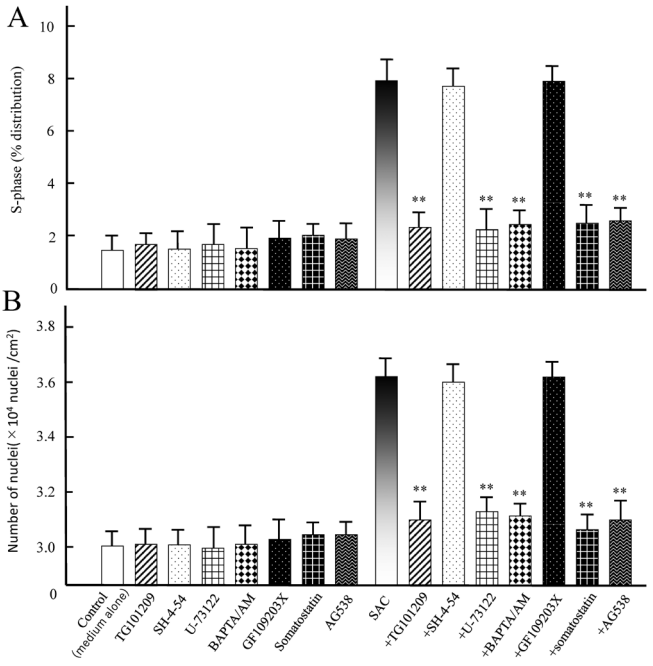

Fig. 3. Effect of SAC on Hepatocyte Proliferation in the Presence of Specific Signal Transduction Factor Inhibitors Related to the JAK2/PLC/Ca^{2+} Pathway

After cell attachment and replacement with serum-free medium as described in Materials and Methods, hepatocytes are cultured in the presence of SAC with or without specific signal transduction factor inhibitors related to the JAK2/PLC/Ca^{2+} pathway. S-phase progression (DNA synthesis [A]) and number of nuclei (cell proliferation [B]) were measured 5 h after addition of SAC (10^{-6}M). Inhibitors are TG101209 (10^{-6}M), SH-4-54 (10^{-6}M), U-73122 (10^{-6}M), BAPTA/AM (10^{-7}M), GF109203X (10^{-7}M), somatostatin (10^{-7}M), and AG538 (10^{-6}M). Values are shown as means ± S.E.M. *($p<0.05$), **($p<0.01$) shows comparison with respective SAC.

intracellular IGF-I of SAC-stimulated hepatocytes decreased compared with the control (Figs. 4B, C). The fluorescence intensity of intracellular IGF-I in the presence of SAC at 20 min of culture was significantly reduced by approximately 65% compared to the control (Fig. 4E). On the other hand, the combined treatment of SAC with TG101209 did not show a decrease in intracellular IGF-I, confirming that TG101209 suppressed IGF-I secretion by SAC (Figs. 4D, E).

Next, to examine the time dependence of IGF-I secretion by SAC, the amount of IGF-I secretion from hepatocytes was measured using ELISA. After exchanging to incubation medium as described in Materials and Methods, the amount of IGF-I secretion increased rapidly at 10 min after SAC (10^{-6}M) stimulation. The peak IGF-I secretion level by SAC stimulation was observed at 20 min, and the IGF-I secretion level was maintained up to 40 min (Fig. 5). The peak IGF-I level in SAC treatment was approximately 140 pg/mL, which was significantly increased by approximately 14-fold compared to control alone. On the other hand, no increase in the amount of IGF-I in the medium of SAC-stimulated hepatocytes in combination with TG101209 (10^{-6}M) was observed (Fig. 5).

Effects of SAC on IGF-I Secretion in the Presence of Specific Signal Transduction Factor Inhibitors Related to the JAK2/PLC/Ca^{2+} Pathway We hypothesized that SAC secretes IGF-I *via* the JAK2/PLC/Ca^{2+} pathway in hepatocytes. Therefore, SAC-induced IGF-I levels in the presence of a specific signal transduction factor inhibitor associated with the JAK2/PLC/Ca^{2+} pathway were measured by ELISA. As shown in Fig. 6, the IGF-secreting effects of SAC were completely inhibited to control level not only by TG101209, but also by the selective PLC inhibitor U-73122, the intracellular Ca^{2+} chelating agent BAPTA-AM, and the granule secretion inhibitor somatostatin (Fig. 6). In contrast, the selective STAT3/5 inhibitor SH-4-54, the selective protein kinase C (PKC) inhibitor GF109203X, and the selective IGF-I RTK inhibitor AG538 did not inhibit the SAC-induced IGF secretion (Fig. 6).

Effects of SAC on p125 kDa JAK2 and p95 kDa RTK Phosphorylation in the Presence of Specific Signal Transduction Factor Inhibitors Related to the JAK2/PLC/Ca^{2+} Pathway Next, the effects of inhibitors used in the previous section on SAC-induced p125 kDa JAK2 and p95 kDa RTK phosphorylation were investigated. As shown in Fig. 7, U-73122, BAPTA-AM, somatostatin, and AG538 did not suppress SAC-induced JAK2 phosphorylation, but they significantly suppressed SAC-induced RTK phosphorylation. On the other hand, TG101209 suppressed both SAC-induced JAK2 and RTK phosphorylation. SH-4-54 or GF109203X did not affect SAC-induced phosphorylation of both JAK2 and RTK (Figs. 7A, B).

Effects of SAC on the Intracellular Ca^{2+} Concentration in Cultured Hepatocytes Next, to examine whether SAC increases the intracellular Ca^{2+} concentration in cultured hepatocytes, the intracellular Ca^{2+} concentration was measured by fluorescence microscope observation using a Ca^{2+} fluorescent probe, Fluo4-AM. As shown in Fig. 8, hepatocytes stimulated with 10^{-6}M SAC showed increased intracellular Ca^{2+} concentration in a time-dependent manner from 0 to 10 min, which

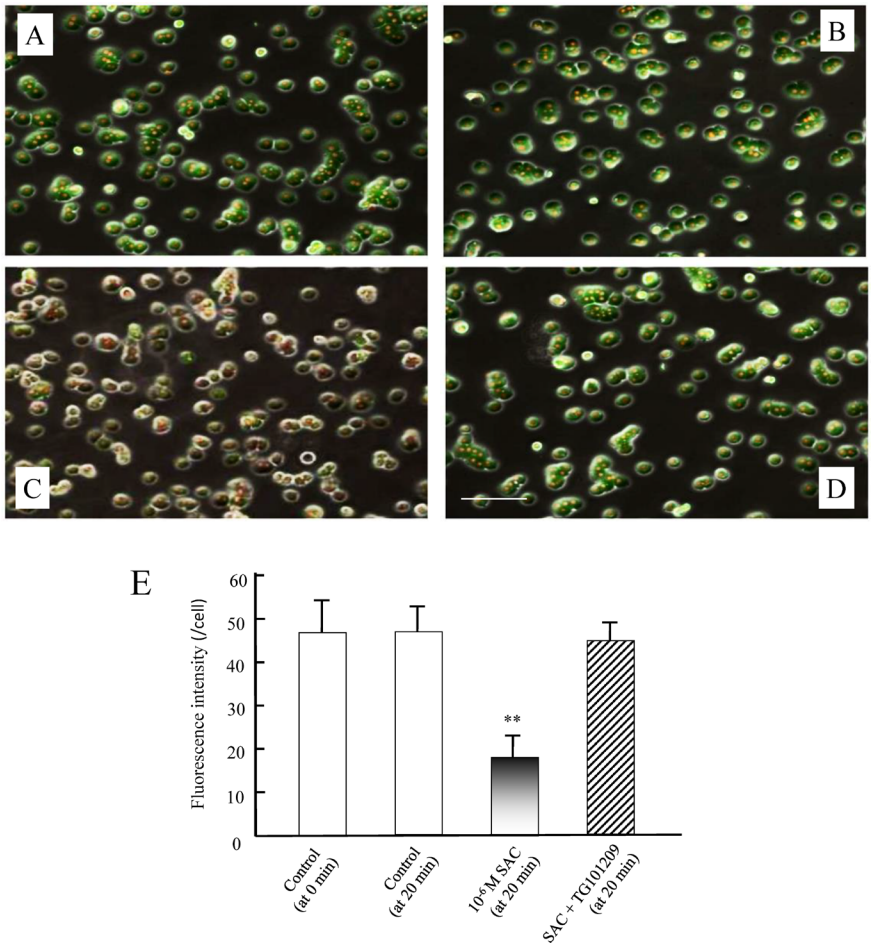

Fig. 4. Typical Fluorescence Image of IGF-I in SAC-Stimulated Hepatocytes with or without TG101209

IGF-I in cultured hepatocytes (3.3×10^4 cells/cm^2) is labeled by immunofluorescence as described in Materials and Methods. (A) Control (at 0 min); (B) control (at 20 min); (C) SAC (10^{-6} M)-treated hepatocytes at 20 min; (D) SAC (10^{-6} M)-treated hepatocytes with presence of TG101209 (10^{-6} M) at 20 min. (E) Fluorescence intensity per cell. Values are shown as means ± S.E.M. *($p < 0.05$), **($p < 0.01$) shows comparison with control. Green signals: labeled IGF-I. Red signals: labeled hepatocyte nuclei. Scale bar: 100 μm.

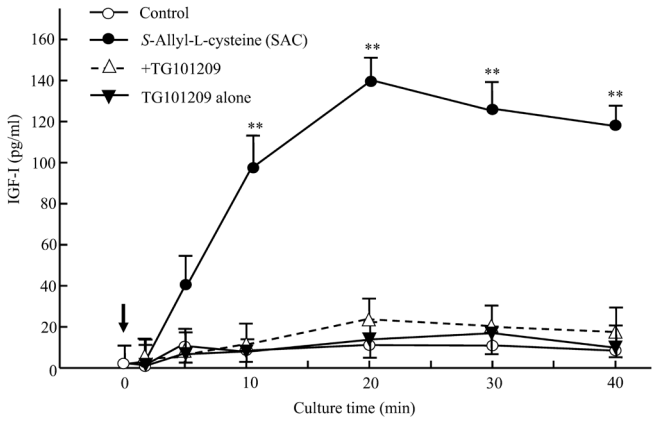

Fig. 5. Effect of SAC on Time-Dependent IGF-I Secretion from Cultured Hepatocytes

As described in Materials and Methods, hepatocytes (3.3×10^4 cells/cm^2) attached to the plate are replaced with incubation medium and then incubated in the presence of SAC (●; 10^{-6} M) or with the co-presence of TG101209 (△; 10^{-6} M) for 0–40 min. Closed triangles represent TG101209 alone (▼; 10^{-6} M). The arrow shows the addition of the drugs. Values are shown as means ± S.E.M. *($p < 0.05$), **($p < 0.01$) shows comparison with respective control.

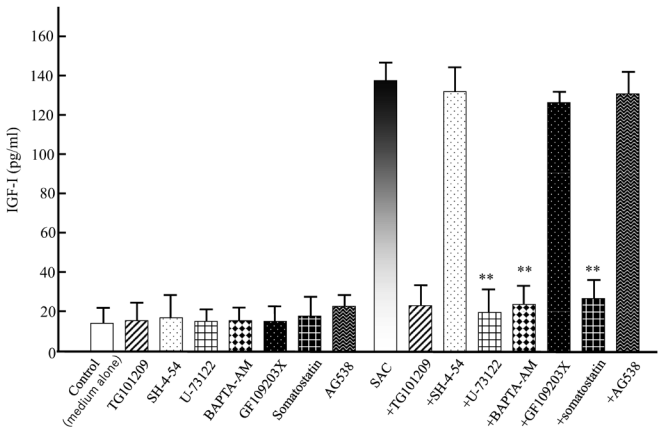

Fig. 6. Effect of SAC on IGF-I Secretion in the Presence of Specific Signal Transduction Factor Inhibitors Related to the JAK2/PLC/Ca^{2+} Pathway

As described in Materials and Methods, hepatocytes (3.3×10^4 cells/cm^2) attached to the plate are replaced with incubation medium and then incubated in the presence of SAC (10^{-6} M) or with the co-presence of growth-related signal-transducing inhibitors for 20 min. Inhibitors are TG101209 (10^{-6} M), SH-4-54 (10^{-6} M), U-73122 (10^{-6} M), BAPTA/AM (10^{-7} M), GF109203X (10^{-7} M), somatostatin (10^{-7} M), and AG538 (10^{-6} M). Values are shown as means ± S.E.M. *($p < 0.05$), **($p < 0.01$) shows comparison with respective SAC-treatment.

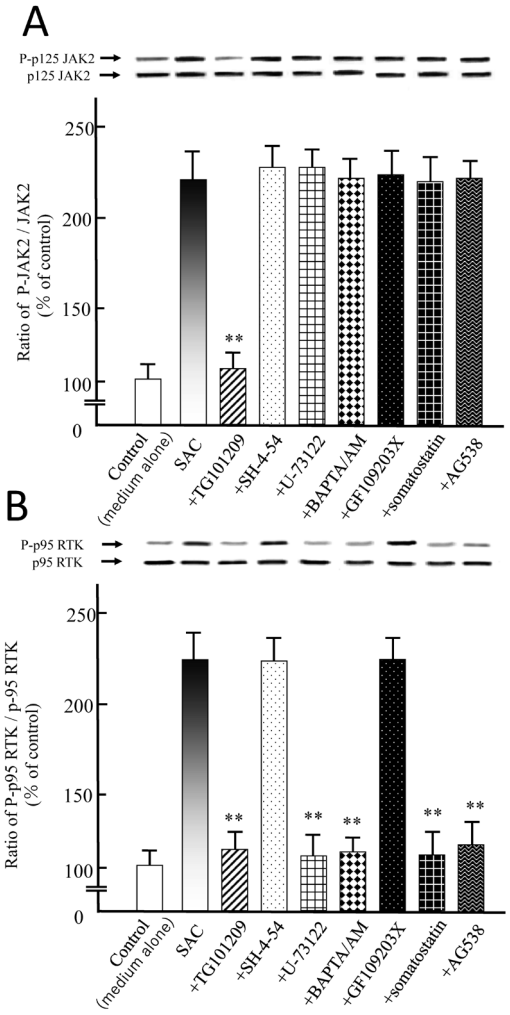

Fig. 7. Effect of SAC on p125 kDa JAK2 and p95 kDa RTK Phosphorylation in the Presence of Specific Signal Transduction Factor Inhibitors Related to the JAK2/PLC/Ca^{2+} Pathway

After cell attachment and replacement with serum-free medium as described in Materials and Methods, hepatocytes were cultured in the presence of SAC with or without specific signal transduction factor inhibitors related to the JAK2/PLC/Ca^{2+} pathway. Phosphorylation of p125 kDa JAK2 and p95 kDa RTK was determined by Western blot analysis as described in Materials and Methods. (A) Phosphorylation of p125 kDa JAK2 5 min after SAC (10^{-6} M) stimulation. (B) Phosphorylation of p95 kDa RTK 20 min after SAC (10^{-6} M) stimulation. Typical Western blot images are shown at the top of each graph. Inhibitors are TG101209 (10^{-6} M), SH-4-54 (10^{-6} M), U-73122 (10^{-6} M), BAPTA/AM (10^{-7} M), GF109203X (10^{-7} M), somatostatin (10^{-7} M), and AG538 (10^{-6} M). Values are shown as means ± S.E.M. *($p < 0.05$), **($p < 0.01$) shows comparison with respective SAC.

then decreased over 20 min (Figs. 8A–H). The fluorescence intensity 10 min after SAC stimulation was significantly increased, to approximately 4.5-fold compared to 0 min (Fig. 8I).

Effects of SAC and GH on Anti-GH Receptor Monoclonal Antibody Binding to GH Receptors in Cultured Hepatocytes In previous studies, we demonstrated that GH stimulates the GH receptors in cultured hepatocytes to stimulate IGF-I secretion *via* the JAK2/PLC/Ca^{2+} pathway.[10] Then, we hypothesized that SAC stimulates the GH receptor in hepatocytes to promote phosphorylation of JAK2 and PLC and subsequent IGF-I secretion. Therefore, to investigate whether SAC binds to GH receptors, the affinity between anti-GH receptor monoclonal antibody (anti-GHR mAb) and GH receptors in the presence of SAC or GH was examined by a GH receptor immunofluorescence technique (GH receptor imaging). Figure 9A shows a typical example of a fluorescence microscope image, and Fig. 9B shows the result of quantifying the fluorescence intensity. In Fig. 9A, the fluorescent green signal represents an anti-GHR mAb bound to the GH receptor. GH and SAC-treated fluorescent green signals tended to decrease compared to control (SAC and GH absent), the effect of which was dependent on GH and SAC doses. As shown in Fig. 9B, the fluorescence intensity of the anti-GHR mAb bound to the GH receptor in the presence of GH decreased from 1 ng/mL GH and suppressed by approximately 80% at 100 ng/mL GH (molar ratio conversion, 0.5×10^{-8} M). On the other hand, the fluorescence intensity in the presence of SAC decreased from 10^{-8} M SAC to 10^{-6} M SAC and showed the same fluorescence intensity as 100 ng/mL GH treatment. In contrast, SMC (10^{-6} M), which is a structural analog of SAC, did not show any decrease in fluorescence intensity (Fig. 9).

DISCUSSION

We previously showed in primary cultures of adult rat hepatocytes that cell proliferation was promoted by SAC *via* activation of the GH receptor/JAK2/PLC pathway followed by activation of the IGF-I RTK/PI3K/ERK2/mTOR pathway. In hepatocytes, it was found that GH promoted IGF-I secretion through the JAK2/PLC pathway.[10] From this, it was inferred that SAC secreted IGF-I *via* the GH receptor/JAK2/PLC pathway, which then resulted in stimulation of the IGF-I RTK signaling pathway. In the present study involving primary cultures of adult rat hepatocytes, the goal was to further investigate the mechanism of SAC-induced IGF-I secretion.

In the present study, the hepatocyte proliferation effect was evaluated mainly using the cell nucleus counting technique, because the attachment between plates or cell-cell contact is tight in cultured hepatocytes, and a single cell cannot be isolated quantitatively. As shown in Fig. 1, in the hepatocytes stimulated by SAC, a significant increase in the number of hepatocytes was observed at 5 h (Figs. 1A, B). This result showed almost the same tendency as the result obtained by the cell nucleus counting technique shown in Fig. 2. It was observed that the increased number of cells mostly consisted of mononuclear and binuclear cells, and that the number of polynuclear (8n genome) cells was minimally increased (Fig. 1D, gray column). Rapid cell proliferation effects on hepatocytes have been demonstrated not only with SAC, but also with growth factors or hormones (*e.g.*, HGF, IGF-I, insulin, and GH) in culture conditions at low dexamethasone (10^{-10} M) and low cell density (3.3×10^4 cells/cm^2).[9,30–33]

Next, the effect on SAC-induced hepatocyte proliferation was investigated using anti-IGF-I mAb and anti-TGF-α mAb. TGF-α is also an autocrine factor stored in hepatocytes, whose secretion is promoted by stimulation with serotonin or interleukin 1β.[23,34] As shown in Fig. 2, the SAC-induced hepatocyte proliferation effect was suppressed depending on the dose of the monoclonal antibody against IGF-I, but not TGF-α. In addition, SAC-induced cell proliferation and DNA synthesis were completely suppressed by somatostatin, which suppresses degranulation of IGF-I and TGF-α, and AG538, which specifically inhibits IGF-I RTK (Fig. 3). These results suggest that SAC induced the secretion of IGF-I contained in hepatocytes, and the secreted IGF-I stimulated IGF-I RTK and promoted cell proliferation.

To further investigate the mechanism of IGF-I secretion by

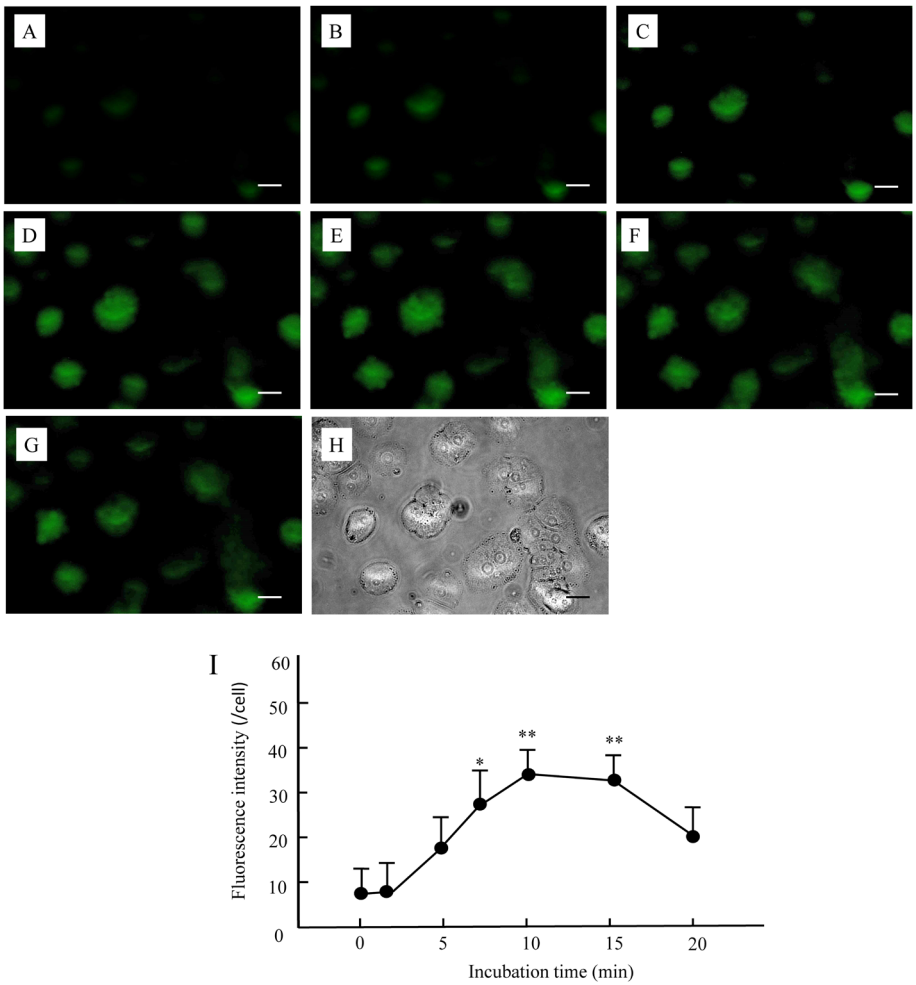

Fig. 8. Time-Dependent Effect of SAC on Intracellular Ca^{2+} Concentration in Cultured Hepatocytes

As described in Materials and Methods, hepatocytes (3.3×10^4 cells/cm^2) attached to the plate are incubated with 3 μM Fluo 4-AM-containing in a recording buffer for 40 min at 37 °C. After washing with PBS, hepatocytes were incubated for 0–20 min in the presence of 10^{-6} M SAC. (A) 0 min, (B) 1 min, (C) 5 min, (D) 7 min, (E) 10 min, (F) 15 min, (G) 20 min, (H) Phase-contrast microscopic image (at 0 min). Scale bar: 25 μm. (I) Fluorescence intensity per cell. Values are shown as means ± S.E.M. *($p < 0.05$), **($p < 0.01$) shows comparison with control (at 0 min).

SAC, immunofluorescence techniques and ELISA were used to measure the localization of IGF-I and amount of IGF-I secretion into culture.

In Fig. 4, fluorescence microscopy confirmed that SAC-stimulated hepatocytes lost the IGF-I stored in the cells after 20 min. In addition, as shown in Fig. 5, it was confirmed that the amount of IGF-I secreted by SAC-stimulated hepatocytes increased significantly after 10 min of stimulation and peaked after 20 min by ELISA. These results suggested that the hepatocyte proliferation effect of SAC is mediated by the secretion of IGF-I from cultured hepatocytes. On the other hand, SAC-induced IGF-I secretion was completely suppressed by TG101209, which specifically inhibits JAK2 (Figs. 4, 5). As shown in Fig. 6, SAC-induced IGF-I secretion was completely suppressed not only by TG101209, but also by U-73122, BAPTA-AM, and somatostatin. The SAC-induced hepatocyte proliferation effects were also suppressed by these inhibitors (Fig. 3). PLC synthesizes the second messengers inositol trisphosphate (IP_3) and diacylglycerol, and IP_3 increases intracellular Ca^{2+} levels.[35] The PKC inhibitor GF109203X, however, had no effect on both SAC-induced hepatocyte proliferation and SAC-induced IGF-I secretion (Figs. 3, 6). Therefore, we hypothesized that the pathway of IGF-I secretion by SAC proceeds in the order of JAK2, PLC, and IP_3, and that the increase in intracellular Ca^{2+} levels induces exocytosis of IGF-I secreted granules.

In Fig. 7, U-73122, BAPTA-AM, somatostatin, and AG538 did not suppress SAC-induced JAK2 phosphorylation, but they significantly suppressed SAC-induced RTK phosphorylation. These results imply that signaling factors PLC/Ca^{2+} and IGF-I secretion are present between JAK2 and IGF-I RTK in the SAC-induced signaling pathway of hepatocyte proliferation.

The STAT family has seven types of molecules, and STAT5b is mainly involved in IGF-I production. STAT5b forms a homodimer by phosphorylated JAK, and when the complex binds to DNA, the expression of STAT5b-dependent genes such as IGF-I and IGF binding protein is promoted.[36] However, it should be noted that SH-4-54 did not suppress the SAC-induced IGF-I secretion, and that IGF-I secretion increased 10 min after SAC stimulation. The IGF-I secretion observed in Fig. 5 is too fast for the process to occur *via* gene expression. In other words, it was found that the IGF-I-secreting effect of SAC (especially in the early secretion of IGF-I) does not occur *via* the JAK/STAT pathway. However, SAC has also been shown to increase IGF-I mRNA expression levels in partially hepatectomized rats.[37] SAC may stimulate

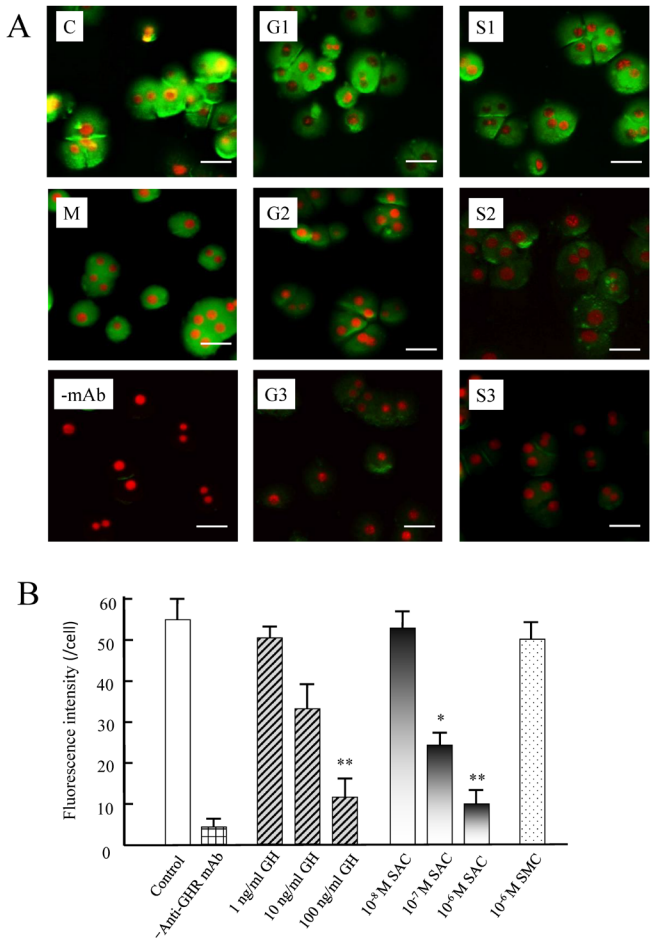

Fig. 9. Effect of SAC and GH on Anti-GH Receptor Monoclonal Antibody Binding to GH Receptors in Cultured Hepatocytes

GH receptors expressed on hepatocytes are labeled by immunofluorescence as described in Materials and Methods. (A) Typical example of a fluorescence microscope image. C, control (anti-GHR mAb alone); M, 10^{-6}M SMC; -mAb, no anti-GHR mAb; G1, 1 ng/mL GH; G2, 10 ng/mL GH; G3, 100 ng/mL GH; S1, 10^{-8}M SAC, S2, 10^{-7}M; S3, 10^{-6}M SAC. Green signals: labeled GH receptor. Red signals: labeled hepatocyte nuclei. Scale bar: 25 μm. (B) Fluorescence intensity per cell. Values are shown as means ± S.E.M. *($p < 0.05$), **($p < 0.01$) shows comparison with control.

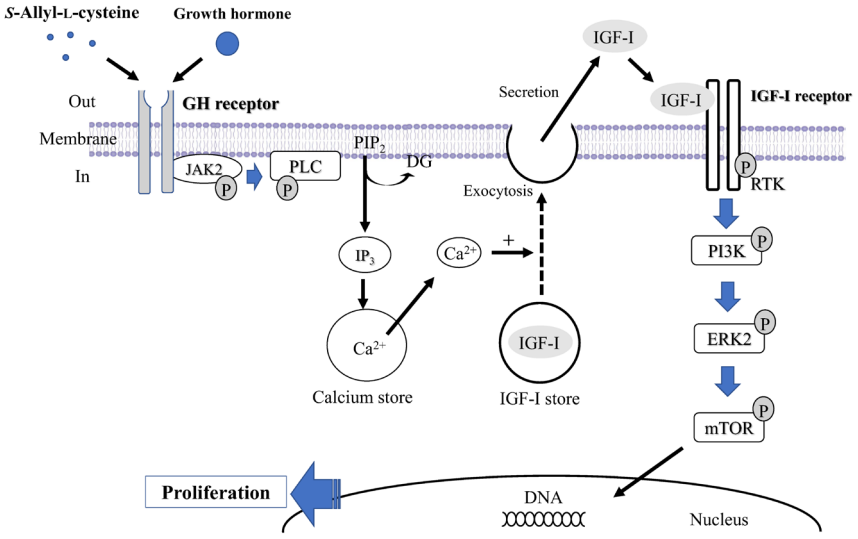

Fig. 10. Mechanism of IGF-I Secretion and Cell Proliferation by SAC in Primary Cultures of Adult Rat Hepatocytes

IGF-I: insulin like-growth factor type-1, GH: growth hormone, JAK2: Janus kinase 2, PLC: phospholipase C, PIP_2: phosphatidylinositol 4,5-bisphosphate, DG: diacylglycerol, IP_3: inositol trisphosphate, RTK: receptor tyrosine kinase, PI3K: Phosphoinositide 3-kinase, ERK2: extracellular signal-regulated kinase, mTOR: mammalian target of rapamycin, P: phosphorylation.

both the JAK/STAT pathway, which promotes IGF-I biosynthesis (late phase), and the JAK2/PLC pathway, which promotes pre-stored IGF-I secretion (early phase).

The release of hormones and cytokines is regulated by the intracellular Ca^{2+} concentration.[38,39] In Fig. 8, the intracellular Ca^{2+} concentration after SAC stimulation was observed to increase in a time-dependent manner from 0 to 10 min. The peak of intracellular Ca^{2+} concentration is observed within 5 min in many cases, although it changes depending on the agonist and culture conditions (ion concentrations, *etc.*).[40,41] The process of phosphorylation of JAK2 by SAC and the subsequent interaction between JAK2 and PLC may lead to the increase in intracellular Ca^{2+} concentration.

IGF-I secretion associated with activation of the JAK2/PLC/Ca^{2+} pathway is observed not only with SAC, but also with GH in primary cultures of hepatocytes.[10] GH binds to GH receptors expressed on hepatocytes and phosphorylates JAK2. We hypothesized that SAC activates the JAK2/PLC/Ca^{2+} pathway *via* binding of GH receptors. In the GH receptor immunofluorescence shown in Fig. 9, the combined use of anti-GH receptor mAb and SAC was observed to reduce the fluorescence intensity compared to SAC alone. Moreover, the effect was dependent on the dose of SAC. The anti-GHR mAb used in these experiments has been shown to suppress GH-induced cell proliferation in a dose-dependent manner. These results indicate that SAC competes for binding of anti-GHR mAb to the GH receptor; in other words, SAC has the same binding site as GH for the GH receptor. The GH receptor is dimerized before the ligand binds, and one GH molecule binds to two GH receptor molecules.[42,43] Binding of GH causes a structural change in the receptor, which causes the intracellular kinase domain to approach JAK2 and lead to phosphorylation.[44] It is unlikely that one molecule of SAC, a small molecule, will bind to two GH receptor sites at the same time. Therefore, it is thought that two or more SAC molecules bind to each site of the GH receptors and cause a structural change of the GH receptor.

In the past, we demonstrated that small molecular compounds such as ascorbic acid or ascorbic acid 2-glucoside bind directly to the receptor for the IGF-I and promote the proliferation of cultured hepatocytes.[45] However, there are few studies on the interaction between small molecular compounds and large molecular compound receptors such as peptides, and there are no reports of small molecular compounds that bind to GH receptors such as SAC and exhibit functional activity. As shown in Fig. 9, SMC did not show GH binding ability despite its high structural similarity to SAC. Sulfur-containing amino acids with allylic groups may be key points for binding to GH receptors. The amino acid sequences of human and rat GH receptors match by more than 70%.[46] Human GH has the ability to bind to GHR in rat hepatocytes, and it is said that there is almost no species difference in the extracellular domain of GHR.[47,48]

Based on these results, the mechanism of the hepatocyte proliferation-promoting action by SAC is shown in the illustration in Fig. 10. SAC phosphorylates JAK2 by binding to GH receptors expressed on the hepatic cell membrane. Then, phosphorylated JAK2 phosphorylates PLC, which increases intracellular Ca^{2+} levels *via* IP_3. Increased intracellular Ca^{2+} levels induce exocytosis of IGF-I secretory granules pre-stored in hepatocytes in an autocrine manner. The secreted IGF-I then stimulates hepatocytes to phosphorylate IGF-I RTK, thereby inducing DNA synthesis and hepatocyte proliferation through activation of the PI3K/ERK2/mTOR pathway.

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Conflict of Interest The authors declare no conflict of interest.

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