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The Growth-Stimulating Effect of α_1-Acid Glycoprotein in Cells in Culture (40751)[1]

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The chemical nature of α_1-acid glycoprotein (AG), also called orosomucoid, has been clarified extensively (1–3). The physiological functions of AG, however, have been clarified only to a limited extent despite its substantial presence in serum (0.3–1.0 mg/ml). The biological functions of AG include inhibition of hemagglutination caused by influenzavirus, effect on blood clotting, binding of progesterone (4), inhibition of platelet adsorption on collagen (5), and inhibition of lymphocyte blastogenesis caused by plant lectin (6). It is, however, hard to rationalize these activities as the intrinsic nature of AG because many other glycoproteins such as those from egg whites (7) or antifreeze glycoproteins from antarctic fish (8) or varieties of saccharides are known to exhibit such properties as inhibition of hemagglutination or cell adhesion caused by a virus, plant lectin, and others, in which interaction of surface glycoprotein and hemagglutinin are interfered with by a similar type of sugar residue. Therefore, such functions of AG may be rather coincidental.

On the other hand, the plasma level of AG is known to increase during the processes of inflammation, pregnancy, malignancy, rheumatism, and pneumonia. Accompanying all these processes is the propagation of malignant, lymphatic, or normal cells. Yamamoto has previously observed that human pleural or ascitic fluids obtained from cancer patients exhibited enhanced colony-forming and growth-promoting activity to the cells in culture (9). Furthermore, we characterized the active component and concluded that AG is responsible for this growth-promoting effect as described previously (10). In this communication, we would like to reconfirm and expand on the growth-promoting effects of AG for various types of cells in a serum-free medium.

Materials and methods. Preparation of AG. Human or bovine serum was diluted twofold with 0.01 *M* phosphate-buffered saline (0.15 *M*) (PBS, pH 7.0) and heated at 85°C for 30 min. By this process albumin, IgG, and a majority of other proteins were removed as precipitates, and the supernatant (called *heat soup* hereafter) after separation by filtration or centrifugation (2500 rpm for 10 min) was applied on a column of CM-cellulose (Whatman, CM-52). The first peak was obtained as a single peak which was identified as AG (10). The AG preparation thus obtained exhibited a single band both on a disc gel electrophoresis containing 10% acrylamide at pH 8.6 and ampholine thin-layer electrophoresis, pH 2.0–6.5 (11). Ouchterlony's agar diffusion analysis, using an authentic sample of AG prepared by Professor Schmid of Boston University School of Medicine as a reference, also confirmed both preparations are identical immunologically and no contamination was detected. The contamination of α_1-antitrypsin was negative as revealed by highly sensitive fluorescence polarization technique (12). The heat soup fraction obtained from the bovine serum was also assayed without further purification.

Preparation of asialo-AG. In order to reveal the role of sialyl residues in AG, which comprises about 12% of AG in weight, sialic acid was removed from AG with a treatment of sialidase (neuraminidase, Behring Werke AG, Marburg–Lahn) at pH 5.5 in 0.1 *M* Na-acetate buffer. To 12 mg of AG about 15 U of the enzyme was added in 4 ml of the buffer solution and incubated for 30 min at 37°C in the presence of Ca^{2+} (15).

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TABLE I. CHEMICAL COMPOSITION OF MODIFIED RITC MEDIUM (17)

MEM (Nissui)[b]	9.4 g	Serine	10.5 mg
Glucose	2.0 g	Glutamine	292.0 mg
Inositol	12.0 mg	Choline-Cl	8.0 mg
Deoxycytidine	0.03 mg	Na-Pyruvate	110.0 mg
Leucovorine	0.5 mg	$FeSO_4-8H_2O$	0.8 mg
Deoxyadenosine	0.25 mg	Insulin	1.0 mg
Putrescine	0.025 mg		

[a] Make up to 1 liter with distilled deionized water.
[b] Eagle's minimum essential medium.

The enzyme treated AG, as well as neuraminidase control, was dialyzed against 2 liters of 2 m*M* $CaCl_2$ overnight, with three changes of dialysis water followed by lyophilization. Subsequently, the content of the sialic acid was determined (16). With this enzyme treatment, more than 95% of the sialic acid was removed from AG, thus designated as asialo-AG. AG, asialo-AG, and the control (neuraminidase alone) were subjected for assay of growth-promoting activity.

Cells and culture. HeLa S_3 (epithelial type) and human embryonic lung cells (normal fibroblast) (HEL) were employed. These cells grow in an attached state. The Epstein–Barr virus genome-carrying, transformed human lymphoblastoid cell line, C-6, and the Yoshida sarcoma cells (YS) obtained from rats as a primary culture, both grown in a floating state, were also tested. The growth-promoting effect was quantified by increments of cell numbers or of protein content with Folin–phenol reagent (13). HeLa and C-6 were cultured in a test tube (1.5 × 11 cm) with a tight screw cap at 37°C containing a basal medium consisting of a RPMI-1640 medium supplemented with fetal calf serum (FCS) at a final concentration of 0.3%. At this serum concentration the cells showed no growth but remained viable for at least 5 days. HeLa, HEL, and YS cells were also grown in a plastic petri dish (Falcon, ϕ = 5.0 cm) under 5% CO_2 with RITC medium and its modified one (Table I) in which no serum or serum component was added (17).

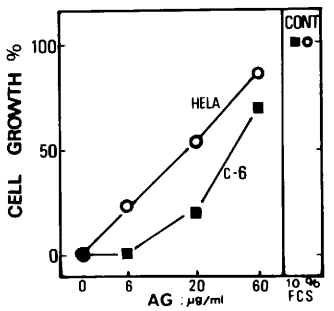

FIG. 1. The growth-stimulating effect of human α_1-acid glycoprotein (AG) on HeLa and C-6 cells. The growth which is based on the protein content was determined by the Folin–phenol method (13) 4 days after inoculation. The inocula of HeLa and C-6 cells were 2 × 10^4 and 2 × 10^5 per tube, respectively. CONT indicates a maximal cell growth attained in the presence of 10% fetal calf serum (FCS). The assay medium contains 0.3% FCS in RPMI-1640 medium in a tight-stoppered test tube.

Results. The effect of AG on the growth of HeLa and C-6 cells which was determined 4 days after inoculation by the protein content is shown in Fig. 1. The growth promoting effect of AG is apparent at more than 6 μg/ml for HeLa and 20 μg/ml for C-6 cells. In this system, a minimum amount of FCS is supplemented to the RPMI-1640 medium but no growth was observed by the medium itself.

In the serum-free RITC media, the effect was tested for AG and heat soup fraction for three different cells (HeLa, HEL, and YS). Without AG or heat soup (Fig. 2) the HeLa cells doubled the cell number, while HEL or YS did not show any appreciable growth (Figs. 3 and 4). When AG was added to the medium, HeLa or HEL exhibited increased cell growth between 1 and 30 μg/ml; however, 100 μg/ml of AG appears to suppress HeLa cells, but the suppressive effect was less marked to HEL cells (Figs. 2 and 3). The heat soup obtained from the bovine serum exhibited higher specific activity between 1 and 10 μg/ml

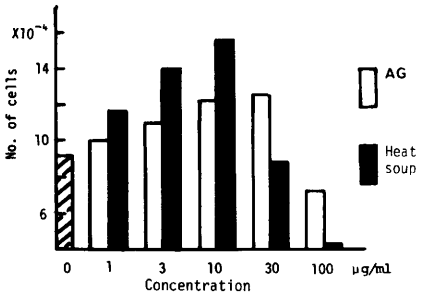

FIG. 2. The growth-stimulating effect of human α_1-acid glycoprotein (AG) and supernatant of heat-denatured serum (heat soup) on HeLa cells in a serum-free medium (RITC). The cells were inoculated at 4×10^4 in a Falcon plastic petri dish (5 cm) under 5% CO_2. The cell numbers were counted on the 6th day after inoculation.

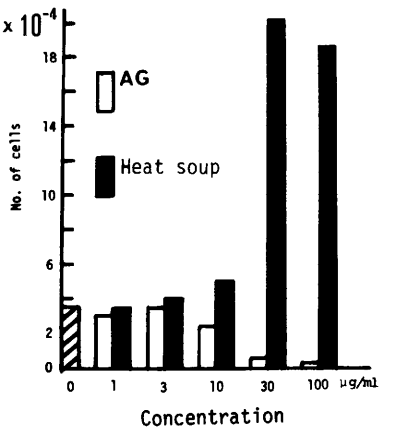

FIG. 4. The growth-stimulating effect of α_1-acid glycoprotein (AG), (blank bars), and supernatant of heat-denatured bovine serum (heat soup) on Yoshida sarcoma cells from rat hepatoma in a serum-free medium. Conditions are similar to Fig. 2.

than AG while at higher concentration (at 30 μg/ml or higher) it suppressed growth activity (Figs. 2 and 3).

The results obtained for YS cells (Fig. 4) were different from those in other cells (Figs. 2 and 3). AG showed no growth-promoting activity, but on the contrary it is rather cytotoxic at higher concentration (30 and 100 μg/ml). The growth-promoting effect of the heat soup on YS cells, however, appears very effective only at higher concentration (30 and 100 μg/ml).

The growth-promoting activity of asialo-AG was tested similarly in HEL cells and the result showed that the activity remained as AG even after removal of the sialyl group from AG.

Discussion. Many proteins are known to stimulate the growth of cells in culture such as protein hormones, thrombin and other proteolytic enzymes, fibroblast growth factor, nerve growth factor, epidermal growth factor, ovarian cell growth factor, fetuin, binding of antibody, and others. Some of them are well characterized and their chemical entities are established while others are, although unique, clarified only in their biological activity.

In the present investigation we have characterized and identified a normal serum component α_1 acid glycoprotein (AG) as a responsible factor for cell growth. This activity is confirmed not only in fibroblast (HEL), epithelial cells (HeLa), but also in floating lymphoblastoid cells (C-6). Their growth is stimulated by AG at a relatively low concentration (less than normal physiological concentration).[3] Furthermore, the AG preparation of Dr. Schmid exhibited similar activity to ours in these cells. YS cells, which are from rats and hepatic in origin, were found unresponsive to AG. Surprisingly, the heat soup of bovine serum exhibited very strong growth-stimulating activity at high concentration to YS which is inhibitory to the other cells. Therefore, these results appear to indicate

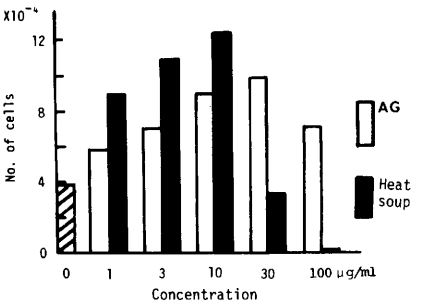

FIG. 3. The growth-stimulating effect of human α_1-acid glycoprotein (AG), and supernatant of heat denatured bovine serum (heat soup) on human lung fibroblast (HEL) cells in a serum free medium. Conditions are similar to Fig. 2.

[3] We found that an addition of serum albumin to the culture medium alters the optimal concentration of AG to a higher concentration than without albumin.

the presence of another growth-stimulating and inhibitory activity. The unresponsiveness of YS cells to AG may be due to variations of species (rat) or different specificity to different origins of the cells (hepatic). Onda *et al.* reported that AG was found suppressive against the regenerating rat liver (16). Although their α_1-glycoprotein needs to establish criteria or an identity with AG such as immunological cross-reactivity with authentic material, content of hexose and the sialic acid, antitryptic activity (negative), as well as chromatographic evidence of a single entity. The present results in YS cells which are, however, similar to that of Onda *et al.* in view of suppressive effect on hepatic cells. In other words growth of cells of hepatic origin may be inhibited by AG.

It is interesting to note two facts: one is the present finding of the growth-stimulating effect of AG and the other is the elevated plasma level of AG in patients with a high cell-propagating state such as pregnancy, malignancy, or inflammation (1–3). Therefore, the mechanism of action of AG is interesting and it should be clarified. An elucidation of the growth effect due to calcium was investigated (known as a growth-stimulating factor (18)) using AG and asialo-AG. Since AG possesses a large number of sialyl residues, it may bind and carry calcium to cells, thus exerting its action. However, this activity of AG as a carrier of calcium to cells seems unrelated to the action of AG because removal of negatively charged sialic acid residues from AG (asialo-AG) did not alter the activity of AG. Another possibility of the action of AG is the binding of progesterone, thus, the uptake of progesterone to cells may be enhanced by AG. Schmid *et al.* suggested that AG possessed a homology in the amino acid sequence with IgG (19). This could be another alternative explanation of the growth stimulation of AG because such a molecule as IgG or β_2-microglobulin or AG, all having sequence homology, may be interacting with cell membranes, thus, triggering a cell division.

We would like to emphasize that one of the biological functions of AG, a normal physiological constituent, is the growth-stimulating activity to certain types of cells in culture at relatively low concentration. In addition there appears to exist an unidentified component(s) which is growth stimulating at low concentration and cytotoxic at high concentration to HeLa or HEL cells in the heat-denatured supernatant of serum, but this factor(s) possess(es) no cytotoxicity to YS cells but on the contrary, exhibits stimulating activity to YS cells at high concentration.

Summary. A highly purified α_1-acid glycoprotein (AG) was found to stimulate the growth of cells in culture above 1 μg/ml in a serum-free medium. This effect was confirmed for the cells which grow as attached on a glass surface or in a floating state, based on the human embryonic lung fibroblast, HeLa, or Epstein–Barr virus-transformed lymphoblastoid cells. The presence of other factor(s) besides AG in the heat-denatured supernatant was indicated by YS (Yoshida sarcoma rat) cells which did not respond to AG at all. Asialo-AG exhibited the same activity as AG which does not carry Ca^{2+}.

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