Cell Growth-Inhibitory Action of SAGP, an Antitumor Glycoprotein from *Streptococcus pyogenes* (Su Strain)

Junko YOSHIDA, Shozo TAKAMURA and Shiro SUZUKI
Department of Pharmacology, Kanazawa Medical University, Uchinada, Ishikawa 920-02, Japan
Accepted June 22, 1987

Abstract—An antitumor acidic glycoprotein (SAGP) from *Streptococcus pyogenes* (Su strain) inhibited the growth of BALB/3T3 cells in culture in a dose-dependent manner (0.03–10.0 μg/ml). This effect of SAGP was abolished by washing the cells, suggesting that SAGP weakly binds to the cell membrane. The ability of the cells to form colonies was unaffected by three days exposure to SAGP. Cell cycle analysis by flow cytometry revealed an accumulation of SAGP-treated cells in the S-phase. SAGP (3 μg/ml) reduced the incorporation of 3H-thymidine into the cells to approximately half of the control level. These results suggest that SAGP inhibits the growth of target cells by acting on their surface membrane and decreasing the rate of DNA synthesis.

We have recently purified an antitumor principle from a soluble fraction of *Streptococcus pyogenes* (Su strain) and identified it as an acidic glycoprotein (termed SAGP) (1). SAGP is composed of three subunits, each with a molecular weight of 50,000 and contains allose (1.5% W/W) as one of the carbohydrate moieties.

In antitumor experiments, SAGP significantly prolonged the life span of mice bearing Ehrlich ascites carcinoma cells and inhibited the proliferation of THEL cells (transformed hamster embryonic lung cells (2)) in culture at a nanomolar order of drug concentration (1). Thus, we suggested that the direct antitumor action of SAGP may be implicated partially in the antitumor effects of streptococci (3–5).

The present study was undertaken to explore the mode of cell growth-inhibitory action of SAGP through the effects on cell growth, DNA synthesis and cell cycle progression of BALB/3T3 cells in culture. This cell line was chosen because experimental methods for investigating the DNA synthesis of these cells have been established and described in the recent literature (6, 7).

Materials and Methods

SAGP was prepared as described earlier (1). Briefly, *Streptococcus pyogenes* (Su strain) cells grown in 60 liters of Wood and Gunsalus broth were collected by continuous centrifugation and washed with Mg²⁺-Tris buffer (pH 7.5). After mechanical disruption of the cells, a supernatant was obtained by centrifugation at 105,000 g for 2 hr. This crude extract was subjected to thermal treatment (45°C, 30 min), streptomycin precipitation, fractionation with ammonium sulfate (55–70%) and column chromatography on octyl-sepharose CL-4B, DE-52 and Sephadex G-200, in that order. The final active fraction was dialyzed against distilled water, and the resulting precipitate was removed. The supernatant thus obtained was lyophilized (SAGP).

BALB/3T3 cells were routinely maintained in Dulbecco's modified Eagle's medium (DME) supplemented with 10% calf serum (CS) and streptomycin (100 μg/ml) and penicillin (100 units/ml) as monolayers in 100-mm Falcon dishes. The cells were grown under a humidified 5% CO₂ atmosphere at 37°C.

For the cell growth assay, the cells (5×10⁴/dish) were placed in 60-mm dishes, containing 5 ml of the culture medium. After the incubation for 24 hr, the medium was renewed, and SAGP dissolved in phosphate-buffered saline (PBS) was added to the
cultures at a designated concentration. The same volume of PBS was added to the control cultures instead of SAGP solution. The cells were cultured for 3 days, then detached by 0.25% trypsin and resuspended in PBS. The cell number was determined on a Coulter counter (model ZBI). The growth inhibition rate (GIR) was calculated as follows:

\[
\text{GIR} (%) = \left(1 - \frac{\text{No. of cells in an experimental dish}}{\text{No. of cells in a control dish}}\right) \times 100
\]

The cytotoxic effect of SAGP on the cells was examined by the colony formation assay. One half milli-liter of cell suspension (400 cells/ml) was added to 60-mm dishes containing 4.5 ml of DME supplemented with 10% CS (10% CS-DME) and SAGP in concentrations ranging from 0.03 to 6.0 \(\mu\text{g}/\text{ml}\). The cultures were incubated at 37°C for 3 days; thereafter, the medium was replaced by the SAGP-free medium. After incubating the cultures for an additional 9 days, colonies formed were rinsed with PBS, fixed with 10% formalin, and stained with 0.1% crystal violet. The plating efficiency of control cultures was approximately 50%.

To measure the cell cycle progression, the cells (5\(\times\)10^4/dish) were cultured in 60-mm dishes, each containing 5 ml of 10% CS-DME. After 24 hr, the medium was renewed, and SAGP was added to the cultures at a concentration of 1.0 \(\mu\text{g}/\text{ml}\). After 48 hr of further incubation, the cells were detached by trypsinization. The single cell suspensions were fixed in cold 70% ethanol, treated with a 0.25% solution of RNase (NBC) in PBS for 30 min at 37°C, and stained with propidium iodide (0.05 mg/ml in PBS). The stained cells were subjected to cytofluorometric analysis using a Cytofluorograf system 50 H (Ortho Instruments) with the laser light at 488 nm (200 mW laser power).

Effect of SAGP on \(^{3}\text{H}\)-thymidine incorporation cells was assayed by the method described by Nishikawa et al. (6, 7) with a slight modification. Subcultured cells (1\(\times\)10^5/dish) were seeded in 60-mm dishes containing 5 ml of 3% CS-DME and allowed to adhere to the bottom of the dishes at 37°C. Five hours later, the medium was replaced by 0.2% CS-DME, and the cells were cultured further for 24 hr at 37°C. Then the medium was replaced by 1% CS-DME. In the experimental cultures, SAGP (1 mg/ml in PBS) was added at final concentrations from 0.3 to 10 \(\mu\text{g}/\text{ml}\). As the control, the cultures without SAGP in both 1% CS-DME and CS-free medium were run in parallel with the experimental cultures. After 16 hr of incubation at 37°C, 1.0 \(\mu\text{Ci}\) of methyl-\(^{3}\text{H}\)-thymidine (specific radioactivity, 40 Ci/mmol, RCC, Amersham, England) was added to all the cultures. After another 3 hr of incubation, the medium was aspirated off. The cells in monolayers were consecutively rinsed with 3 ml of PBS and next with 3 ml of 10% trichloroacetic acid to remove free radioactive thymidine. The cells were digested with 1 ml of 1N NaOH for 30 min, and 0.5 ml of this digest was mixed with a Triton X-100-toluene scintillator which contained trichloroacetic acid (final concentration of 0.85%) for neutralizing NaOH, and the radioactivity in the acid-insoluble fraction was counted in a liquid scintillation spectrometer (Aloka LSC-653). The rate of \(^{3}\text{H}\)-thymidine incorporation was calculated by the following expression:

\[
\text{\(^{3}\text{H}\)-thymidine incorporation (\%) = } \frac{T - C_0}{C - C_0} \times 100
\]

where T, C and C_0 are dpm of \(^{3}\text{H}\) of the cells exposed to SAGP in 1% CS-DME, dpm of the intact cells in 1% CS-DME and dpm of the intact cells in CS-free DME, respectively.

Results

As shown in Fig. 1, SAGP in concentrations ranging from 0.1 to 3.0 \(\mu\text{g}/\text{ml}\) inhibited the growth of BALB/3T3 cells in a dose-dependent manner. Figure 2 shows the time-course of the growth-inhibitory effect of SAGP (1 \(\mu\text{g}/\text{ml}\)). The GIRs (%) on the first, second and third days were 30, 48 and 51, respectively. Thus the inhibitory effect became more prominent with increasing ex-
Fig. 1. Inhibition of growth of BALB/3T3 cells in culture by SAGP. Each point represents the mean ± S.E. of 3 determinations.

Fig. 2. The cell growth-inhibitory effect of SAGP and its abolishment by removal of SAGP from the culture-medium. BALB/3T3 cells were grown in the presence (Δ—Δ) and absence (●—●, ▲——▲) of SAGP (1 μg/ml). Each point represents the mean of 2 determinations.

Fig. 3. Effect of SAGP on cell cycle progression in BALB/3T3 cells. The cells were cultured for 48 hr in the absence (A) or presence (B) of SAGP (1 μg/ml).
the rate of DNA synthesis.

SAGP at concentrations from 1.0 to 10.0 μg/ml inhibited the incorporation of 3H-thymidine into the acid-insoluble fraction of BALB/3T3 cells (Fig. 4). In this experiment, apparent difference in the DNA content between the control and the experimental dishes seems to be unlikely, since there was no difference in number between both dishes at the end of the experiment. Therefore, the decrease of 3H-thymidine incorporation into the acid-insoluble fraction per dish may be mainly due to the inhibitory effect of SAGP on DNA synthesis.

Discussion

SAGP prolonged the population doubling time of BALB/3T3 cells in culture. This means the inhibition of the cell cycle progression by SAGP. Since we were interested in determining whether the inhibition is phase dependent or not, the effect of SAGP on the cell cycle progression was examined by the use of cytofluorometry. The cytofluorometric analysis revealed an accumulation of the cells in the S-phase. Since the S-phase is defined as the period for active synthesis of DNA (8), the accumulation of the cells in the S-phase will reflect the decreased rate of DNA synthesis. This assumption was supported by the decrease of 3H-thymidine incorporation into the acid-insoluble fraction.

In the present study, the maximal inhibitory effect of SAGP on the proliferation of BALB/3T3 cells was observed at a SAGP concentration of 3.0 μg/ml (GIR=ca. 50%). However, the IC50 values of SAGP on THEL and L1210 cells were 0.06 μg/ml (1) and 0.08 μg/ml (J. Yoshida et al., unpublished data), respectively. THEL is tumorigenic in hamsters (2) and L1210, also in mouse. Lower sensitivity of the BALB/3T3 cell line to SAGP might be related to its nontumorigenic property.

Acknowledgments: We thank Professor K. Nishikawa, Second Department of Biochemistry of this University, for providing the BALB/3T3 cell line and for critical advice. We also wish to thank Dr. K. Fujikawa-Yamamoto, Central Laboratory of this University, for his valuable discussions in the study on the cell cycle progression. We are grateful to Miss Y. Shinzawa for typing the manuscript. This study was supported in part by the Science Research...
Promotion Fund from the Japan Private Promotion Foundation.

References
1 Yoshida, J., Yoshimura, M., Takamura, S. and Kobayashi, S.: Purification and characterization of an antitumor principle from *Streptococcus hemolyticus*, Su strain. Japan. J. Cancer Res. 76, 213–223 (1985)
2 Yamada, T. and Hatano, M.: Lowered transplantability of cultured tumor cells by persistent infection with paramyxovirus (HVJ). Gann 63, 647–665 (1972)
3 Nauts, H.C., Swift, W.E. and Coley, B.L.: The treatment of malignant tumors by bacterial toxins as developed by the late William B. Coley, M.D., reviewed in the light of modern research. Cancer Res. 6, 206–216 (1946)
4 Okamoto, H., Shoin, S. and Koshimura, S.: Streptolysin S-forming and antitumour activities of Group A streptococci. In *Bacterial Toxins and Cell Membranes*, Edited by Jeljaszewicz, J. and Wadstrom, T., p. 259–289, Academic Press, London (1978)
5 Shoin, S.: Isolation and fractionation of cell-free extract from streptolysin S-forming streptococci. Gann 67, 661–667 (1976)
6 Nishikawa, K. and Okitsu, C.: Control of BALB/3T3 growth by factors present in tumor extract. In *Hormones and Cell Culture*, Edited by Ross, R. and Sato, G., Vol. 6, p. 441–452, Cold Spring Harbor Press, New York (1979)
7 Nishikawa, K., Yoshitake, Y., Okitsu, C. and Adachi, K.: Growth factors localized in tumor nuclei. In *Control Mechanisms in Animal Cells*, Edited by Jimenez de Asua, L., Shields, R. and Lacobelli, S., p. 279–284, Raven Press, New York (1980)
8 Tobey, R.A. and Crissman, H.A.: Comparative effects of three nitrosourea derivatives on mammalian cell cycle progression. Cancer Res. 35, 460–470 (1975)
9 Hickman, J.A., Scanlon, K.J. and Tritton, T.R.: Membrane targets in cancer chemotherapy. Trends Pharmacol. Sci. 5, 15–17 (1984)
10 Marx, J.L.: Biochemistry of cancer cells: Focus on the cell surface. Science 183, 1279–1282 (1974)
11 Lazarus, H., Raso, V. and Samy, T.S.A.: *In vitro* inhibition of human leukemic cells (CCRF-CEM) by agarose-immobilized neocarzinostatin. Cancer Res. 37, 3731–3736 (1977)
12 Kunimoto, T., Hori, M. and Umezawa, H.: Macromomycin, an inhibitor of the membrane function of tumor cells. Cancer Res. 32, 1251–1256 (1972)
13 Farkas-Himsley, H. and Cheung, R.: Bacterial proteinaceous products (bacteriocins) as cytotoxic agents of neoplasia. Cancer Res. 36, 3561–3567 (1976)