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Inhibitory Effect of a Somatostatin Analogue (SMS 201-995) on the Growth of Androgen-dependent Mouse Mammary Tumor (Shionogi Carcinoma 115)

Shinzaburo Noguchi,[1,4] Yasuko Nishizawa,[2] Kazuyoshi Motomura,[1] Hideo Inaji,[1] Shingi Imaoka,[1] Hiroki Koyama[1] and Keishi Matsumoto[3]

[1]Department of Surgery and [2]Department of Experimental Pathology, The Center for Adult Diseases, Osaka, 3-3 Nakamichi 1-chome, Higashinari-ku, Osaka 537 and [3]Osaka Medical Center for Maternal and Child Health, Murodoh-cho 840, Izumi, Osaka 590-02

The influence of a somatostatin analogue, SMS 201-995 (SMS), on the growth of an androgen-dependent mouse mammary tumor, Shionogi carcinoma 115 (SC115), was studied. Treatment of SC115 tumor-transplanted male mice with s.c. injections of SMS (0.04, 0.2, 1, and 5 μg twice a day) resulted in a dose-dependent inhibition of tumor growth. The growth-inhibitory effect of SMS reached its peak at a dose of 1 μg twice a day. SMS was found not to elicit its growth-inhibitory effect through lowering plasma testosterone levels or down-regulating androgen receptor of SC115 tumors. Since specific binding sites for somatostatin were not observed in the membrane fractions of SC115 tumors and SMS did not inhibit the proliferation of primarily cultured SC115 tumor cells, a direct inhibitory mechanism of SMS on SC115 tumors was unlikely to be operative. Since SMS is a very potent inhibitor of growth hormone (GH) secretion, it was speculated that SMS might inhibit the growth of SC115 tumors indirectly through down-regulation of plasma GH levels. This possibility was evaluated by studying the influence of GH replacement on the growth of SC115 tumors grown in SMS-treated mice. GH replacement was done both in a male secretory pattern (intermittent injection, human GH 500 μg/kg twice a day) and in a female secretory pattern (continuous infusion, 1000 μg/kg/day). Intermittent injections of GH fully restored the growth of SC115 tumors in the SMS-treated mice to that in the normal controls but continuous infusion of GH was without effect. These results suggest that SMS inhibits the growth of SC115 tumors through suppression of GH secretion, and that the mode of GH administration is an important determinant of its action on SC115 tumor growth.

Key words: Mammary tumor — Androgen — Somatostatin — Growth hormone

Somatostatin is a tetradecapeptide which was originally isolated from the ovine hypothalamus.[1] It was found to inhibit the secretion of growth hormone (GH) and, under certain conditions, PRL (prolactin) release.[2] Later studies have shown that somatostatin inhibits the secretion of almost all known gastrointestinal hormones from normal and neoplastic tissues.[3,4] These general inhibitory functions of somatostatin prompted clinical application of this substance in the treatment of a variety of diseases with hypersecretion of peptide hormone(s).[5] The clinical usefulness of somatostatin, however, is limited by its short plasma half-life (1 to 3 min). This drawback has been overcome by development of a long-acting, synthetic octapeptide analogue of native somatostatin, SMS 201-995 (SMS), which has a long plasma half-life of approximately 113 min.[6] SMS has been successfully applied in the treatment of patients with GH-secreting pituitary adenoma, carcinoid tumor, gastrinoma, VIPoma, and glucagonoma.[5] In some of these patients, SMS not only inhibited the secretion of specific hormones, but also reduced the tumor size. This antineoplastic activity of SMS seems not to be specific to the endocrine tumors, since experimental studies have shown that SMS inhibits the growth of human pancreatic and gastric cancer xenografts in nude mice and that of murine colon cancer autografts in mice.[5]

In addition to these gastrointestinal tumors, recent studies have disclosed the inhibitory effect of SMS on the growth of sex-steroid dependent cancers such as breast cancer[7,8] and prostate cancer.[9-11] It has been reported that SMS inhibits the growth of estrogen-dependent human breast cancer both *in vivo*[8] and in cell culture.[7] Bogden *et al.* reported that SMS exerted its growth-inhibitory effect on an androgen-dependent rat prostate tumor, and that this growth-inhibitory effect was not mediated through suppression of androgen production in the testes.[10] The mechanism through which SMS exerts its growth-inhibitory effect still remains largely unknown. A direct antiproliferative effect mediated through specific, high-affinity somatostatin receptors on tumor cells and/or an indirect effect via suppression of release of GH, PRL, and other growth factors (epidermal growth factor (EGF), insulin-like growth factor-I (IGF-I)) may be responsible for the growth-inhibitory action of SMS.[5]

Demonstration of the growth-inhibitory effect of SMS on sex-steroid-dependent cancers prompted us to evalu-

[4] To whom correspondence should be addressed.

ate its effect on an androgen-dependent mouse mammary tumor, Shionogi carcinoma 115 (SC115). SC115 was established as a transplantable, androgen-dependent mouse mammary tumor in 1964[12] and has been used as a model of androgen-dependent cancer. The mechanism of action of androgen in growth stimulation has been extensively studied with this tumor both *in vivo*[13-15] and in cell culture.[16-19] In the present paper, the influence of SMS on the growth of SC115 tumors was studied in mice. We have found that SMS inhibits the growth of SC115 tumors, and its growth-inhibitory effect can be reversed by GH supplemented in a male, but not female, secretory pattern.

MATERIALS AND METHODS

Animals and tumors Two- to 3-month-old male DS mice raised in our laboratory were used. When tumors were grafted in castrated mice, the castration was carried out at least 1 week in advance. A fragment of tumor (1 μl) was inserted beneath the dorsal skin, using a specially devised needle.[12] Seed tumors were taken from SC115 tumors maintained in male DS mice.

Chemicals [1,2,6,7-^{3}H]Testosterone (99 Ci/mmol) and [^{125}I]Try^1-somatostatin (2200 Ci/mmol) were purchased from New England Nuclear (Boston, MA). Unlabeled somatostatin was from Sigma Chemical Co. (St. Louis, MO). Human GH and SMS were generous gifts from Yamanouchi Pharmaceutical Co. Ltd. (Tokyo) and Sandoz Ltd. (Basel, Switzerland), respectively. The other chemicals were of analytical grade.

Injection of hormones SMS were dissolved in 0.1 ml of saline and injected s.c. twice a day at 8:00 and 20:00. Since the GH secretory pattern is sex-differentiated and the mode of GH administration is crucial for the biological effects obtained, GH was given in two different schedules according to the method of Jansson *et al.*,[20] i.e., intermittent and continuous administration, mimicking the male and female secretory patterns, respectively. Intermittent administration of GH was performed by s.c. injections of GH (500 μg/kg) twice a day at 8:00 and 20:00 h. Continuous infusion of GH (1000 μg/kg/day) was accomplished using osmotic minipumps (model 2002, Alza Corp., Plo Alto, CA) implanted intraperitoneally under ether anesthesia. The filling volume of these pumps is 200 μl and the pumping rate at 37°C is 0.5 μl/h. The minipumps were implanted one day in advance of tumor transplantation. The growth rate of SC115 tumors in intact mice and that in mice implanted with the minipumps filled with saline were identical.

Determination of tumor growth Length and width of each tumor were measured once a week, and the mean of the length and width was used as an index of tumor size.

Cell growth experiments Primary cell culture from SC115 tumors was carried out according to the method previously described.[21] In brief, SC115 tumors grown in normal males were removed 4 weeks after the transplantation of seed tumors. The tumors were minced and digested in Hanks' balanced salt solution (Ca^{2+} and Mg^{2+}-free) containing 0.1 mg/ml trypsin, 0.5 mg of collagenase type IV, 1 mg/ml bovine serum albumin (BSA), and 5 m*M* N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES) for 30 min at 37°C. The dispersed cells were washed once with Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS) and 3 times with Hanks' balanced salt solution and then resuspended in 2 ml of 10% FCS-MEM, and plated onto 35 mm dishes (2×10^4 cells/dish). On the following day, the medium was aspirated and the cells were rinsed three times with phosphate-buffered saline (PBS). Then, 2 ml of growth experiment medium composed of Ham's F12: MEM (1:1) and 0.1% BSA was added to each dish and the cells were allowed to grow in the presence or absence of 10^{-8} *M* testosterone with or without 10^{-10}–10^{-6} *M* SMS. The medium was changed every 2 days and the cells were harvested 8 days after plating. Cell number was counted by a Coulter counter after dispersing the cells by incubating them with PBS containing 0.02% EDTA and 0.05% trypsin for 15 min at 37°C.

Androgen receptor (AR) assay SC115 tumors grown in males were removed 24 h after the castration. AR in tumor cytosols was assayed as we have described previously.[22] In brief, tumor cytosols were obtained by ultracentrifugation (105,000*g* for 60 min) of the tumor homogenates. The cytosols were incubated with various concentrations of [^{3}H]testosterone in the presence or absence of a 500-fold molar excess of cold testosterone 18 h at 4°C. Free and bound [^{3}H]testosterone were separated by a dextran-coated charcoal method. The maximum number of binding sites and K_d were calculated according to the method of Scatchard.[23]

Somatostatin receptor assay Determination of somatostatin receptor in the membrane fractions of SC115 tumors and human breast cancers was carried out according to the method of Srkalovic *et al.*[24] Human breast cancers (infiltrating ductal carcinoma) were included as positive controls for somatostatin receptor. SC115 tumors grown in males were removed 4 weeks after the transplantation. Human breast cancers and SC115 tumors were stored at −80°C until processed. All the following procedures were carried out at 4°C unless otherwise specified. After removal of necrotic tissue, the tumors were homogenized in 5 volumes of 50 m*M* HEPES, 0.32 *M* sucrose, 1 m*M* EDTA, and 0.5 m*M* phenylmethylsulfonyl fluoride (PMSF), pH 7.4, in a Teflon-glass homogenizer. The homogenate was centri-

fuged at 500*g* for 10 min. The supernatant containing the membranes was centrifuged at 70,000*g* for 45 min and the resultant pellet was gently resuspended in the buffer (50 m*M* HEPES and 0.5 m*M* PMSF, pH 7.4).

Membrane preparations (50 μg protein) were incubated in 150 μl of the binding assay buffer (50 m*M* HEPES, 150 m*M* NaCl, 0.5 m*M* PMSF, and 0.5% BSA; pH 7.4) containing various concentrations of [^{125}I]somatostatin (0.01–4 n*M*) for 120 min at 21°C. Non-specific binding was assessed by adding a 500-fold molar excess of unlabeled somatostatin to parallel tubes. After the incubation, the reaction mixtures were filtered through Whatman GF/B filters (Whatman, Inc., Maidstone, Kent, UK) with two additional washings with 10 ml of the buffer containing 50 m*M* HEPES and 0.5 m*M* PMSF, pH 7.4. The radioactivity on the filters was measured in a Beckman Gamma 5500 counter (Beckman, Palo Alto, CA). The maximum number of binding sites and K_d were estimated according the method of Scatchard.[23]

Miscellaneous Total testosterone levels in serum were determined by radioimmunoassay according to the method previously described.[25] The protein concentrations were assayed by using a protein assay kit (Bio-Rad, Richmond, CA).

RESULTS

Influence of SMS treatment on the growth of SC115 tumors in mice Seed SC115 tumors were grafted into normal and castrated male mice and the mice were injected s.c. with various doses of SMS or vehicle twice a day starting from the day of transplantation (Fig. 1). Growth of SC115 tumors was rapid in normal males but quite slow in castrated males, indicating strict androgen-dependent growth of SC115 tumors. Treatment of normal males with SMS resulted in a dose-dependent inhibition of SC115 tumor growth.

In order to study the time of onset of the growth-inhibition by SMS, three treatment schedules with SMS (1 μg, twice a day) were started, beginning one week prior to transplantation of SC115 tumors or from the day of transplantation or one week following transplantation (Fig. 2). SMS treatment significantly inhibited the growth of SC115 tumors when it was started one week in advance of transplantation or from the day of transplantation, but was without effect when it was started one week after transplantation.

Influence of SMS treatment on serum testosterone levels and weight of seminal vesicles and ventral prostates The mice used in the study presented in Fig. 1 were killed 4 weeks after the transplantation of seed SC115 tumors, and serum testosterone levels, and weights of seminal vesicles and ventral prostates were measured (Table I).

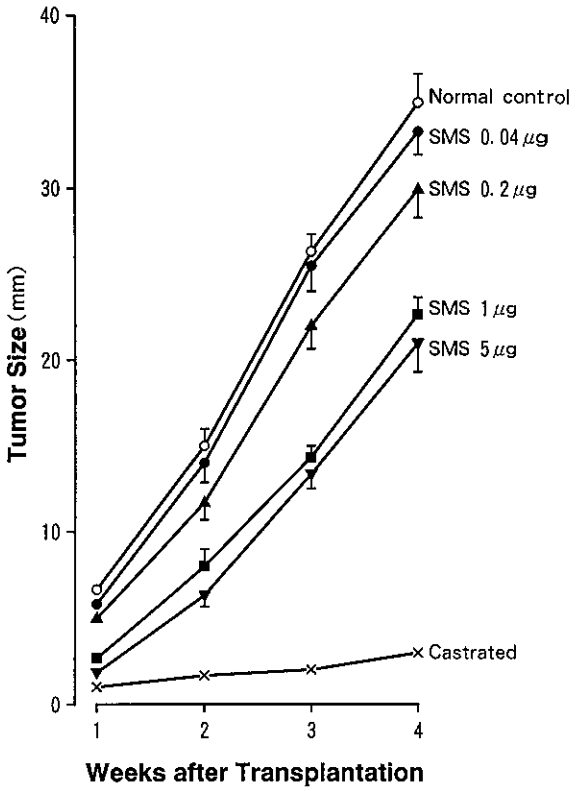

Fig. 1. Effects of various doses of SMS on the growth of SC115 tumors in male mice. Seed SC115 tumors were transplanted into normal or castrated males, and the mice were s.c. injected with various doses of SMS (0.04–5 μg) or vehicle twice a day, starting from the day of transplantation. Each point represents the mean of 6–8 mice; bars, SE.

Castrated males showed a significant reduction in serum testosterone levels and weights of seminal vesicles and ventral prostates as compared with normal males. In comparison with normal males, treatment with SMS did not significantly affect serum testosterone levels or weights of the androgen-target organs at any of the doses tested. Also, body weights were not affected by SMS treatment. These results indicate that the growth-inhibitory effect of SMS on SC115 tumors is not mediated through suppression of serum testosterone levels.

Influence of SMS on AR levels in SC115 tumors SC115 tumors grown in normal males for 4 weeks were removed to be assayed for AR, 24 h after castration. SC115 tumors grown in castrated mice were removed 8 weeks after the transplantation as growth of the tumors was so slow (it took 8 weeks to obtain a tumor volume of adequate size for AR assay). The maximum number of binding sites and K_ds of AR of SC115 tumors grown in

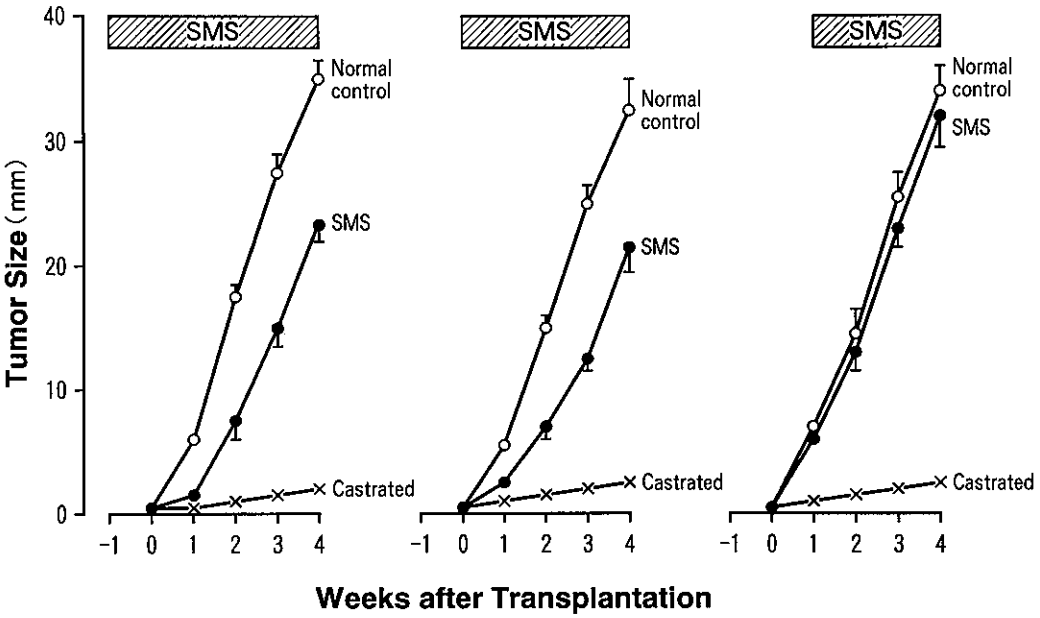

Fig. 2. Influence of starting time of SMS treatment on the growth of SC115 tumors in male mice. Seed SC115 tumors were transplanted into normal male mice, and the mice were s.c. injected with 1 μg of SMS or vehicle twice a day. Treatment with SMS was started from 1 week prior to the transplantation (left panel) or from the day of transplantation (middle panel) or from 1 week after the transplantation (right panel). Each point represents the mean of 6–8 mice; bars, SE.

Table I. Influence of SMS Treatment on the Weights of Seminal Vesicles and Ventral Prostates and on the Levels of Serum Testosterone and Androgen Receptor of SC115 Tumors

	Body weight (g)	Weight (mg)		Serum testosterone (ng/dl)	Androgen receptor	
		Seminal vesicle	Ventral prostate		MBS (fmol/mg.p)	K_d (nM)
Normal male	30±2[a]	82±3	12±1	337±25	45±3	0.6±0.1
Normal male +0.04 μg SMS	31±1	83±4	12±1	356±30	42±4	0.7±0.1
Normal male +0.2 μg SMS	30±2	80±4	13±2	321±30	40±4	0.7±0.1
Normal male +1 μg SMS	29±2	79±3	11±1	354±44	42±4	0.8±0.11
Normal male +5 μg SMS	28±2	78±4	11±2	309±38	41±5	0.7±0.1
Castrated male	29±1	12±1[b]	2±1[b]	57±3[b]	12±3[b]	0.7±0.2

Seed SC115 tumors were transplanted into normal or castrated males, and the mice were s.c. injected with various doses of SMS (0.04–5 μg) twice a day, starting from the day of transplantation. The mice were killed by exsanguination under ether anesthesia 4 weeks after transplantation. Blood was collected from the right atrium and centrifuged at 8,700g for 10 min to obtain serum. Testosterone levels in the serum were assayed by radioimmunoassay according to the method previously described.[25] The seminal vesicles and ventral prostates were removed and weighed. Androgen receptor of SC115 tumors were assayed by a dextran-coated charcoal method.[22]

a) Mean±SE of 6–8 mice.

b) $P<0.01$ when compared with the normal males.

Abbreviations used in this table are: MBS, maximum number of binding sites; K_d, equilibrium dissociation constant; mg.p, mg protein.

normal males with SMS treatment were not significantly different from those in normal males without SMS treatment (Table I). The maximum number of binding sites of AR of SC115 tumors grown in castrated mice was significantly lower than that in SC115 tumors grown in normal males without SMS treatment, although there was no significant difference in K_d values between them.

Effect of testosterone and/or SMS on the proliferation of primarily cultured SC115 cells Primarily cultured SC115 cells were grown in serum-free culture as

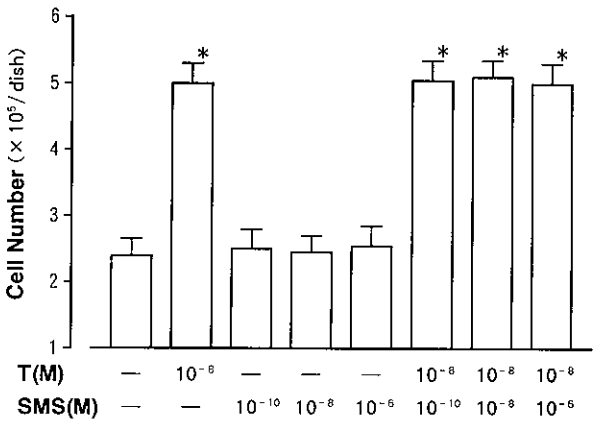

Fig. 3. Effects of SMS on the proliferation of primarily cultured SC115 tumor cells. Primarily cultured SC115 tumor cells were allowed to grow in the presence or absence of testosterone (T, 10^{-8} *M*) with or without SMS (10^{-10}–10^{-6} *M*). Cells were harvested 8 days after plating. Cell number was counted by a Coulter counter. Each bar represents the mean ± SE of 4 separate determinations. * $P<0.01$ when compared with the T-free and SMS-free controls.

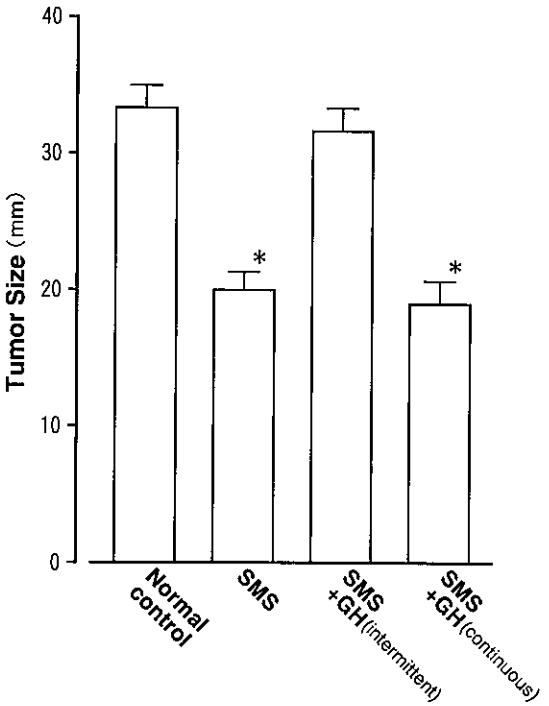

Fig. 4. Effects of GH replacement on the growth of SC115 tumors in SMS-treated male mice. Seed SC115 tumors were transplanted into normal male mice, and the mice were s.c. injected with 1 μg of SMS or vehicle twice a day, starting from the day of transplantation. Some of SMS-treated mice were also treated with intermittent s.c. injection of GH (500 μg/kg, twice a day) or with continuous infusion of GH (1000 μg/kg/day, osmotic minipumps). Tumor size at 4 weeks after the transplantation is shown in this figure. Each bar represents the mean ± SE of 6–8 mice. * $P<0.01$ when compared with the normal controls.

described in "Materials and Methods." Testosterone (10^{-8} *M*) increased the cell number by 2-fold as compared with the control (testosterone- and SMS-free) (Fig. 3). SMS alone did not affect the cell number at any concentration (10^{-10} *M*–10^{-6} *M*) when compared with the control group. Also, testosterone-stimulated growth of SC115 cells was not inhibited by SMS.

Somatostatin receptor of SC115 tumors and human breast cancers High-affinity somatostatin-binding sites were demonstrated in two out of three cases of breast cancer. Maximum numbers of binding sites in these two cancers were 58 and 87 fmol/mg protein, and their K_d values were 0.9 and 0.7 n*M*, respectively. Five SC115 tumors were assayed for somatostatin receptor but specific binding was not observed in any of them.

Reversal of growth-inhibitory effect of SMS on SC115 tumors by GH Seed SC115 tumors were transplanted into normal male mice, and the mice were injected s.c. with SMS (1 μg) or vehicle twice a day from the day of transplantation. Some of the SMS-treated mice were also given GH through intermittent injection (twice a day) or continuous infusion with nimipumps (Fig. 4). SMS treatment resulted in a significant inhibition of SC115 tumor growth and this inhibition was completely reversed by intermittent injection of GH, but not by continuous infusion of GH.

Treatment of SC115 tumor-transplanted normal males or castrated males with intermittent injection of GH did not affect the tumor growth (data not shown).

DISCUSSION

In the present study, we have found that SMS inhibits the growth of SC115 tumors in mice. In an attempt to elucidate the mechanism of action of SMS in the growth-inhibition of SC115 tumors, we have firstly investigated the possibility that SMS inhibits the growth of SC115 tumors indirectly through suppression of serum testosterone levels, because the growth of SC115 tumors is strictly dependent on androgen. This indirect mechanism of SMS, however, is not likely to operate since SMS treatment affected neither serum testosterone levels nor the weights of androgen-target organs such as seminal vesicle or ventral prostate. Our findings are consistent with the reports of Bogden *et al.*[10] and Murphy *et al.*[9] that the growth-inhibitory effect of somatostatin analogues on prostate tumors is not attributable to the suppression of testicular function. They reported that treatment of tumor-bearing rats with somatostatin analogues did not

result in a decrease in serum testosterone levels or organ weights of ventral prostates.[9, 10] We have also investigated the influence of SMS on AR levels of SC115 tumors, i.e., whether SMS has the potential to inhibit tumor growth through down-regulating AR and rendering cancer cells less sensitive to androgens. SMS treatment, however, was found not to affect AR levels of SC115 tumors grown in male mice, thereby providing evidence that the growth-inhibitory effect of SMS is not mediated through down-regulation of AR.

Though a direct growth-inhibitory effect of SMS on human breast cancer cells[7] and pancreatic cancer cells[26] via somatostatin receptor on the cell surface has been demonstrated in cell culture, SMS is unlikely to elicit its inhibitory effect directly on the growth of SC115 tumors for the following reasons: SC115 tumors lack specific, high-affinity somatostatin-binding sites in the membrane fraction and SMS does not affect the proliferation of primarily cultured SC115 tumor cells.

Since SMS is a very potent inhibitor of GH secretion (19 times more potent than native somatostatin), we have studied whether or not SMS inhibits the growth of SC115 tumors through suppression of GH secretion. The GH secretory pattern has been shown to be sex-differentiated, i.e., males display large GH pulses at 3–3.3 h intervals interrupted by low baseline secretion while females have smaller and more frequent pulses and higher baseline levels.[27, 28] The mode of GH secretion (female or male pattern) has been implicated in the regulation of EGF receptor (EGFR) expression[20] and the activities of some enzymes involved in sex-steroid hormone metabolism in the liver.[29] The GH secretion pattern is also reported to play an important role in promoting longitudinal bone growth,[30] i.e., a male secretory pattern is more effective than a female secretory pattern. In view of these secretory pattern-dependent effects, GH was given to the SMS-treated mice in a male secretory pattern (intermittent injection) and female secretory pattern (continuous infusion). We have found that GH replacement in a male secretory pattern could fully restore the growth of SC115 tumors in male mice treated with SMS, but GH replacement in a female secretory pattern was without such effects. Since androgens determine the male secretory pattern of GH, it is conceivable that the growth-stimulative effect of androgens on SC115 tumors is at best partially mediated through regulation of GH secretory pattern. These results are consistent with the recent report of Jansson *et al.* that androgens may exert their stimulatory effect on longitudinal bone growth mainly by altering the secretory pattern of GH.[30]

The reason why GH given in a male, but not female, secretory pattern was effective in reversing the inhibitory effect of SMS remains to be established. Most actions of GH, if not all, are believed to be mediated through IGF-I which is produced in the liver and peripheral tissues under the control of GH and stimulates the growth of various types of cells.[31] Recent studies have suggested that androgens increase IGF-I levels in plasma through modulating the GH secretory pattern in cattle.[32] However, plasma IGF-I levels are reported not to be increased by androgens in mice, though androgens can modulate the GH secretory pattern,[33, 34] suggesting that the mode of GH secretion does not affect plasma IGF-I levels in mice. In addition, IGF-I does not stimulate the growth of SC115 cells in cell culture.[35, 36] Thus, IGF-I appears not to mediate the action of GH in the reversal of the inhibitory effect of SMS on the growth of SC115 tumors. However, there remains a possibility that local production of IGF-I is GH secretory pattern-dependent and IGF-I affects the growth of SC115 tumors indirectly through stimulating the proliferation of stromal cells and angiogenesis.

Although SMS is reported to lower plasma levels of EGF,[4] the growth-inhibitory effect of SMS is unlikely to be mediated through suppression of plasma EGF levels since EGF is not implicated in the growth stimulation of SC115 tumors. We studied the influence of EGF on the growth of SC115 tumors by removing the submandibular glands which produce and secrete EGF into the blood. Removal of the submandibular glands resulted in a significant decrease in plasma levels of EGF but did not affect the growth of SC115 tumors in male mice, indicating that the growth of SC115 tumors is not dependent on EGF (data not shown). In addition, Jansson *et al.* reported that GH did not affect plasma levels of EGF in mice.[20] Thus, EGF seems not to mediate the secretory pattern-dependent growth stimulation by GH. An inhibitory effect of SMS on the PRL secretion[2] seems not to be involved in the growth-inhibitory mechanism of SMS because the growth of SC115 tumors, unlike that of other mammary tumors of rodents, is not dependent on PRL.[22]

SMS inhibited the growth of SC115 tumors in mice when SMS treatment was started from the day of transplantation or one week in advance of transplantation but not when it was started one week after transplantation. These results suggest that SMS retards the process of implantation of tumor grafts rather than inhibiting the growth of established tumors. This observation is inconsistent with the reports of Weber *et al.*[8] and Bogden *et al.*[10] that SMS treatment resulted in a significant inhibition of growth of human breast cancer xenografts (MCF-7) in nude mice and rat prostate tumors (R-3327) in rats when SMS treatment was started after the grafted tumors were well established. We have shown that SMS is unlikely to inhibit the growth of SC115 tumors through a direct mechanism since SC115 tumors lack somato-

statin receptors and SMS does not inhibit the proliferation of SC115 cells in cell culture. On the other hand, both MCF-7 cells and R-3327 tumors have somatostatin receptors[24] and SMS can directly inhibit the growth of MCF-7 cells through somatostatin receptors in cell culture.[7] The differential responsiveness to a direct effect by SMS may help explain why SMS treatment is effective in well-established MCF-7 and R-3327 tumors, but not in well-established SC115 tumors.

In conclusion, we have shown that the growth-inhibitory effect of SMS on SC115 tumors is mediated through suppression of GH, and that GH replacement in a male, but not female, secretory pattern can fully reverse this inhibition. Further studies are necessary to elucidate the influence of the mode of GH secretion on the growth of other types of cancer.

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