Effects of Testosterone and Estradiol on Serum Somatomedin A and Growth Rate of Rats

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Abstract

The mode of involvement of sex steroids in the growth spurt during adolescence was studied in Wistar rats with a special reference to the level of serum somatomedin A (SMA) determined by radioreceptor assay. Intramuscular administration of testosterone propionate (T; 1 mg/day, alternately for 10 days) to female or gonadectomized male rats provoked a small but significant increase in their body weight or body length without affecting the serum SMA level. In contrast, in hypophysectomized (hypox) male rats T caused a considerable increase in body weight and the serum SMA level only when administered concurrently with bovine growth hormone (bGH; 0.2 U/day, ip). T did not affect the sulfation activity *in vitro*. These results suggest that androgen participates in the growth spurt during adolescence by enhancing the SMA effect and/or potentiating the SMA production by GH.

Estradiol benzoate (E₂; 100 µg/day, alternately for 10 days) caused a decrease in the serum SMA level and the growth rate in normal male rats. However, E₂ produced an increase in the SMA level when administered to hypox male rats, although the growth was retarded and sulfation potency of the serum was sharply reduced. These results indicate that E₂ may suppress the growth by lowering SMA generation in normal rats and cause a production of biologically inactive SMA in hypox male rats.

Although growth hormone (GH) plays a major role in the stimulation of the growth of bone, skeletal muscle and several other tissues, it is also possible that hormones of the thyroid, parathyroids, adrenals, gonads and pancreatic islets are involved in the growth (Daughaday *et al.*, 1975). The marked pubertal increase in the growth velocity has been ascribed to the concurrent enhancement of the secretion of sex steroids in addition to sustained GH secretion (Daughaday *et al.*, 1975). Testosterone

was shown in animals to interact with sub-maximal doses of GH in increasing epiphyseal cartilage growth (Geschwind and Li, 1955). On the other hand, pharmacological doses of estrogens have been reported to retard the skeletal growth both in man and in animals (Almqvist *et al.*, 1961; Berntsen, 1968).

It is established that the growth-promoting effect of GH on the skeletal system is mediated by somatomedins (Daughaday *et al.*, 1972). Therefore, elucidation of the relationship between somatomedins and sex steroids appears crucial for understanding the influence of sex

steroids on the growth response. Although several papers have already dealt with this problem by using the bioassay of somatomedin activity, the relationship between the growth spurt, gonadal activity and the serum somatomedin level remains yet to be settled. The uncertainty appears to stem in part from the poor reliability of available bioassay systems for somatomedins. The assay suffers from serious interference by several serum constituents, for example free fatty acids and glucocorticoids (Eisenbarth *et al.*, 1973). The radio-receptor assay (RRA) is an alternate and is considered more specific in measuring somatomedins in serum.

In the present study, the effects of gonadal steroids were examined in rats in terms of the growth rate and of the serum somatomedin activity by RRA and bioassay. The *in vitro* action of steroids on somatomedin bioassay was also studied. From these experiments, it was shown that testosterone stimulated the skeletal growth mainly by potentiating the effect of SMA. Estradiol, on the other hand, caused an inhibition of growth by diminishing the SMA production or by producing abnormal SMA in the presence or absence of the pituitary, respectively.

Materials and Methods

Materials:

Testosterone propionate in sesame oil (ENARMON), estradiol benzoate in water suspension (OVAHORMON) and the respective vehicles were kindly donated by Teikokuzoki Co. (Tokyo). Testosterone propionate and estradiol benzoate used in *in vitro* experiment were purchased from Sigma (St. Louis, MO). Bovine GH was supplied from the National Pituitary Agency (NIH-GH-B18, The biological activity is 0.8 IU/mg). It was dissolved in a slightly alkaline solution of 0.9% NaCl at a concentration of 2.5 mg/ml. Radioactive sulfate (^{35}S) was obtained from the Radiochemical Center (Amersham, UK).

Animals:

Wistar strain rats of both sexes were used. They were fed *ad libitum* with a standard laboratory rat chow (Oriental Yeast Industry Co. Tokyo) and tap

Fig. 1. * and ▼ indicate the day when sacrificed and injected, respectively.

water. Some rats underwent transaural hypophysectomy at 21 days of age. They were used when hypopituitarism was confirmed by growth retardation. As shown in Fig. 1, the animals were randomly allocated into four groups at the age of 25 days (the number of each group is described in the figures and tables). From 30 through 39 days, they received 1 mg of testosterone propionate (T), 100 µg of estradiol benzoate (E_2) or vehicle of T im on alternate days (5 times). Some of the hypox rats concurrently received 0.2 U bGH ip every day. Twenty four hours after the last injection, under anesthesia with pentobarbital the body weight and length (nose to tail tip) were measured and the blood was withdrawn from the abdominal aorta. In group 1, the above procedures were performed at 30 days of age. Serum was separated and kept frozen at -70°C until assay.

Radioreceptor assay of SMA:

Rat serum SMA was determined by a radioreceptor assay (Takano *et al.*, 1975). Pooled serum from two healthy males and two healthy females ages between 20 and 25 was used as an arbitrary reference. One unit (U) of SMA is arbitrarily defined as the SMA content in 1 ml of this pooled serum. The data are presented on this arbitrary unit.

Bioassay of somatomedins:

Sulfation activity of the rat serum was measured with embryonic chick cartilage using a slight modification of the method by Hall (1970) as described previously (Kawai and Ogata, 1981). Half pelvic cartilage from 12-day-old chick embryo was added to the tube containing 1 ml of the assay mixture which consisted of the Minimum Essential Medium with Hank's salts (GIBCO, U.S.A.) supplemented with 150 µg glutamine, 100 µg kanamycin, 30 IU of penicillin, 0.2 µCi of carrier-free ^{35}S -sulfate and serum. Serum was added in

final concentrations of 5% and 20% for a four point assay. Following the incubation the cartilage was boiled for 10 min, placed in saturated Na_2SO_4 for 3 hr and then rinsed with distilled water. The cartilage was solubilized in a scintillation vial with 1 ml of 0.5 N quarternary ammonium hydroxide in toluene (Soluene-350, from Packard, U.S.A.) and diluted with 9 ml of toluene-Triton X-100 (2 : 1, V/V) containing 0.4% 2,5-diphenyloxazole (PPO) and 0.008% 1,4-bis[2-(5-phenyloxazolyl)] benzene (POPOP) (Dry Blend 1, from Eastman, U.S.A.). Radioactivity was measured in a liquid scintillation spectrometer (Packard). The samples were analysed in quadruplicate. One unit is arbitrarily defined as the sulfation activity in 1 ml of the pooled normal human serum described above.

Serum estradiol and testosterone levels were determined with CIS-radioimmunoassay kits (Green-Cross Co. Tokyo).

Statistical analysis of results was carried out using Student's *t* test. Results were compared only among groups of animals from the same batch. Mean values were expressed as mean $\pm$ SEM.

Results

Changes in serum SMA with aging

The body length and the serum SMA content were examined in male rats as function of age. The growth spurt was recognized following the onset of puberty beginning at about 4 weeks of age. The SMA increased in rough correlation with the increase in body length (Fig. 2).

Effect of sex steroids on serum SMA and growth of normal male rats

As shown in Table 1, administration im of T (1 mg/day, alternately for 10 days) tended to increase the serum SMA and the body weight although statistically not significant.

Fig. 2. Increase in the body length and serum SMA level of the rat following the onset of puberty. Under ether anesthesia, the body length (nose to tail tip) was measured and the blood was withdrawn by heart puncture. The change was studied in the bled rat. Values are mean $\pm$ SEM ($n=4-6$).

The serum testosterone contents of the T group and the vehicle group were 3.6 ng/ml and 0.284 ng/ml, respectively (by radioimmunoassay). Administration im of E_2 (100 $\mu\text{g/day}$, alternately for 10 days) diminished the increase in body weight, length and food intake. The age-related increase in the serum SMA was also suppressed. The effects of vehicles for both T and E_2 were compared in a preceeding series of experiments. They showed no significant difference. Therefore, only the vehicle of T was examined as a control. Serum E_2 content of the E_2 group and the vehicle group was 294 pg/ml and 15.6 pg/ml, respectively (by radioimmunoassay). Smaller doses of T (10

Table 1. Effect of sex steroids on growth of normal male rat and serum SMA levels.

Groups	No.	SMA (U/ml)	Body length (cm)	Body weight (g)	Food intake (g/day/rat)
Before	5	1.94 $\pm$ 0.02	28.4 $\pm$ 0.3	132 $\pm$ 4	11.8 $\pm$ 2.2
Testosterone propionate (1 mg/day)	5	4.81 $\pm$ 0.40 ^a	32.9 $\pm$ 0.3 ^a	181 $\pm$ 3 ^a	14.9 $\pm$ 1.0 ^a
Estradiol benzoate (100 $\mu\text{g/day}$)	5	2.45 $\pm$ 0.30 ^b	30.5 $\pm$ 0.5 ^c	124 $\pm$ 5 ^c	10.2 $\pm$ 0.9 ^c
Vehicle	4	3.91 $\pm$ 0.49	33.1 $\pm$ 0.2	172 $\pm$ 12	14.7 $\pm$ 0.4

Values are mean $\pm$ SEM. Difference compared to vehicle of each column; ^aNS, ^b $P < 0.05$, ^c $P < 0.001$.

Fig. 3. Effect of various amounts of sex steroids on SMA content in normal rats. Values are mean $\pm$ SEM (n=5).

μ g or 100 μ g/day, alternately for 10 days) or E_2 (1 μ g or 10 μ g/day, alternately for 10 days) did not affect the increase in serum SMA (Fig. 3) and growth rate during 10 days.

Effect of sex steroids on serum SMA and growth of gonadectomized male rats

To exclude the influence of endogenous testicular androgens in male rats, sex steroids were administered to gonadectomized rats whose testes were removed at 22 days. The

administration of E_2 suppressed the increase in the serum SMA. The increase in body weight and length tended to be suppressed as well (Table 2). The administration of T did not exhibit a significant effect on the increase in the serum SMA. However, the increase in body weight was larger than that of the vehicle group accompanying greater food intake (Table 2).

Effect of sex steroids on serum SMA and growth of female rats

In female rats, the administration of T did not affect the increase in the serum SMA but enhanced the increase in body length significantly. On the other hand, E_2 suppressed the increase in the serum SMA and food intake, although the growth was not retarded significantly (Table 3).

Effect of sex steroids on serum SMA and growth of hypox male rats

The serum SMA content of hypox male rats was low, as expected. The serum SMA of the hypox male rats increased with E_2 and not with T. The body length and weight were not effected by T but was decreased significantly by E_2 (Table 4).

When 0.2 U of bGH was injected concurrently for 9 days, the serum SMA was higher in the rats with T than without. The body length and weight were highest in this group although the body length was not significantly greater than in the vehicle group. The serum SMA of the E_2 group was the highest. Again,

Table 2. Effect of sex steroids on the growth of orchietomized rat and serum SMA levels.

Groups	No.	SMA (U/ml)	Body length (cm)	Body weight (g)	Food intake (g/day/rat)
Before	4	4.47 $\pm$ 0.47	27.7 $\pm$ 0.2	126 $\pm$ 4	9.1 $\pm$ 0.5
Testosterone propionate (1 mg/day)	4	5.76 $\pm$ 0.61 ^a	33.8 $\pm$ 0.6 ^a	182 $\pm$ 5 ^b	15.2 $\pm$ 1.6 ^c
Estradiol benzoate (100 μ g/day)	4	3.84 $\pm$ 0.31 ^d	30.8 $\pm$ 0.5 ^a	143 $\pm$ 8 ^a	12.2 $\pm$ 1.7 ^a
Vehicle	4	6.48 $\pm$ 0.30	32.1 $\pm$ 0.7	148 $\pm$ 10	11.8 $\pm$ 0.4

Values are mean $\pm$ SEM. Difference compared to vehicle of each column; ^aNS, ^bP<0.05, ^cP<0.01, ^dP<.0001.

Table 3. Effect of sex steroids on the growth of female rats and serum SMA levels.

Groups	No.	SMA (U/ml)	Body length (cm)	Body weight (g)	Food intake (g/day/rat)
Before	4	3.15±0.33	27.0±0.4	120±4	11.2±1.3
Testosterone propionate (1 mg/day)	4	4.53±0.57 ^a	31.7±0.3 ^c	154±9 ^a	12.6±0.6 ^c
Estradiol benzoate (100 µg/day)	4	2.87±0.22 ^b	30.3±0.4 ^a	133±7 ^a	11.1±1.4 ^c
Vehicle	4	4.72±0.57	29.9±0.3	145±2	14.3±0.5

Values are mean±SEM. Difference compared to vehicle of each column; ^aNS, ^bP<0.05, ^cP<0.01, ^dP<0.005.

Table 4. Effect of sex steroids on the growth of hypox or hypox rats concurrently treated with bGH (0.2 U, every day) and serum SMA levels.

Groups	No.	SMA (U/ml)	Body length (cm)	Body weight (g)	Food intake (g/day/rat)
Before	4	1.13±0.04	24.7±0.2	82.0±1.4	5.3±0.9
Testosterone propionate (1 mg/day)	4	0.75±0.07 ^a	25.4±0.5 ^a	89.5±4.2 ^a	6.4±0.5 ^a
+bGH	4	1.65±0.01 ^{b'}	27.3±0.5 ^{a'}	114.3±5.6 ^{d'}	4.3±0.5 ^{e'}
Estradiol benzoate (100 µg/day)	4	2.37±0.31 ^c	23.7±0.3 ^b	67.8±3.3 ^c	3.9±0.0 ^e
+bGH	4	3.09±0.48 ^{d'}	24.2±0.2 ^{d'}	75.3±4.3 ^{d'}	1.7±0.7 ^{e'}
Vehicle	4	0.77±0.03	26.3±0.6	90.8±3.0	6.3±0.3
+bGH	4	1.46±0.01	27.1±0.5	103.8±2.5	7.2±0.8

Values are mean±SEM. Difference compared to vehicle of each column; ^aNS, ^bP<0.05, ^cP<0.01, ^dP<0.005, ^eP<0.001. Difference compared to vehicle+bGH of each column; ^{a'}NS, ^{b'}P<0.05, ^{c'}P<0.01, ^{d'}P<0.005, ^{e'}P<0.001.

Table 5. Sulfation activity of the serum from male normal, hypox and hypox+bGH rats injected sex steroids.

Groups	Normal		Hypox		Hypox+bGH	
	Sulfation activity (U/ml)	SMA level (U/ml)	Sulfation activity (U/ml)	SMA level (U/ml)	Sulfation activity (U/ml)	SMA level (U/ml)
Before	1.18	2.53	0.28*	1.13	0.28*	1.13
Testosterone propionate (1 mg/day)	1.29	4.99	0.31*	0.72	0.63	1.48
Estradiol benzoate (100 µg/day)	0.98	2.85	0.28*	2.68	0.13*	3.31
Vehicle	1.27	4.24	0.26*	0.76	0.68	1.44

Sulfation activity and SMA level (by RRA) of pooled sera used in the experiments shown in Table 1 and 4 were measured as described in Materials and Methods. *Values estimated graphically.

the increase in body weight and length were considerably suppressed. The food intake of this group was extremely reduced.

Sulfation activity of the rat serum

In the hypox rats treated E₂, the elevated serum SMA was not accompanied by an increase in the growth rate. Therefore, the bio-activity of somatomedins in the serum

Table 6. Effect of sex steroid on ^{35}S incorporation into embryonic chick cartilage.

Hormone added	Final concentrations	Incorporation of $^{35}\text{SO}_4$ (% of vehicle)	
		without normal human serum	with 25% normal human serum
0.008% Ethanol (Vehicle)		100 $\pm$ 8.0	100 $\pm$ 14.0
Testosterone propionate	2.5 ng/ml	106 $\pm$ 5.8	101 $\pm$ 7.8
	25	99 $\pm$ 7.7	95 $\pm$ 2.7
	250	72 $\pm$ 3.7 ^b	111 $\pm$ 8.1
Estradiol benzoate	5 pg/ml	101 $\pm$ 4.8	104 $\pm$ 13.4
	50	114 $\pm$ 6.7 ^a	106 $\pm$ 14.2
	500	92 $\pm$ 7.5	121 $\pm$ 11.9 ^a
H ₂ O		95 $\pm$ 2.4	99 $\pm$ 5.2

T and E₂ were dissolved with ethanol and added to the assay mixture. Their vehicle (finally 0.008% ethanol) had no effect on the sulfation activity by itself. Values are mean $\pm$ SEM. Difference compared to vehicle;

^aNS, ^bP<0.02.

was assayed by ^{35}S incorporation into embryonic chick pelvic cartilage. Table 5 shows that the biological potency in hypox rat serum of E₂ group was very low. It remained low after concurrent injection of bGH. It is also shown that the sulfation activity of normal male rat serum was nearly the same as that of the normal human serum. This is in contrast to the observation that the value measured by RRA was significantly higher in the rat than that in the human serum.

Effect of sex steroids on ^{35}S incorporation into embryonic chick cartilage

The direct effect of sex steroids on sulfation activity by serum somatomedins was examined. Although a very high concentration of T (250 ng/ml) inhibited ^{35}S incorporation into embryonic chick pelvic cartilage in the absence of serum somatomedins, physiological doses of T or E₂ did not affect the sulfation activity either in the presence or absence of serum *in vitro* (Table 6).

Discussion

The present study showed that the serum SMA level clearly increased in parallel with the pubertal growth spurt (Fig. 2). Testo-

sterone caused a significant acceleration of the increase in body weight of gonadectomized male rats and body length of normal female rats without a change in the serum SMA level (Table 2 and 3). Testosterone did not exhibit any effect on growth or the serum SMA level in hypox male rats, while it significantly augmented the increase in body weight and serum SMA level in hypox male rats supplemented with bGH (Table 4). These results are in line with the earlier observations that T stimulated the sulfate uptake into costal cartilages of castrated rats with intact pituitary (Salmon *et al.*, 1963) and that the administration of T to hypox male or female rats supplemented by bGH caused an increase in the thymidine uptake in the cartilages (Matsuzaki, 1974). From these results it seems almost certain that testosterone or its metabolites exhibits a growth stimulating activity *in vivo* in a manner dependent on the presence of GH. In the *in vitro* system, testosterone did not activate sulfate incorporation by chick cartilage either in the presence or absence of the serum. Phillips *et al.* (1975) reported the same results. The effect of androgens on growth hormone synthesis and secretion is controversial (Hang and Gautvik, 1978). In rats no difference was found between the prepubertal and the pubertal levels of daily

GH secretion (Thompson *et al.*, 1972 and Plotnick *et al.*, 1974). These data and the present results indicate that the growth promoting effect of testosterone evolves through the production of SMA via a GH dependent reaction under subnormal GH secretion, and that testosterone potentiates the effect of SMA which is not able to be elucidated by its sulfation activity *in vitro*.

The serum SMA of group 1 (before) of gonadectomized rats was higher than that of group 1 of normal male rats (Table 1 and 2). In the subsequent repetitive type of experiments, the serum SMA level of 30 days orchietomized rat was 4.09 U/ml, that of sham operation 4.31 U/ml and that of non-treatment 3.82 U/ml. Thus it was concluded that the difference was due to the difference in animal batches rather than to the effect of gonadectomy.

The administration of E_2 to normal rats lowered the serum SMA level (Table 1). E_2 exhibited no effect on sulfate incorporation by cartilage *in vitro* (Table 6). These results are compatible with the previous reports by Wiedemann and Schwarz (1972) and Phillips *et al.* (1974, 1975). They showed that the administration of E_2 lowered the level of serum somatomedin activity of hypox rat treated concurrently with bGH. They also showed that in a patient with acromegaly a short term estrogen therapy (7–18 days) caused a prompt reduction in the elevated sulfation factor without affecting the plasma growth hormone levels and that E_2 (0.1–100 ng/ml) *in vitro* had no effect upon the bioassay system of sulfation factors. Food intake was significantly suppressed by E_2 . Malnutrition maybe partly contribute to the reduced increase in growth and serum SMA level reported by Takano *et al.* (1978).

In this study, it was recognized that serum SMA levels of normal male rats measured by RRA were about 5 times as high as those of normal adult men and increased further with aging (Fig. 2). The sulfation potency of these rat sera were almost the same as that of the

human serum (Table 5). The serum sulfate was about 3 times higher in rat than in man (95.7 $\mu\text{g/ml}$ and 35.1 $\mu\text{g/ml}$, respectively, by the method of Kleeman *et al.*, 1956). Thus, the specific activity of $^{35}\text{SO}_4$ is diluted only by a factor of 1.43. Therefore, reasons other than the contamination of cold sulfate should be considered. Although the binding of somatomedin A to its receptor on the placental membrane is very specific (Takano *et al.*, 1975), it might be due to the existence of somatomedins without sulfation potency which retain binding capacity to the somatomedin receptor on human placenta. It was already clarified that somatomedin C, insulin like growth factors I and II, and multiplication stimulating activity displace ^{125}I -labeled SMA on the placental receptor (Hall *et al.*, 1978). However, their sulfation potency in chick cartilage was not fully demonstrated. The existence of SMA without sulfation activity was further suggested by the result that serum SMA in hypox rats injected with E_2 was significantly higher than the other groups but that their growth was extremely retarded (Table 5).

Thus it has become evident that gonadal steroids are intimately involved in the regulation of pubertal growth spurt. Although both androgens and estrogens exhibited their effects in a GH dependent manner, analyses of serum SMA by RRA as compared with serum somatomedins-level obtained by the sulfation activity indicate the multiplicities of GH-dependent growth factors induced by the steroids and effector sites of steroid hormones; the steroids appear to act at the level of somatomedin production and somatomedin function.

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