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Original Article

SUBMAXILLARY GLAND FACTORS ALTER UTERINE WEIGHT AND PEROXIDASE ACTIVITY IN MICE

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To elucidate a functional significance of submaxillary gland, a simple in vivo biological assay for uterine growth inhibitory activity of submaxillary gland extract has been developed based on its inhibition of serum gonadotropin induced uterine growth of immature mice. Regression of weight gain after administration of serum gonadotropin was found sigmoidal against protein concentration of submaxillary extract. The inhibitory activities of submaxillary glands were also confirmed in rats, mice, and goats. Parotid and sublingual glands had very little inhibitory activity. Surgical removal of submaxillary glands from female mice increased both uterine weight and peroxidase activity and mice submaxillary extract administration attenuated the increased effect of submaxillary removal, while rat submaxillary extract had less effect on both parameters. Using ammonium sulphate fractionation, the factor was partially purified over crude extract. The submaxillary gland factor(s) is soluble protein, non-dialyzable, heat labile, susceptible to trypsin digestion in crude form, and present in 40-70% ammonium sulphate fraction. The present data suggest that submaxillary gland contains inhibitory factor(s), which inhibits the uterine weight and peroxidase activity in mice.

Key words: submaxillary gland, gonadotropin, bioassay, uterus, peroxidase

Rat, rabbit, and guinea pig uteri have been generally used as the biomodel for the study of hormone action. Uterine stimulation by estrogenic hormones in ovariectomized or immature rats causes elevation in peroxidase activity of the uterus (Lyttle and DeSombre, 1977a) and peroxidase activity is the marker enzyme of uterus (Lyttle and DeSombre, 1977b). Previously, we have shown some quantitative data on the histological and biochemical changes in uterus after submaxillariectomy (Banerjee *et. al.,* 1987). To substantiate that submaxillary glands play an important role in the development of uterus, the effect of submaxillariectomy on the uterine peroxidase activity as well as the incorporation of radioactive phenylalanine into nuclear protein of uterus was studied. Inoue (1990) studied the effect of sialoadenectomy on the aromatase activities in rat ovaries. Later, Tanaka et al. (1995) studied effects of submaxillariectomy on ovarian androgen and estrogen production in female rats and suggested, furthermore, that submaxillary gland may inhibit the production of ovarian estrogen mainly because of the inhibition of ovarian androgen production. They concluded that the submaxillary gland might play a physiological role in female reproductive systems caused by the change in ovarian steroidogenesis (Tanaka et al., 1995). Our objective of the present work is to study the uterine weight and peroxidase activity of immature submaxillariectomized mice and effect of submaxillary gland extract on those

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operated mice. However, these results would confirm our previous finding (Banerjee et al 1987). Another objective is to develop a suitable bioassay of the submaxillary extract using immature mice as model.

Materials and Methods

Animals

Immature female Balb/c mouse of 4 weeks were obtained from our Institute colony. Mice were maintained under controlled air and temperature as well as 12 h. of light and 12 h. of darkness. Animals had free access to food and water all the time. To prepare submaxillariectomized mice, immature mice were operated bilaterally to remove the submaxillary glands (Smith et al., 1979). Sham operated mice served as controls. After 15 days of operation, both sham operated and submaxillariectomized animals were sacrificed.

Tissue extract preparation

Submaxillary glands were excised from mice, weighed, and homogenized (5% w/v) in cold 10 mM Tris-HCl buffer, pH 7.6. Submaxillary glands from goats and rats were taken and homogenized in the same way as mice. For comparison with other salivary glands, parotid and sublingual glands were taken and 5% homogenates were prepared as with submaxillary glands (unpublished data). All the tissue homogenates were centrifuged at 105,000x g for 1 h at 4°C in a Beckman ultracentrifuge (model L5- 50B). The supernatants were carefully collected and stored at -20°C until use.

Uterine weight and peroxidase assay

The mouse submaxillary extract containing 1 mg of protein was injected intraperitoneally to all submaxillariectomized mice on the 4^{th}, 8^{th}, and 12^{th} day, repeatedly and sacrificed on the 15^{th} day after removal of submaxillary glands for uterine weight and peroxidase activity. Sham operated mice received vehicle only.

Uterine peroxidase activity from mice uterus was measured using potassium iodide as substrate (Alexander, 1962) and followed by our previously report (Banerjee et al., 1987). The reaction mixture (3 ml) contained 50 mM acetate buffer, pH 5.0, 1.7 mM KI, 0.33 mM hydrogen peroxide and suitable amount of extract protein. Hydrogen peroxide solution was added last to start the reaction and an initial increase in absorbance at 353 nm was recorded in a dual beam Hitachi spectrophotometer. Peroxidase activity was expressed as units per ml of tissue extract.

Biological assay

After randomization, immature mice were weighed and caged in groups of 5 to 6 per cage. Various concentrations of PMSG (Antex, DeCruz) starting from 1.25-20 IU were injected subcutaneously per mouse in a final volume of 0.1 ml of normal saline. Control groups received the same volume of normal saline and animals of all the groups were sacrificed 24 h after the injection of the hormone. For experiment with the submaxillary gland factor, mice submaxillary gland extract of different concentrations (0.15-0.6 mg protein) were injected intraperitoneally 90 min prior to the administration of gonadotropin. Twenty-four h after the injection of hormone, the animals were killed by decapitation, uteri removed, trimmed, and weighed. Same amount of extract proteins from goat and rat submaxillary glands were also injected separately into another group of mice treated with PMSG in the same manner and uteri were collected and weighed. Parotid and sublingual extracts of increasing concentrations were also administered in the hormone treated immature mice, for comparison the effect.

Partial purification of submaxillary gland factors

As the inhibitory factor is present in submaxillary gland of rats, mice, and goats, purification of the factor from the submaxillary gland of rat was undertaken for easy availability of the animals. The starting material was 105,000x g supernatant of 20% homogenate of rat submaxillary gland in 10mM tris-HCl buffer, pH 7.6. A dose response curve with increasing concentration of the above crude preparation was obtained. This 105,000x g supernatant was then subjected to precipitation with solid ammonium sulphate at 4°C to collect between 0-40% and 40-70%, respectively. The precipitates obtained from different fractions were dissolved in the homogenizing buffer and dialyzed against the same buffer for 24 h with three changes. The obtained materials were then tested for biological activity. The same material was taken for heat treatment at 45°C and 65°C for five minutes and trypsinization.

Statistical analyses

All data were expressed as mean ± standard deviation (SD) and Student's t-test was used for statistical significance ($p < 0.05$).

Results

Effect of submaxillariectomy on uterine growth and peroxidase activity in mice

Surgical removal of submaxillary gland in immature female mice caused increased uterine growth as compared to sham-operated control (Table 1). The peroxidase activity also three folds increased 15 days after the removal of submaxillary glands. Administration of mouse submaxillary extract to submaxillariectomized mice brought back the uterine weight and peroxidase activity almost to the control level (Table.1). Submaxillary extract of rats, instead of mice, was also effective but to a lesser extent than the mouse submaxillary extract.

Table 1. Effect of submaxillariectomy on wet weight and uterine peroxidase activity in immature mice.

Treatment	Uterine weight (mg) 15th day after submaxillariectomy	Peroxidase Δ OD/min/ml
Sham	9.00 ± 0.11	0.11 ± 0.02
Submaxillariectomy	14.00 ± 0.14 [1]	0.33 ± 0.03*
Submaxillariectomy+mice submaxillary extract	8.20 ± 0.11 [2]	0.09±0.025**
Submaxillariectomy + rat submaxillary extract	8.60 ± 0.23 [3]	0.25 ± 0.10***

Values are means ± SD of six determinations; 1, P<0.001 against sham, 2, P<0.001 against submaxillariectomy, 3, P<0.01 against submaxillariectomy, *, p<0.001 against sham, **, p<0.001 against submaxillariectomy, ***, p<0.001 against submaxillariectomy

Biological assay of uterine growth inhibitory activity of submaxillary extract

Administration of PMSG at increasing doses caused a linear increase in uterine wet weight (Figure 1). The uterine weight of control mice 24 h after the vehicle injection was 6.2 ± 0.2 mg (mean ± SD). The increase in uterine weight was almost linear up to 20 IU of gonadotropin and at this dose, the increase in uterine weight was 12.5 ± 0.6 mg. Therefore, a single dose of 20 IU of PMSG was employed routinely to determine the uterine growth inhibitory activity of submaxillary extract. We have used the term 'growth' to mean 'wet-weight' of uterus.

When soluble supernatants (105,000x g supernatant) of mouse submaxillary gland containing 0.15-0.60 mg protein was injected intraperitoneally and followed by a single injection of 20 IU PMSG, uterine wet weights decreased about 60% when compared to PMSG administration alone (Figure 2). Doses of lower than 0.15 mg had no effect whereas those of higher than 0.6 mg of protein were lethal.

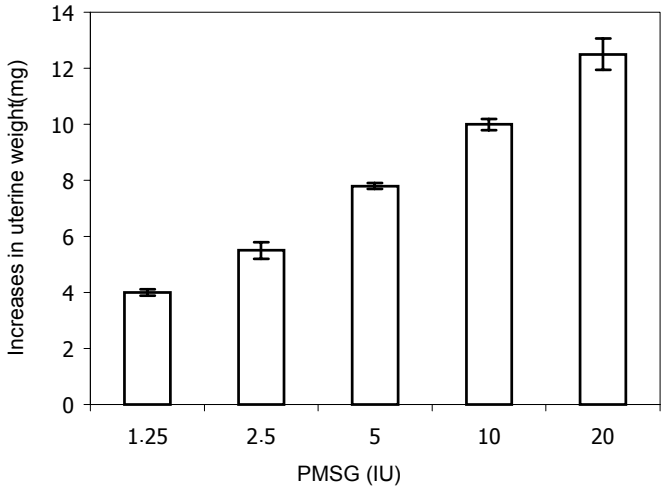

Figure 1. A dose dependent increase in uterine weight of immature mice after PMSG treatment. All values are means ± SD of six determinations. Each point is statistically significant ($p<0.001$) in all concentrations used when compared to control values.

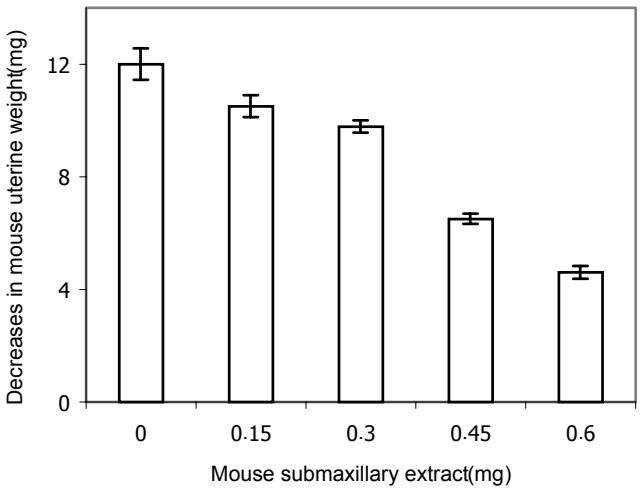

Figure 2. Inhibition of PMSG-induced uterine growth by increasing concentrations of mouse submaxillary gland extract. All values are means ± SD of six experiments and are statistically significant ($p<0.001$) when compared to PMSG-treated uterus. PMSG (20 IU) treated uterine weight is 12.0 ± 0.56 mg (marked zero in the figure).

A combination of 2.5 IU each of PMSG and human chorionic gonadotropin (HCG, Organon) also produced significant uterine growth but the reduction of uterine growth after administration of submaxillary extract was not linear. Administration of diethylstilboestrol (Sigma), the synthetic estrogen, produced an increase in uterine growth as expected. However, in this case, the increase was not linearly reversed by submaxillary extract (results not shown). The specificity of submaxillary extract was confirmed by testing the biological activity of parotid and sublingual gland extracts prepared in the same manner (as in the case of submaxillary); and a slight inhibition was However, in this case, the increase was not linearly reversed by submaxillary extract (results not shown). The specificity of submaxillary extract was observed. This indicates that the active factor is present in parotid and sublingual glands to a much lesser extent compared to submaxillary gland. The inhibitory activity of the factor in mice is more or less similar in submaxillary gland of goat and rat (Table 2).

Table 2. Effect of goat and rat submaxillary gland extract on PMSG-induced uterine growth in mice.

	Decreases in mouse uterine weight (mg)*	
	Goats	**Rats**
Control without factor	10.0 ± 0.10	10.0 ± 0.12
Factor (0.10mg)	9.3 ± 0.11	7.0 ± 0.10
Factor (0.20mg)	7.0 ± 0.90	6.6 ± 0.90
Factor (0.30mg)	5.0 ± 0.10	6.0 ± 0.11

Values are means ± SD of six determinations. *All the values are statistically significant ($p<0.01$) when compared to PMSG-treated uterus without factor.

Partial purification of the rat submaxillary factor

Preliminary results indicated that the factor is non-dialyzable, loses its biological activity on heating for 5 min at 65°C, susceptible to trypsin digestion. After ammonium sulphate precipitation of the crude extract prepared from rat submaxillary gland, the inhibitory activity was studied and found that the inhibition of uterine growth was effective in 40-70% fraction. The other fractions, 0-40% and 70-90%, displayed little inhibitory activity. In addition, the results indicated that, with 0.1 mg of 40-70% ammonium sulphate fractioned extract, the decrease in uterine weight observed was only 8.48± 0.25 mg, whereas with a half dose (0.05 mg), possessed little inhibition. In contrast, a dose of 0.1 mg of crude rat submaxillary extract decreased uterine weight (9.5± 0.2 mg).

Discussion

Many lines of evidence indicate that a submaxillary gland extract inhibits uterine growth (Banerjee et al, 1987; Minamida et al, 1996; Tanaka et al, 1995). The present data in mice, rat, and goats confirm previous studies. However, its mechanism of inhibition at the molecular level remains to be solved. The submaxillary factor might act at three levels to cause inhibition. First, it may interfere the binding of gonadotropins to their target organ, mainly ovary. Several non-steroidal modifiers of gonadotropin function have been identified in the follicular fluid of various species (Channing et. al., 1982; Darga and Reichert, 1972; Grady et al., 1982; Hillensjo et al., 1978;

Reichert et al., 1979; Tsafriri and Channing, 1975). Second, it may act at the level of ovarian cells producing estrogens (Tanaka et al., 1995). Inhibitors and stimulators of granulosa cell maturation and luteinization in the follicular fluid has been known for a long time (Ledwitz et al., 1977). Third, it may mimic the action of antiestrogen factor and prevents the stimulation of uterine growth by ovarian estrogens.

We reported previously that the submaxillariectomy elevated peroxidase activity and incorporation of radioactive phenylalanine into uterine nuclear fraction in female rats (Banerjee et al., 1987). Inoue (1990) studied the effect of submandibular gland removal on aromatase activity in ovarian microsomes and found increased ovary and uterus weights and enzyme activity. This effect was also supported by other studies (Koshika et. al., 1998; Minamida et. al., 1996; Tanaka et. al., 1995). Minamida et al. (1996) reported an inhibition of estradiol-17β secretion in ovarian granulosa cells by an extract from rat submandibular gland. It was hypothesized that the enzyme activated by the submaxillariectomy might be C17-20 lyase. Koshika et al. (1998) also studied the submaxillary gland factor and proposed that submaxillary might contain a high molecular weight, heat labile soluble factor(s) that affects testosterone secretion by inhibiting leutinizing hormone action in testicular cells. The present findings are quite similar to previous observation by Koshika et al. (1998). The submaxillary gland factor isolated by the present study is also a soluble, non-dialyzable, heat labile and trypsin sensitive protein.

The use of PMSG-induced growth of the uterus, as a biological assay offers several advantages. It does not require any expensive radioactive agents, is not species specific but is very much organ specific. A limitation of this bioassay could be false negatives that may be produced in the test sample containing both inhibitors and stimulators of growth. Several monoclonal antibodies of growth factors and their receptors have been reported, based on the induction of DNA synthesis and/or the stimulation of tyrosine phosphorylation. These assays can be subject to false positive results and do not measure the full biologic activity of a mitogen. The capacity of a factor to inhibit uterine growth induced by serum gonadotropin would seem to constitute a more convincing demonstration of full bioactive properties as a growth factor.

The present results suggest that the mouse submaxillary gland contains a factor that regulates the uterine growth and development of immature mouse. In addition, an in vivo biological assay of this factor has been developed based on its inhibition of PMSG-induced uterine growth in immature mouse.

References

1. Alexander NM. A spectrophotometric assay for iodide oxidation by thyroid peroxidase. *Anal Biochem* 4:341-345, 1962.

2. Banerjee PN, Bhattacharyya J, Datta A, and Datta AG. The effect of submaxillriectomy on the uterine peroxidase activity and [^{14}C]-phenylalanine incorporation into uterine proteins in rats. *Acta Endocrinol* 114**:**208-13, 1987.

3. Channing CP, Anderson LD, Hoover DJ, Kolena J, Osteen KJ, Pomerantz SH, and Tanabe K. The role of nonsteroidal regulators in the control of oocyte and follicular maturation. *Rec Prog Horm Res* 38:331-408, 1982.

4. Darga NC and Reichert LE (Jr). Some properties of the interaction of follicle stimulating hormone with bovine granulosa cells and its inhibition by follicular fluid. *Biol Reprod* 19:235-41, 1978.

5. Grady RR, Chariesworth MC, and Schwartz NB. Characterization of the FSH suppressing activity in follicular fluid. *Rec Prog Hor Res* 38:409-456, 1982.

6. Hillensjo T, Batta SK, Schwartz-Kripper A, Wentz AC, Sulewski J, and Channing CP. Inhibitory effect of human follicular fluid upon the maturation of porcine oocyte in culture. *J Clin Endocrinol Metab* 47:1332-35, 1978.
7. Inoue K. Effect of sialoadenectomy on the aromatase activities in ovary of rat. *Bull Kanagawa Dent Coll* 18:7-14, 1990.
8. Koshika S, Izukuri K, Kato Y, Saito S, and Hoshaka M. Inhibitory effects by a submandibular gland extract on leutinizing hormone-stimulated testosterone production by testicular cells. *Biochem Mol Biol Int* 46:165-173, 1998.
9. Ledwitz-Rigby F, Rigby BW, Gay VL, Stetson M, Young J, and Channing CP. Inhibitory action of porcine follicular fluid upon granulosa cell leutininization in vitro: assay and influence of follicular maturation. *J Endocrinol* 74:175-84, 1977.
10. Lyttle CR and DeSombre ER. Generality of oestrogen stimulation of peroxidase activity in growth responsive tissue. *Nature* 268:337, 1977a.
11. Lyttle CR and DeSombre ER. Uterine peroxidase as a marker for oestrogen action. *Proc Natl Acad Sci, USA* 14:3162-3166, 1977b.
12. Minamada G, Izukuri K, and Koshika S. Inhibition of estradiol-beta secretion in ovarian granulosa cells by an extract from the submandibular gland of the rat. *Biochem Mol Biol Int* 39:1099-1108, 1996.
13. Reichert LE, Sanzo MA and Darga NS. Studies on a low molecular weight follicle stimulating hormone binding inhibitor from human serum. *J Clin Endocrinol Metab* 49: 866-72, 1979.
14. Smith S, Mazur A, Voyles N, Bathena S, and Reeant L. Is submaxillary gland immunoreactive glucagon important glucagon important in carbohydrate homeostasis? *Metabolism* 28**:**343-347, 1979.
15. Tanaka M, Watnabe M, Kumai T, Nakura H, and Kobayashi S. Effects of submaxillariectomy on ovarian androgen and estrogen production in adult female rats. *Biochem Biophys Res Commun* 213:550-54, 1995.
16. Tsafriri A and Channing CP. An inhibitory influence of granulosa cells and follicular fluid upon porcine oocyte meiosis in vitro. *Endocrinology* 95:922-27, 1975.