Hormonal regulation of retinoic acid-binding proteins in the mammary gland

Rajendra G. MEHTA and Richard C. MOON

Laboratory of Pathophysiology, Life Sciences Division, IIT Research Institute, Chicago, IL 60616, U.S.A.

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A cytosolic retinoic acid-binding (RAB) protein that sediments specifically as a 2S component on sucrose density gradients was detected in the mammary glands of virgin, pregnant and lactating rats. Mammary cytosol from pregnant rats contained significantly higher concentrations of cytosolic RAB protein than did cytosol from either virgin or lactating rats. The glands of pregnant animals exhibited increased concentration of cytosolic RAB protein during the first 5 days of pregnancy, and a steady decline was observed thereafter. The concentration of cytosolic RAB protein dropped to the value observed during lactation on the day 20 of pregnancy. Moreover, throughout lactation, low concentrations of cytosolic RAB protein were maintained. Daily treatment of virgin and lactating animals with 5 µg of oestradiol-17β for 1 week increased cytosolic RAB protein to concentrations comparable with those seen in pregnant rats. Progesterone, however, did not affect the mammary cytosolic RAB protein content of virgin rats. These results suggest hormonal involvement in the regulation of cytosolic RAB protein concentration of mammary gland during differentiation.

The maintenance, differentiation, and function of many epithelial tissues can be influenced by retinol, retinoic acid and their natural and synthetic analogues (retinoids) (DeLuca *et al.*, 1972; Wolbach & Howe, 1925). Unlike retinol, retinoic acid and its analogues are not stored in the liver, but are rapidly metabolized (Moore, 1967; Roberts & DeLuca, 1967; Roberts *et al.*, 1979). This suggests that retinoic acid or one of its metabolites may be an active form of vitamin A. Although previous reports from our laboratory and elsewhere have shown that dietary supplementation with certain retinoids significantly retards chemically induced mammary carcinogenesis (Moon *et al.*, 1977, 1979; McCormick *et al.*, 1980; Sporn & Newton, 1979), surprisingly little is known of the influence of retinoids on either the normal differentiation of the mammary gland or on the differentiation that accompanies pregnancy and lactation.

There is a growing body of evidence to indicate that retinoids may exert their influence through retinoid-binding proteins. Two retinoid-binding proteins, one associated with retinol (retinol-binding protein) and the other with retinoic acid [retinoic acid-binding (RAB) protein] have been isolated from

several tissues (Chytil & Ong, 1979), including mammary tumours (Mehta *et al.*, 1980; Moon & Mehta, 1981; Trown *et al.*, 1980). Additionally, low concentrations of cytosolic RAB protein have been reported in the cytosol of mammary glands from young rats (Mehta *et al.*, 1980) and in the disease-free normal mammary tissue of breast-cancer patients (Palan & Romney, 1980; Kung *et al.*, 1980). The present study was undertaken to determine the concentration of cytosolic RAB proteins in the mammary gland at various stages of differentiation with an attempt to correlate cytosolic RAB protein content with the alteration in hormonal concentrations during structural and functional differentiation.

Experimental

Female Sprague–Dawley rats were obtained from ARS/Sprague–Dawley, Madison, WI, U.S.A., and were mated with males of the same strain. The day that sperm appeared in the vaginal washing was designated as day 1 of pregnancy; days of lactation were monitored as days *post partum*. All animals were housed in a room artificially illuminated for 14 h each day and maintained at 22 ± 2°C. Wayne lab chow (Allied Mills, Chicago, IL, U.S.A.) and tap water were given *ad libitum*.

Abbreviation used: RAB protein, retinoic acid-binding protein.

To investigate the influence of steroid hormones on the concentrations of cytosolic RAB protein, young virgin rats were treated daily for 1 week with either 5 µg of oestradiol-17β (Steraloids, Pawling, NY, U.S.A.), 5 mg of progesterone (Syntex Corp., New York, NY, U.S.A.) or a combination of both hormones in 0.1 ml of sesame oil; controls received carrier only. The effect of oestradiol-17β on the mammary cytosolic RAB protein concentrations in the lactating rats was investigated by the same protocol. The litter of each lactating mother was adjusted to six pups on day 2 *post partum*.

For cytosolic RAB protein assays, the abdominal–inguinal mammary glands were excised and were either used immediately or frozen at –80°C for later use. Freezing of the mammary tissue did not result in any degradation of cytosolic RAB protein. The mammary glands were thawed, minced and homogenized in 0.01 M-Tris buffer, pH 7.4, containing 1.5 mM-EDTA and 1 µM-dithiothreitol, with a Polytron homogenizer (Brinkman Instruments, Westbury, MA, U.S.A.). Cytosol was prepared by centrifuging the homogenate at 105 000 g for 30 min at 0°C and retaining the supernatant. For the measurements of cytosolic RAB protein, portions of cytosol were made to react with 1 mM [³H]retinoic acid (specific activity 1.14 Ci/mmol) either alone or in the presence of a 25-fold excess of unlabelled all-*trans*-retinoic acid for 16 h at 0°C in the dark. The incubation mixtures were then treated for 5 min with a pellet containing 1 ml of dextran-coated charcoal, and a portion (0.2 ml) was layered on a preformed linear 5–20% (w/v) sucrose gradient. The gradients were centrifuged at 400 000 g for 2 h in a vertical-tube rotor (DuPont Instruments, Newton, CT, U.S.A.) at 0°C and fractionated by collecting 10-drop fractions from the bottom of each tube. Scintillation fluid [4 ml; 4 g of Omnifluor (New England Nuclear, Boston, MA, U.S.A.) in 700 ml of toluene and 300 ml of Triton X-100] was added to each fraction, which was then counted for radioactivity in a liquid-scintillation spectrometer (Tracor Analytical Mark III). Myoglobin and haemoglobin were used as marker proteins. The protein concentration in the cytosol was measured by the procedure of Waddell (1956).

Results

Retinoic acid-binding proteins were separated as specifically binding 2S components when the mammary-gland cytosol fractions from virgin, pregnant or lactating rats were made to react with [³H]-retinoic acid and subsequently subjected to sucrose-density-gradient analysis (Fig. 1). Unlabelled retinoic acid at a 25-fold excess concentration inhibited the binding of [³H]retinoic acid to cytosolic RAB protein in the 2S region. The mammary

cytosol from pregnant rats contained significantly higher concentrations of cytosolic RAB protein compared with lactating or virgin rats (Table 1); the concentration of mammary cytosolic RAB protein in pregnant rats ranged from 2 to 4 pmol per mg of cytosolic protein, whereas in lactating rats it was less than 1 pmol per mg of protein. The protein concentration in the cytosol of pregnant and lactating rats was determined before the binding assay and was standardized to a concentration of approx. 10 mg per ml of cytosol by appropriate dilutions. The protein concentration of mammary glands from virgin female rats was considerably lower (3–5 mg/ml of cytosol) than that of either pregnant or lactating animals.

In order to analyse the differences observed in the cytosolic RAB protein content between pregnant and lactating rats, the cytosolic RAB protein concentrations were measured in the mammary cytosol fractions at different times during gestation and lactation. The results are shown in Fig. 2. An increased amount of cytosolic RAB protein was observed during the first 5 days of pregnancy. A steady decline in the cytosolic RAB protein content was noted from day 5 to day 15 of pregnancy. Even though the cytosolic RAB protein concentration was lower on day 15 of pregnancy compared with day 5, the cytosolic RAB protein concentrations were significantly higher on day 15 of pregnancy than at any time during lactation ($P < 0.05$). However, just before parturition (day 20 of pregnancy), the cytosolic RAB protein concentration approached that found in lactation. During the entire lactation period thereafter the concentration of cytosolic RAB protein remained rather constant.

Since a primary hormonal difference between pregnancy and lactation is an elevated concentration of circulating oestrogen during pregnancy (Turner & Baglioni, 1976), experiments were conducted to investigate the influence of oestrogen

Table 1. *Distribution of retinoic acid-binding proteins in the mammary gland during differentiation*

Glands from early-, mid- and late-pregnant and lactating rats were analysed for cytosolic RAB protein concentration. Retinoic acid-binding proteins were determined by using sucrose-density-gradient analysis. Specific binding under the 2S region was calculated. ^a Significantly different from ^b ($P < 0.05$); ^b significantly different from ^c ($P < 0.025$).

Physiological stage	No. of expts.	Retinoic acid-binding proteins
		(pmol/mg of protein; mean ± s.d.)
Virgin	4	1.02 ± 0.23 ^a
Pregnant	24	2.83 ± 0.76 ^b
Lactating	19	0.72 ± 0.11 ^c

Fig. 1. Sucrose-density-gradient analysis of cytosolic RAB protein in mammary glands of virgin, pregnant and lactating rats

Portions of mammary-gland cytosol prepared from virgin (a), pregnant (b) or lactating (c) rats were incubated with [3 H]retinoic acid ($1\mu\text{M}$) either alone (●) or in the presence of $25\mu\text{M}$ unlabelled all-*trans*-retinoic acid (○) for 16 h at 0°C in the dark. Retinoic acid-binding proteins were separated on sucrose density gradients as described in the Experimental section. Myoglobin (Mb) and haemoglobin (Hb) were used as marker proteins.

Fig. 2. Concentration of cytosolic RAB protein during pregnancy and lactation

Mammary glands obtained from rats 2, 5, 10, 15, 18 and 20 days pregnant as well as rats 2, 5, 12 and 19 days *post partum* were selected for the study. Cytosol fractions were separated on sucrose density gradients. Specific binding under the 2S region was determined (●). Each time point with random error represents four experiments, each from mammary glands of different animals. Influence of exogenous oestradiol- 17β on the lactating rats was determined by injecting $5\mu\text{g}$ of oestradiol- 17β in oil into rats either 5 or 12 days *post partum* for 5–7 days and cytosolic RAB protein concentration was determined as described above (○).

administration on the cytosolic RAB protein content of mammary glands in lactating rats. As shown in Fig. 2, treatment with oestradiol- 17β resulted in increased concentrations of cytosolic RAB protein in the mammary tissue. Mammary cytosol from untreated animals contained less than 1 pmol of cytosolic RAB protein per mg of cytosol protein throughout the lactation period; however, a 2–3-fold increase in cytosolic RAB protein concentration was apparent after oestrogen administration, irrespective of whether oestrogen administration occurred in early or late lactation. Similarly, treatment of virgin rats with exogenous oestradiol- 17β ($5\mu\text{g}$ daily for 5 days) resulted in a 4-fold increase in the cytosolic RAB protein concentration, whereas progesterone had little effect (results not shown). Furthermore, the cytosolic RAB protein content of mammary glands at different stages of the oestrous cycle did not differ except at metoestrus. During metoestrus the cytosolic RAB protein content of the glands was 2–3 times greater than that of glands removed during either of the other stages of the oestrus cycle.

Discussion

Retinoic acid retains the overall growth-promoting activity of vitamin A; however, the role of retinoic acid in mammary-gland differentiation has not been reported. The presence of cytosolic RAB protein in the vitamin A-responsive tissues is thought to be a mediator of retinoic acid action (Chytil & Ong, 1978, 1979). Cytosol fractions prepared from testes, ovary, brain and embryonic skin contain such retinoic acid-binding components, whereas cyto-

solic RAB proteins are absent in the cytosol of liver, kidney and muscle (Ong & Chytil, 1975; Sani & Hill, 1976). Although previous studies indicated absence of cytosolic RAB protein in the normal mammary tissues from breast-cancer patients (Ong *et al.*, 1975; Huber *et al.*, 1978), cytosolic RAB protein has recently been detected in these tissues (Kung *et al.*, 1980; Palan & Romney, 1980). In the present paper, we have demonstrated the presence of cytosolic RAB protein in the mammary glands of virgin, pregnant and lactating rats, although the mammary glands of virgin and lactating rats contained considerably lower concentrations of cytosolic RAB protein compared with the mammary glands of pregnant animals. It is apparent from the present study that detectable amounts of cytosolic RAB protein were present in the mammary gland throughout various stages of physiological differentiation.

It is well established that the structural (e.g. during pregnancy) and functional (e.g. during lactation) differentiation of mammary gland is governed by an alteration in steroid and protein hormones (Banerjee, 1976). Determinations of serum concentrations of steroids during pregnancy and lactation have indicated that the concentrations of circulating oestrogens are elevated during pregnancy, whereas those of plasma oestrogen decrease dramatically at the onset of lactation (Turner & Bagnara, 1976). In addition, Beattie *et al.* (1977) and Beattie (1979) have reported that, on day 3 of pregnancy, serum oestradiol concentrations were as high as 75 pg/ml of serum and decreased thereafter. In the present study we observed a significant difference in the concentrations of cytosolic RAB protein when the results obtained from pregnant rats on any day of pregnancy (except day 20) were compared with any day during lactation. Moreover, the concentrations of cytosolic RAB protein were found to be maximal on day 5 of pregnancy and decreased steadily thereafter. Thus it is possible that a correlation exists between alteration in circulating concentrations of hormones and the occurrence of cytosolic RAB protein.

The influence of steroid hormones on the plasma concentration of vitamin A has been of interest in recent years, since the plasma concentrations of vitamin A remain significantly elevated in women taking oral contraceptives (Gal *et al.*, 1971; Smith *et al.*, 1975). In addition, it has also been reported that the utilization of vitamin A is enhanced as a result of oestrogen in the birth-control drugs, whereas progesterone was ineffective in enhancing the vitamin A utilization (Yeung & Chan, 1975; Vahlquist *et al.*, 1979). These workers suggested that the oestrogen-induced increase in the utilization of vitamin A is attributable to the increased concentrations of vitamin A-binding proteins in the

target tissues. Since the concentration only of retinol was determined in both of these studies (Yeung & Chan, 1975; Vahlquist *et al.*, 1979), little is known of the effects of steroid hormones on the utilization of retinoic acid. However, in the present study we observed enhancement in the cytosolic RAB protein content of lactating mammary glands as a result of oestrogen treatment. Similarly, the concentration of cytosolic RAB protein in the mammary glands of virgin rats was increased significantly by administration of oestradiol. Progesterone under similar conditions was ineffective. These results suggest a possible regulation of cytosolic RAB protein concentration in the mammary tissues by oestrogen. However, oestrogen can markedly increase prolactin secretion in a dose-related manner (Chen & Meites, 1970; Yanai & Nagasawa, 1980); it is thus possible that the observed stimulatory effects of exogenous oestradiol on the mammary cytosolic RAB protein concentration in the present study may be mediated by an increase in prolactin secretion.

At present the role of retinoids in the differentiation of the mammary gland is unknown; however, the altered concentrations of cytosolic RAB protein during different physiological stages of the gland may reflect a difference in the utilization of vitamin A during mammary-gland differentiation. Although it is premature to postulate a mechanism by which hormones regulate the concentration of cytosolic RAB protein and possible utilization of retinoic acid, the results clearly indicate the involvement of hormones in this process.

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