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# IMPAIRED DEVELOPMENT OF MAMMARY GLANDS IN SCORBUTIC RATS UNABLE TO SYNTHESIZE ASCORBIC ACID

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**Summary**—The effects of ascorbic acid (AsA)-deficiency on the development of mammary glands were investigated using mutant rats (osteogenic disorder syndrome rats; ODS rats) with hereditary inability to synthesize AsA. Female ODS rats of 21 days old were castrated and divided into two groups. One group was given AsA in their drinking water, and the other was not. All the rats received a daily injection of oestradiol-17$\beta$ and progesterone (EP) from day 28 to day 49 of age. After EP treatment, the concentrations of AsA in the mammary glands of rats not given AsA were less than one tenth of those of rats given AsA and the contents of hydroxyproline in the mammary glands of the former rats were about half of those in the latter. Furthermore, the concentration of serum prolactin in rats not given AsA was reduced to about one third of that in rats given AsA. After EP treatment, whole mounts of mammary glands showed that in rats not given AsA the development of ducts was impaired and there was extensive accumulation of endbuds. Consistent with this finding, EP injections did not increase the area of parenchyma in the mammary glands of rats not given AsA, whereas they increased it about 2-fold in rats given AsA. Moreover, after EP treatment the amount of $\alpha$-lactalbumin was significantly less in the mammary parenchyma of rats not given AsA than in that of rats given AsA. On the other hand, AsA deficiency did not impair the response of the mammary cells to insulin or prolactin in terms of DNA synthesis and $\alpha$-lactalbumin production. These findings indicate that AsA deficiency impaired the development of mammary glands. This effect may be partly attributable to a defect in collagen synthesis in the mammary glands and a decrease in the concentration of serum prolactin.

## INTRODUCTION

Collagen, a major component of the extracellular matrix, plays an important role in the development and differentiation of mammary glands. The *in vivo* study of Wicha *et al.* [1] showed that inhibition of collagen synthesis resulted in an involution-like structural alteration of the mammary glands of rats in which mammary gland proliferation was induced by perphenazine. Wakimoto and Oka [2] also found in an *in vitro* study using mammary explants that inhibition of collagen synthesis brought about decrease in the synthese of DNA and milk protein by mammary cells. Furthermore, many studies using cell culture systems have shown that mammary cells proliferate and differentiate better on collagen gel than on a plastic surface [3–5] and that mammary morphogenesis occurs when mammary cells are cultured within a collagen gel matrix [6, 7].

Ascorbic acid (AsA) is required for collagen synthesis because collagen contains much hydroxyproline and hydroxylysine, and AsA is required for optimal activation of proline and lysine hydroxylase [8, 9]. Furthermore, AsA has been shown to increase collagen production of fibroblasts [10, 11]. Thus, as AsA is involved in collagen synthesis, it presumably takes part in the development and differentiation of the mammary gland, although its exact role(s) in these processes is unknown.

Most animals other than guinea-pigs and primates can synthesize AsA in the liver and so do not become scorbutic when not supplied with AsA. However, Mizushima *et al.* [12] and

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Hori *et al.* [13] established a mutant strain of Wistar rats with a hereditary osteogenic disorder that involves a deficiency of gulonolactone oxidase. Like guinea-pigs, this mutant strain becomes scorbutic when not supplied with AsA. The disorder is controlled by a single autosomal recessive gene and homozygotes of the strain (*od/od*), which are named osteogenic disorder syndrome (ODS) rats, manifest the disorder. In the present study, using these ODS rats, we investigated the effects of AsA deficiency on the development and differentiation of the mammary glands.

## EXPERIMENTAL

### *Chemicals*

Chemicals were obtained from the following sources: Medium 199 (Hanks' salt) from Handai-biken (Suita, Osaka, Japan); [methyl-$^3$H]thymidine (79.8 Ci/mmol), [$^{14}$C]UDP-galactose (272.8 mCi/mmol) and protosol (tissue solubilizer) from New England Nuclear (Boston, Mass, U.S.A.); cortisol, oestradiol-17$\beta$, progesterone, bovine $\alpha$-lactalbumin and galactosyl transferase from Sigma Chemical Co. (St Louis, Mo., U.S.A.), and ascorbic acid from Fuso Co. (Osaka, Japan). Bovine prolactin (NIH/B5) was obtained through the Hormone Distribution Program, NIADDK, and porcine zinc insulin was kindly provided by Eli Lilly Co. (Indianapolis, Ind., U.S.A.). All other reagents were of analytical grade.

### *Animals*

Female ODS rats of 21 days old which had been kept on a diet containing AsA were purchased from Crea Japan Inc. (Osaka, Japan). These rats were promptly ovariectomized under pentobarbital anesthesia and divided into two groups: one group was given drinking water containing 0.2% AsA and the other group was given drinking water without AsA. All the rats were kept at 25°C under controlled lighting (12 h light; 12 h darkness). They were given AsA-deficient chow (CL-2; Crea Japan Inc.) which had initially been autoclaved at 121°C for 10 min to destroy AsA, and was autoclaved again just before use to ensure complete destruction of AsA. At the age of 28 days, 6 rats in each group were killed by cervical dislocation and whole mounts of the right mammary glands were prepared. The remaining 21 rats in each group were given a daily injection of oestradiol-17$\beta$ (4 $\mu$g) and progesterone (5 mg) in 0.1 ml of sesame oil from day 28 to day 49 of age. Then all the rats were killed by cervical dislocation, and the abdominal mammary glands, liver and adrenal glands were removed.

### *Whole mounts of mammary glands*

Whole mounts of the right abdominal mammary glands were prepared by the method of Freeman and Topper [14].

### *Measurement of parenchymal areas of mammary glands*

The areas of parechyma in the abdominal mammary glands were determined in whole mounts. A photograph of the whole mount was taken at 4 magnifications and the margin of the mammary gland parenchyma was marked on a photograph with ink. The area enclosed by ink was then calculated using a PIAS LA-500 personal image analyzer system (PIAS Co., Tokyo, Japan).

### *Concentration of AsA*

The liver, adrenal glands and abdominal mammary gland were homogenized in 20–320 vols of ice-cold 5% metaphosphoric acid, and the homogenate was centrifuged at 1880 *g* for 10 min. AsA in the supernatant was measured by the dinitrophenylhydrazine method [15]. The minimum detectable concentration of AsA was 0.5 $\mu$g/tube.

### *Concentration of $\alpha$-lactalbumin in mammary glands*

Mammary tissues containing mammary parenchyma were homogenized with 5 vols of 20 mM Tris–HCl (pH 7.4) containing 10 mM $MgCl_2$ and 1% Triton X-100, and centrifuged at 3000 *g* for 15 min at 4°C. The concentration of $\alpha$-lactalbumin in the supernatant was measured by a modification of the method of Fitzgerald *et al.* [16] with exogenous bovine galactosyl transferase and bovine $\alpha$-lactalbumin as a standard, as described by Ono and Oka [17].

### *Response of mammary cells to insulin or prolactin in culture*

Mammary explants (about 1 mm$^3$) were prepared from abdominal mammary glands of ODS rats given drinking water with or without AsA after treatment with oestradiol-17$\beta$ and progesterone. The mammary explants were cultured as described by Topper *et al.* [18]. For estimation of the response of mammary cells to insulin, mammary explants were cultured

for 48 h in 3 ml of Medium 199 containing [$^3$H]thymidine (1 $\mu$Ci/ml) with or without insulin (5 $\mu$g/ml), and then the radioactivity incorporated into their DNA was determined as described by Hori and Oka [19]. The radioactivity incorporated in cultures with insulin was expressed as a percentage to that in cultures without insulin. For determination of the response to prolactin, mammary explants were cultured for 48 h in 3 ml of medium containing insulin (5 $\mu$g/ml) and cortisol (0.001 $\mu$g/ml) with or without ovine prolactin (5 $\mu$g/ml), and then the amount of $\alpha$-lactalbumin in the explants was determined as described above. The amount of $\alpha$-lactalbumin in cultures with prolactin was expressed as a percentage to that in cultures without prolactin.

*Hydroxyproline content of mammary glands*

The whole abdominal mammary gland was defatted in acetone, and homogenized with 4 ml of saline. An aliquot (80 $\mu$l) of the homogenate was hydrolyzed in 1 ml of 6 N HCl at 110°C for 22 h, the solution was evaporated, and the residue was dissolved in 4 ml of 0.02 N HCl. The amount of hydroxyproline in 30 $\mu$l of the solution was then measured in a Hitachi L-8500 High Speed Amino Acid Analyzer (Hitachi, Tokyo, Japan).

*Serum prolactin concentration*

Serum prolactin was measured with a radioimmunoassay kit for rat prolactin according to the protocol of NIADDK of the National Institutes of Health (Bethesda, Md., U.S.A.)

*Statistic analysis*

Statistic significances ($P < 0.05$) of differences were analysed by Student's $t$-test.

## RESULTS

*Body weights and concentrations of AsA*

Increases in the mean body weights of ODS rats given drinking water with and without AsA are shown in Fig. 1. The body weights of the two groups increased similarly for the first 7 days. However, the body weight of the AsA-deficient group did not increase further, whereas that of the group given AsA continued to increase steadily. At the age of 49 days, when all the rats were killed, the AsA-deficient rats showed hemorrhage around the eyes and nose, whereas the control group did not. The concentrations of AsA in the adrenal glands, liver and mammary gland of the AsA-deficient rats were extremely low or undetectable (Table 1).

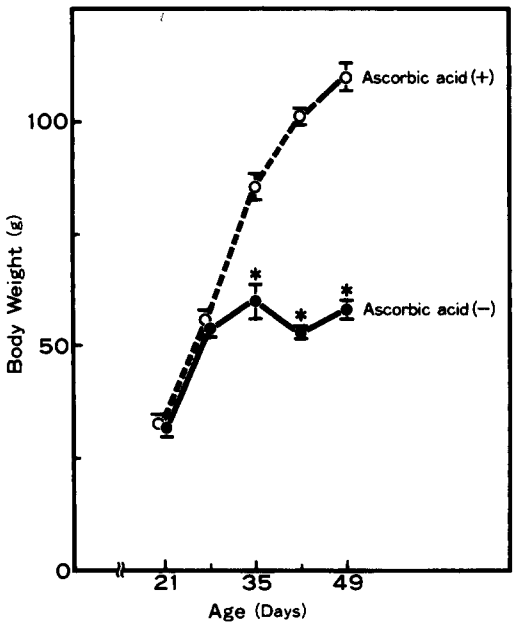


Fig. 1. Mean body weights of ODS rats given drinking water with and without ascorbic acid (AsA). Female ODS rats of 21 days old were divided into two groups: One group (○—○) was given AsA in their drinking water and the other group (●—●) was not. Points and bars represent mean body wt ± SE for 10 rats. Asterisks indicate significant differences between groups at $P < 0.01$.

*Whole mounts of mammary glands*

To examine the effects of oestradiol-17$\beta$ and progesterone (EP) on the development of the mammary glands, we prepared whole mounts of mammary glands before and after EP treatment. As shown in Fig. 2, there was no apparent difference in the morphology of the mammary glands of control as AsA-deficient ODS rats before EP treatment. EP treatment induced marked growth of ducts in the mammary glands of ODS rats given AsA. But, in the mammary glands of ODS rats not given AsA, the development of ducts was impaired and endbuds were very numerous. There was no significant difference in the area of parenchyma of mammary

Table 1. Concentrations of AsA in various tissues

| Tissue | No. | Concentration of AsA (mg/100 g wet tissue) ODS rats given AsA | ODS rats not given AsA |
|---|---|---|---|
| Adrenal gland | 15 | 104 ± 9.5 | <1.6 |
| Liver | 15 | 7.6 ± 0.3 | <0.1 |
| Mammary gland | 6 | 3.4 ± 0.7 | <0.3 |

ODS rats of 21 days old were divided into 2 groups; one group was given drinking water containing 0.2% ascorbic acid (AsA), and the other group drinking water without AsA. The rats received daily injections of oestradiol-17$\beta$ (4 $\mu$g) and progesterone (5 mg) from day 28 of age and were killed on day 49.

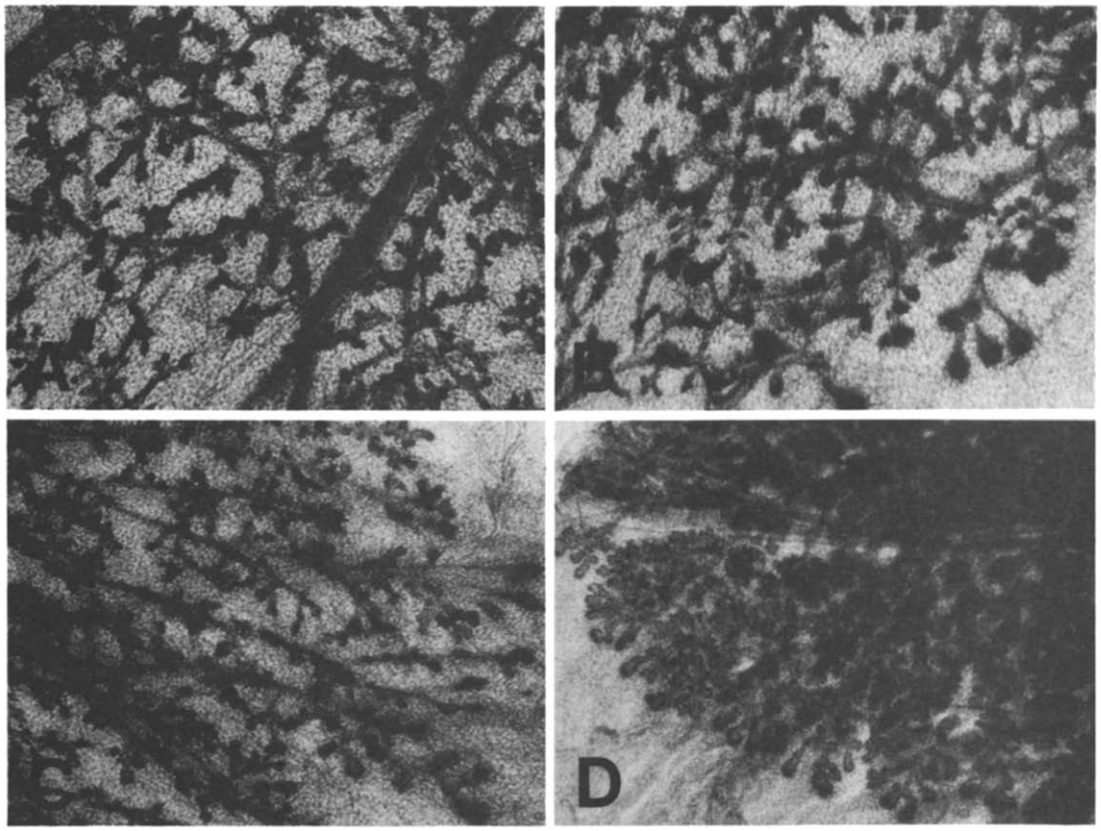


Fig. 2. Whole mounts of mammary glands of ODS rats given drinking water with and without ascorbic acid (AsA) before and after treatment with oestradiol-17$\beta$ and progesterone (EP). × 18. (A) and (B) show the mammary glands of ODS rats given drinking water with and without AsA, respectively, before EP treatment, and (C) and (D) show those of rats in the respective groups after EP treatment. In the mammary gland of the ODS rat not given AsA, the development of ducts is impaired and marked accumulation of endbuds is seen after EP treatment.

glands of the ODS rats in the two groups before EP treatment, but EP treatment increased the area in ODS rats given AsA about 2-fold, whereas it caused no significant increase in the area in AsA-deficient ODS rats (Fig. 3).

*Amount of α-lactalbumin*

We measured the concentrations of α-lactalbumin in the mammary glands of the two groups after treatment with oestradiol-17$\beta$ and progesterone. The amount of α-lactalbumin in mammary tissue was significantly less in ODS rats not given AsA ($7.7 \pm 1.9$ ng/mg wet tissue; $n = 7$, mean ± SE) than in those given AsA ($16.8 \pm 1.5$ ng/mg wet tissue; $n = 5$, mean ± SE).

*Responses of mammary cells to insulin and prolactin*

Results on insulin-induced DNA synthesis in mammary explants are shown in Fig. 4. Insulin increased DNA synthesis in mammary explants from ODS rats of both control and AsA-deficient rats about 2-fold, and there was no significant difference in the increases in the two groups. Prolactin increased the amount of α-lactalbumin in mammary explants from ODS rats, and again there was no significant difference in the increases in explants of the two groups (Fig. 5).

*Hydroxyproline content*

The amount of hydroxyproline in whole mammary glands was determined after EP injections. The amount of hydroxyproline per whole mammary gland was about twice as high in ODS rats given AsA ($430.8 \pm 39.8$ $\mu$g; $n = 5$, mean ± SE) as in ODS rats not given AsA ($205.6 \pm 17.9$ $\mu$g; $n = 5$, mean ± SE).

*Serum prolactin concentration*

The concentration of serum prolactin after EP injections in ODS rats not given AsA ($10.0 \pm 1.6$ ng/ml; $n = 12$; mean ± SE) was

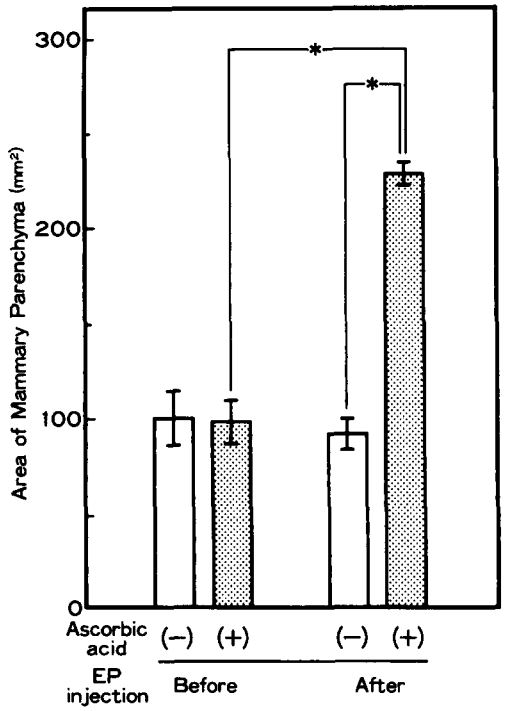


Fig. 3. Areas of mammary gland parenchyma in control and AsA deficient ODS rats. The area of parenchyma in the mammary gland was determined from whole mounts of mammary gland. Open columns and bars show mean ± SE for areas before and after treatment with oestradiol-17$\beta$ and progesterone (EP) in the AsA-deficient group and dotted columns and bars show those in the AsA-supplemented group. Values were determined in groups of 6 animals, and asterisks indicate significant differences ($P < 0.01$).

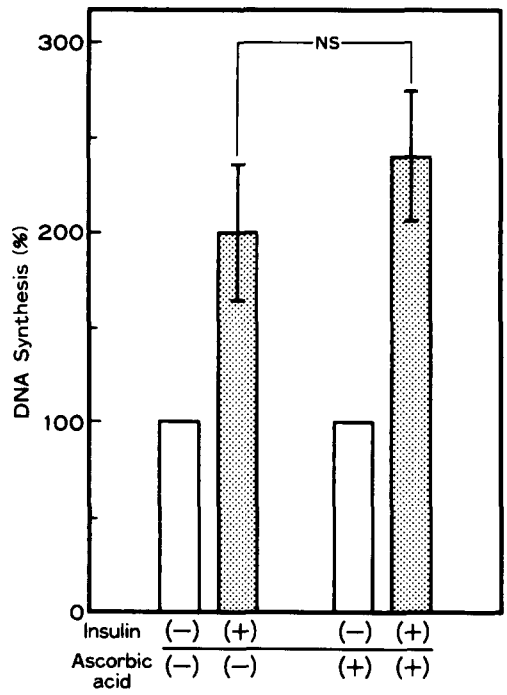


Fig. 4. DNA synthesis in mammary cells in cultures of mammary explants from ODS rats given drinking water with and without ascorbic acid (AsA). Mammary explants from ODS rats given drinking water with and without AsA after treatment of oestradiol-17$\beta$ and progesterone were cultured for 48 h in medium containing [$^3$H]thymidine with or without insulin (5 $\mu$g/ml), and then radioactivity incorporated into DNA was determined. Mean ± SE for DNA synthesis in cultures from 4 rats with insulin (dotted columns and bars) are shown as percentages to those in cultures without insulin (open columns). NS, not significant.

about one third of that in ODS rats given AsA (30.5 ± 4.1 ng/ml; $n = 11$; mean ± SE).

*Uterine wet weight*

There was no significant difference between the uterine wet weights of ODS rats given AsA (19.8 ± 0.7 mg; $n = 5$; mean ± SE) and those of ODS rats not given AsA (20.0 ± 1.1 mg; $n = 5$; mean ± SE) at the age of 28 days. EP injections increased the uterine wet weights of ODS rats given AsA about 10-fold (200 ± 12 mg; $n = 21$; mean ± SE) while they increased those of ODS rats not given AsA about 7.5-fold (154 ± 7 mg; $n = 21$; mean ± SE).

## DISCUSSION

The amounts of AsA in tissues of ODS rats not given AsA were extremely low and these rats showed scorbutic symptoms such as hemorrhage around the eyes and nose. However, ODS rats given AsA did not show these symptoms and their body weights increased steadily. As ODS rats became scorbutic when not supplied with AsA, they are useful for studies on the physiological roles of AsA. Guinea-pigs also become AsA-deficient when not given AsA, but far more biological and biochemical data are available on rats than on guinea-pigs, and so for studies on AsA-deficiency ODS rats seems preferable to guinea-pigs.

We found that the amount of hydroxyproline was reduced in AsA-deficient mammary glands. AsA is required in the hydroxylation of proline and lysine of procollagen and it increases collagen synthesis by fibroblasts [8–11]. Thus, our finding suggests that AsA-deficiency caused the accumulation of unhydroxylated collagen, or that it resulted in decrease in accumulation of collagen. Irrespective of the mechanism involved, this result suggests that AsA-deficiency brought about a defect in collagen synthesis in the mammary glands. Since collagen in both the stroma and basement membrane is important for the proliferation of mammary cells [1–3, 6, 7], the defect of collagen synthesis may be partly responsible for the impairment of

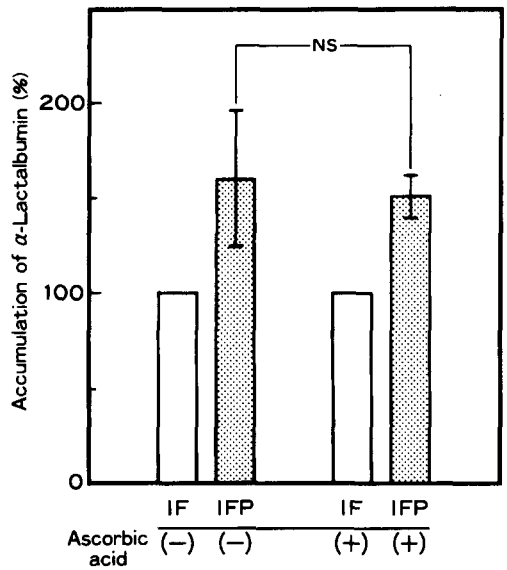


Fig. 5. Accumulation of α-lactalbumin in cultures of mammary explants from ODS rats given drinking water with and without ascorbic acid (AsA). Mammary explants from ODS rats given drinking water with and without AsA after treatment of oestradiol-17β and progesterone were cultured in medium containing insulin and cortisol with (IFP) or without (IF) prolactin (5 μg/ml), and the amount of α-lactalbumin in explants were determined. The amount of α-lactalbumin is expressed as a percentage to that in cultures without prolactin. Dotted columns and bars represent mean ± SE of 4 rats. NS, not significant.

ductal growth in the mammary glands of AsA-deficient ODS rats.

AsA deficiency resulted in a decrease in the concentration of serum prolactin. Prolactin is required for the growth-promoting actions of oestrogen and progesterone on the mammary glands [14]. Thus, a decrease in the concentration of serum prolactin may also be responsible in part for the impaired ductal growth of mammary glands. The reduced amount of α-lactalbumin in AsA-deficiency is also attributable to decrease in the concentration of serum prolactin. The mechanism of the decrease in the concentration of serum prolactin is unknown. Shin and Stirling [20] reported that AsA potentiated the inhibitory effect of dopamine on prolactin release from primary cultures of rat pituitary cells. They found that AsA deficiency caused increased secretion of prolactin if release of dopamine from the hypothalamus was not altered. Therefore, the decrease in the concentration of serum prolactin in AsA-deficient rats may be caused by increase in the release of dopamine from the hypothalamus. This possibility requires further investigation.

The responses of mammary cells to insulin and prolactin were not altered significantly by AsA deficiency. In our culture system, mammary explants from AsA-deficient and AsA-supplemented rats were cultured in Medium 199, which contains AsA at a concentration of 50 μg/l. As AsA is present in the medium, the result reflected only the state of mammary cells at the beginning of culture. Thus, the present results suggest that AsA deficiency does not impair the ability of mammary cells to proliferate and differentiate in response to hormones.

EP injections increased the uterine wet weights of ODS rats not given AsA about 7.5-fold while they increased those of ODS rats given AsA about 10-fold. The less increase in the uterine wet weights of ODS rats not given AsA may be related to the disturbance of the collagen production in the uterus or may reflect non-specific effects of weight loss. In contrast, EP injections did not increase the area of parenchyma of the mammary glands in ODS rats not given AsA although they increased that of ODS rats given AsA about 2-fold. The marked difference in the responsiveness to oestrogen and progesterone between the mammary glands and uterus of ODS rats not given AsA shows that the impaired development of the mammary glands in these ODS rats does not reflect non-specific effects of weight loss but specific effects of AsA deficiency on the mammary glands.

In conclusion, the present results suggest that AsA at a physiological concentration plays a role in development of the mammary glands.

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