Differential effects of retinoids on proliferation of bovine mammary epithelial cells in collagen gel culture

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Summary. The effects of increasing concentrations of retinol, retinal and retinoic acid on proliferation of bovine mammary epithelial cells were investigated in collagen gel cultures. All retinoids significantly inhibited proliferation of mammary epithelial cells. The relative inhibitory potency of the retinoids was: retinoic acid > retinal > retinol. Maximal inhibition at 10 μg/ml corresponded to a 75–95% inhibition of proliferation obtained in basal medium. Retinol, retinal and retinoic acid also inhibited proliferation of cells growth-stimulated with insulin-like growth factor-I (IGF-I). Retinoids in highest concentrations (10 μg/ml) inhibited 68–85% of proliferation of cells obtained in culture medium containing 25 ng IGF-I/ml. Retinol and retinoic acid also inhibited proliferation of cells growth-stimulated by insulin and other growth factors from the IGF growth factor family (des(1-3)IGF-I and IGF-II), as well as growth factors from the epidermal growth factor family (EGF and TGF-α), with retinoic acid being more effective than retinol. At a concentration of 100 ng/ml, retinol and retinoic acid inhibited respectively 24–38 and 44–52% of mammary cell proliferation stimulated by growth factors of the IGF family, and at 10000 ng/ml, 61–71% of cell proliferation was inhibited. The growth-stimulating effect of insulin, EGF and TGF-α was inhibited 42–64% by retinol and retinoic acid at 100 ng/ml, and 64–84% at 10000 ng/ml. The present results show that retinol, retinal and retinoic acid are potent inhibitors of bovine mammary epithelial cell proliferation. It is suggested that retinoids may have concentration-dependent roles in regulation of pubertal mammary growth and development, indicating that the milk yield potential of heifers may be affected by vitamin A status.

Keywords: Retinoids, bovine mammary epithelial cells, collagen cell culture, growth factors.

Vitamin A and its metabolites, collectively known as retinoids, are important for growth and differentiation of epithelial tissues. In cattle, vitamin A is present in blood and tissue mainly as all-*trans*-retinol, 9-*cis*-retinol and as their palmitate esters. In the target tissue vitamin A is metabolized into retinal and retinoic acid. The concentration of vitamin A in plasma from cows is strongly regulated between 200 and 400 ng/ml (Jensen *et al.* 1999). In grazing cattle, the vitamin A content in the liver can be as high as 350 µg/g. Knowledge about the effects of retinoids in mammary epithelium is limited, but retinoids have been shown to inhibit proliferation of a number of mammary carcinoma cell lines (Lacroix & Lippman,

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1980). In heifers, changes in mammary growth in relation to puberty can have important consequences for the subsequent milk yield potential (Sejrsen & Purup, 1997). This implies that variation in vitamin A status at this stage of development may influence the milk yield potential of heifers. The objective of the present study was to investigate the possible effect of retinol, retinal and retinoic acid on proliferation of pubertal mammary epithelial cells. Preliminary data were presented at the IX International Symposium on Ruminant Physiology, Pretoria, South Africa (Purup *et al.* 1999).

materials and methods

Materials and cell culture

Mammary epithelial cells isolated from prepubertal Friesian heifers were cultured in three-dimensional collagen gels as described previously (Weber *et al.* 1999). Briefly, tissue pieces excised aseptically from mammary parenchyma from two prepubertal Friesian heifers (both 188 kg live weight) were digested in basal medium (M199 with Earles salts; Sigma, St Louis, MO, USA) supplemented with collagenase (type II; Worthington Biochemical Corp., Freehold, NJ, USA), hyaluronidase, DNase and bovine insulin (all Sigma). Organoids were isolated by filtration, centrifugation and precipitation, and subsequently stored in liquid nitrogen until used in cell culture experiments. From each of the heifers, two preparations of organoids were prepared corresponding to harvest of cells at different times. For cell culture experiments, a mixture (1 : 1) of cells from the two heifers was prepared. Previous validation of sensitivity of different cell preparations has shown that organoids from different heifers at approximately the same live weight respond equally to growth factors, serum and mammary tissue extracts (Purup *et al.* 2000*b*).

Collagen gels were prepared essentially as described by Shamay *et al.* (1988). Cells were cultured for 24 h in basal serum-free medium 199 containing BSA (2·6 g/l), transferrin (5 mg/l), reduced glutathione (1 mg/l), soyabean trypsin inhibitor (1 mg/l), bovine insulin (10 μg/l), selenium (1 μg/l) and antibiotic solution (0·2 %) containing penicillin (50000 IU/l), streptomycin (50 mg/l) and amphotericin (125 μg/l), followed by 4 d in treatment media containing insulin-like growth factor (IGF)-I (1·56–50 ng/ml; Austral Biologicals, San Ramon, CA, USA), all *trans*-retinol, -retinal or -retinoic acid (0·1 pg/ml–10 μg/ml; Sigma) or the combination of IGF-I (25 ng/ml), des(1-3)IGF-I (25 ng/ml; GroPep Pty. Ltd, Adelaide, Australia), IGF-II (25 ng/ml; GroPep Pty. Ltd), insulin (INS; 100 ng/ml; Sigma), epidermal growth factor (EGF; 25 ng/ml; Austral Biologicals), transforming growth factor-α (TGF-α; 5 ng/ml; R&D Systems Europe Ltd, Abingdon, UK), and retinoids (0·01, 0·1, 1 and 10 μg/ml).

The different retinoids tested were solubilized in a 960 ml/l ethanol solution. The final ethanol concentrations never exceeded 3·3 ml/l. Medium 199 as manufactured (Sigma), also contained retinol acetate (140 ng/ml). However, based on results with retinol palmitate (0·01–10000 ng/ml; S. Purup, S. K. Jensen and K. Sejrsen; unpublished results), the concentration of retinol esters in the media is not supposed to have any significant effect on cell proliferation. Culture media were changed every 2 d and 1 μCi [methyl-^{3}H]thymidine was added for the last 24 h of the culture period. Proliferation of epithelial cells was determined using incorporation of thymidine (d.p.m./well) as a measure of DNA synthesis (Weber *et al.* 1999). The experimental design included at least three replicates per treatment and all experiments were repeated at least once. Because variation was observed in the mitogenic response to basal medium among the six independent assays (mean 122504 $\pm$ 17254 d.p.m.),

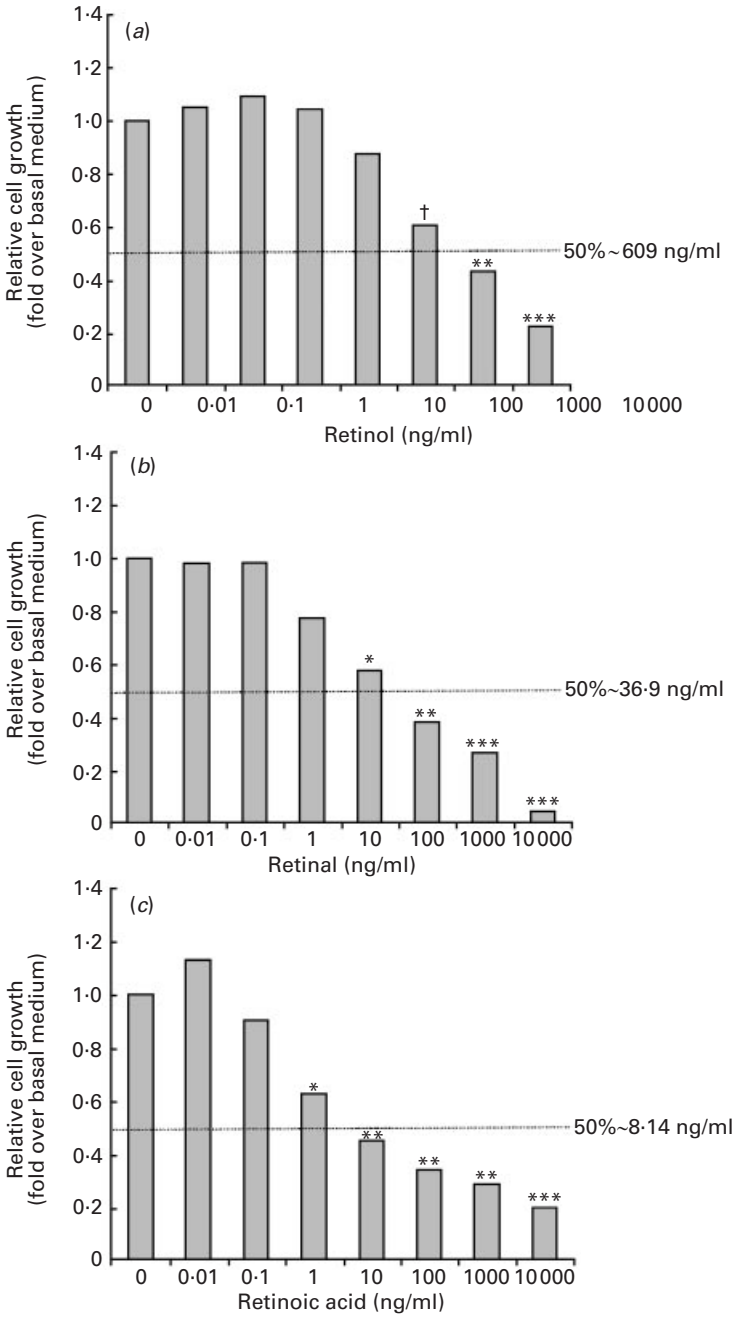

Fig. 1. Effect of increasing concentrations of (*a*) retinol, (*b*) retinal and (*c*) retinoic acid on proliferation, measured by incorporation of [methyl-^{3}H]thymidine into mammary epithelial cells prepared from 8–9-month-old prepubertal heifers and grown in 3-dimensional collagen gels. Values are least square means obtained from cultures with triplicate samples and presented as relative to proliferation obtained in basal medium (BM ~ 111388 d.p.m./well). Concentrations of retinoids corresponding to 50% inhibition of basal response are shown (calculated by linear regression of data including concentrations of 1–10000 ng/ml of retinoids). Values significantly different from proliferation obtained in basal medium are indicated: † $P < 0{\cdot}06$, * $P < 0{\cdot}05$, ** $P < 0{\cdot}01$, *** $P < 0{\cdot}001$.

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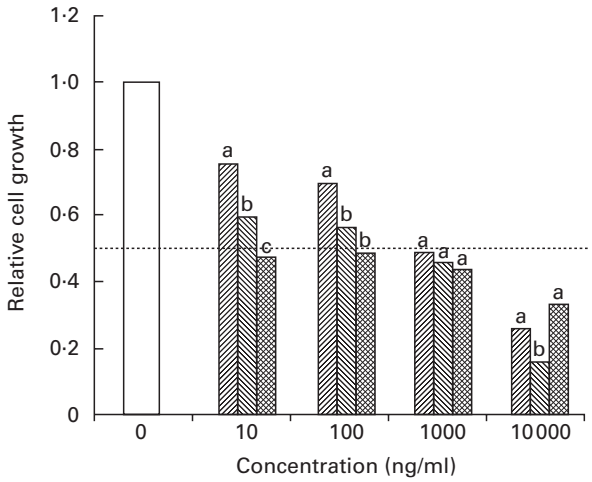

Fig. 2. Effect of increasing concentrations of retinol (▨), retinal (▧) and retinoic acid (⊠) on insulin-like growth factor (IGF)-I (25 ng/ml)-stimulated proliferation, measured by incorporation of [methyl-^{3}H]thymidine into mammary epithelial cells prepared from 8–9-month-old prepubertal heifers and grown in 3-dimensional collagen gels. Values are least square means obtained from cultures with triplicate samples and presented as relative to proliferation obtained in basal medium (BM ~ 107010 d.p.m.). Dotted lines represent 50% of proliferation obtained by IGF-I alone (□). Significant ($P < 0{\cdot}05$) differences within concentration of retinoids are indicated by different letters.

Table 1. *Inhibition of mammary cell proliferation (%) by retinol and retinoic acid as measured by incorporation of [methyl-^{3}H]thymidine into epithelial cells stimulated by growth factors of the insulin-like growth (IGF) and epidermal growth factor (EGF) families. Values were calculated relative to proliferation obtained by growth factors alone (basal medium ~ 122551 d.p.m./well; IGF-I ~ 543970 d.p.m./well; des(1-3)IGF-I ~ 536372 d.p.m./well; IGF-II ~ 505767 d.p.m./well; insulin ~ 278200 d.p.m./well; EGF ~ 183131 d.p.m./well; transforming growth factor-α (TGF-α) ~ 168429 d.p.m./well)*

	Growth factor					
Inhibitor (ng/ml)	IGF-I (25 ng/ml)	des(1-3)IGF-I (25 ng/ml)	IGF-II (25 ng/ml)	INS (100 ng/ml)	EGF (25 ng/ml)	TGF-α (5 ng/ml)
Retinol						
100	25	24	38	42	63	43
1000	40	37	49	57	72	62
10000	61	71	68	77	83	80
Retinoic acid						
100	44	47	52	55	64	63
1000	51	44	55	61	78	73
10000	66	62	64	64	84	82

results were adjusted to a similar scale by dividing values for [^{3}H]thymidine incorporation per well by the average basal value for incorporation in each assay.

Statistical analysis

Statistical analyses were performed using the general linear models procedure (PROC GLM) of SAS Institute Inc. (SAS, 1989). Different concentrations of the three retinoids (Fig.1) were tested using a model including the systematic effects of source of retinoid ($n = 3$), concentration of retinoid (eight levels) and their interactions. The

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inhibitory effects of the three retinoids on IGF-I stimulated cell proliferation (Fig. 2) were tested in a model including the systematic effects of source of retinoid ($n = 3$), concentrations of retinoids (five levels) and their interactions. The inhibitory effects of the two retinoids on different growth-stimulating factors (Table 1) were tested in a model including source of mitogen ($n = 6$), source of retinoids ($n = 2$), concentration of retinoids (four levels) and all 2- and 3-way interactions. The effect of assay ($n =$ 2 or 3) was included as a blocking factor in all models. The residual mean error was used as the error term for F-tests.

RESULTS AND DISCUSSION

The present study demonstrates the effect of retinoids on proliferation of primary mammary epithelial cells from prepubertal heifers. Overall, proliferation in response to retinoids was significantly ($P < 0{\cdot}01$) affected by the source of retinoid added to cell culture medium. The effects of the three different retinoids on cellular proliferation are seen in Fig. 1. Concentrations of retinol below 100 ng/ml were not significantly different ($P > 0{\cdot}44$) from proliferation obtained in basal medium. However, higher concentrations (> 100 ng/ml) significantly ($P < 0{\cdot}01$) inhibited cellular proliferation. Linear regression analysis of proliferation in response to retinol in concentrations between 1 and 10000 ng/ml, showed that a concentration of 609 ng/ml corresponded to 50% inhibition of the basal response. Retinal was more potent in inhibiting cellular proliferation than retinol. Concentrations below 10 ng/ml did not affect proliferation significantly ($P > 0{\cdot}24$), but in contrast to retinol, a concentration of 10 ng/ml significantly ($P < 0{\cdot}05$) inhibited proliferation. The concentration estimated to produce 50% inhibition of cellular proliferation was also lower than for retinol (36·9 *v.* 609 ng/ml). The most potent inhibitor of the retinoids tested was retinoic acid. A concentration of 1 ng/ml caused a significant ($P < 0{\cdot}05$) decrease in cellular proliferation compared with proliferation cultured in basal medium. The estimated concentration causing a 50% reduction in proliferation was 8·14 ng/ml. The maximal inhibiting effect of all retinoids was observed at 10000 ng/ml and corresponded to a 75–95% inhibition of proliferation obtained in basal medium.

The effect of retinoids has previously been studied in mammary carcinoma cell lines and in the bovine mammary epithelial cell line (MAC-T) (Lacroix & Lippman, 1980; Fontana *et al.* 1991; Adamo *et al.* 1992; Halter *et al.* 1993; Shemer *et al.* 1993; Woodward *et al.* 1996; Cohick & Turner, 1998). These studies all showed retinoids to inhibit mammary cell proliferation. The present study, however, is the first to show that retinoids are potent inhibitors in primary mammary epithelial cells. In fact, the different potencies of the metabolites on regulation of mammary epithelial cells in vitro suggest that retinoids may have concentration-dependent roles in regulation of pubertal mammary growth and development.

IGF-I has been proposed as a key regulator of mammary epithelial growth and differentiation (Cohick & Turner, 1998). Therefore, the effect of retinol, retinal and retinoic acid on mammary epithelial cells stimulated by IGF-I was investigated (Fig. 2). As shown previously (Weber *et al.* 1999; Purup *et al.* 2000*a*, *b*), proliferation was stimulated in a dose-dependent manner by IGF-I (data not shown). At an IGF-I concentration of 25 ng/ml, proliferation was stimulated more than four times relative to proliferation in basal medium.

Retinol, retinal and retinoic acid also inhibited proliferation of cells growth-stimulated with IGF-I (Fig. 2), and the inhibition was significantly ($P < 0{\cdot}001$) affected by the source of retinoid added to cell culture medium. At concentrations of

10 and 100 ng/ml the relative potency of the retinoids investigated was retinoic acid > retinal > retinol. At 1000 ng/ml, no significant difference was observed between the retinoids, while retinal inhibited proliferation more than retinol and retinoic acid at 10000 ng/ml. Thus, retinol and retinal are needed in higher concentrations than retinoic acid to cause a 50% reduction in proliferation. Retinoids in highest concentrations (10000 ng/ml) inhibited 68–85% of proliferation of cells obtained in culture medium containing IGF-I alone. Woodward *et al.* (1996) showed similarly, that proliferation of MAC-T cells that were growth-stimulated by IGF-I was inhibited 64% by retinoic acid.

A number of hormones and growth factors have been shown to be involved in mammary gland development and function (Purup *et al.* 2000*a*). These factors include growth factors of the IGF family and the EGF family. The effect of increasing concentrations of retinol and retinoic acid on proliferation of cells stimulated by growth factors from the IGF family as well as INS and growth factors from the EGF family was therefore investigated. Cell growth was stimulated 4·4-, 4·4- and 4·1-fold over basal medium by IGF-I, des(1-3)IGF-I and IGF-II at a concentration of 25 ng/ml. INS, EGF and TGF-α exhibited lower activity than growth factors of the IGF family, and only stimulated cell growth 2·2-, 1·5- and 1·4-fold over basal medium, respectively. These results are in accordance with our previously reported results for mammary epithelial cells (Purup *et al.* 2000*a*), except for EGF which in the previous results exhibited higher activity (3–4 fold over basal medium) than in the present study. This could possibly be due to different sources of human recombinant EGF used in the culture medium. The differential effects of growth factors have been discussed in the previous paper (Purup *et al.* 2000*a*).

In Table 1, the inhibition of mammary cell proliferation, calculated as a percentage of proliferation obtained by the growth factors alone, is shown. Retinol and retinoic acid at concentrations of 100, 1000 and 10000 ng/ml significantly ($P < 0{\cdot}001$) inhibited cell proliferation stimulated by the different growth factors. Retinoic acid was significantly ($P < 0{\cdot}01$) more effective than retinol. However, there was no significant ($P > 0{\cdot}28$) interaction between the growth-stimulating factor and retinoid, suggesting that the inhibiting effect of retinoids is unaffected by the growth factor stimulating proliferation. As shown, retinol and retinoic acid at a concentration of 100 ng/ml inhibited 24–38 and 44–52% of mammary cell proliferation stimulated by growth factors of the IGF family, whereas retinoids at the highest concentration (10000 ng/ml) caused an inhibition of 61–71% of cell proliferation. Proliferation stimulated by INS and growth factors of the EGF family was inhibited to approximately the same extent by retinol and retinoic acid, i.e. 42–64% at 100 ng/ml and 64–84% at 10000 ng/ml. These results suggest retinol and retinoic acid to be generally potent inhibitors of mammary epithelial cell proliferation, when cells are stimulated by a number of growth factors.

Retinoids have been demonstrated in previous studies to be potent regulators of IGF binding protein (IGFBP) production by various cell types (Adamo *et al.* 1992; Sheikh *et al.* 1993; Martin *et al.* 1995; Woodward *et al.* 1996), suggesting that retinoids inhibit growth through a pathway involving IGFBP. The proposed hypothesis is that local IGFBP levels govern the proliferation of cells by regulating the level of free IGF available to interact with IGF receptors, or by an IGF independent mechanism via specific cell surface receptors (Conover, 1992; Oh *et al.* 1995; Woodward *et al.* 1996; Leal *et al.* 1997; Mohseni-Zadeh & Binoux, 1997). Woodward *et al.* (1996) showed that growth of cells stimulated by des(1-3)IGF-I was only inhibited by 27% by retinoic acid, in contrast to 64% when stimulated

with IGF-I. Since des(1-3)IGF-I has reduced affinity for IGF binding proteins, this suggests that the mechanism of retinoic acid inhibition of cell growth could involve regulation by IGFBP. In accordance with these results, we recently showed that addition of IGFBP-3 or IGFBP-2 to cell culture medium significantly inhibited the proliferative effect of IGF-I on primary mammary epithelial cells (Weber *et al.* 1999; Purup *et al.* 2000*a*). However, the observed inhibitory effect of retinol and retinoic acid greatly exceeds the inhibition observed with very high concentrations of IGFBP-2 (250 ng/ml) and IGFBP-3 (800 ng/ml) (Weber *et al.* 1999; Purup *et al.* 2000*a*). The present results, showing that the mitogenic effect of IGF-I and des(1-3)IGF-I is inhibited to approximately the same extent by retinol and retinoic acid, also support the idea that the effect of retinoids is independent of IGFBP.

The present experiment shows an inhibitory effect on cell proliferation of all the retinoids: retinol, retinal and retinoic acid. However, it remains unclear whether retinol and retinal are directly involved in the inhibitory effect themselves, or whether they are metabolized into retinoic acid, leaving retinoic acid as the only inhibitor.

In summary, the results of the present study support a role for retinoids in the regulation of pubertal mammary development in heifers. However, more information is needed concerning the suggested presence and concentrations of retinoids in mammary tissue. Further studies on how retinoids affect IGFBP synthesis, IGF receptors etc. are also needed to elucidate the mechanism of action of retinoids on growth and development of the mammary gland, and to investigate whether milk yield potential of heifers is affected by vitamin A status.

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