Regulation of growth in cultured mammary epithelium from beef and dairy heifers

T.B. McFADDEN AND D.C. COCKRELL[1]

Dairying Research Corporation, Ruakura Agricultural Centre, Private Bag 3123, Hamilton, New Zealand.

ABSTRACT

Growth of mammary epithelium is regulated by endocrine signals, local factors and sensitivity of epithelial cells to mitogens and inhibitors. This study was designed to identify how these factors interact in regulating mammogenesis and determine if breed differences exist among these factors. Mammary epithelial cell organoids were isolated from five pairs of beef and dairy heifers (7 to 9 months of age) and cultured within collagen gels. Sera were collected from all heifers and pooled within breed for use in culture. At slaughter, explants of mammary fat pad were also prepared for culture. Culture treatments were arranged in a 2x2x3x4 factorial design. Factors included source of cells (beef or dairy), addition of growth factors (10 ng/ml IGF-I + 50 ng/ml EGF or control), source of mammary fat pad explants (beef, dairy or none) and source of serum added to culture media at 5% final concentration (beef, dairy, foetal calf serum, or none). Mammary growth was assessed as incorporation of [3H]thymidine into DNA. Mammary epithelial cells from beef heifers grew more rapidly than dairy cells (2518 vs 1192 cpm/ug; P<0.01). Growth of epithelia was enhanced by addition of growth factors (2265 vs 1445 cpm/ug; P<0.01). Sera from both beef and dairy heifers stimulated growth 2-fold compared to no serum (P<0.01), but addition of mammary fat inhibited epithelial growth (P<0.05). Cells from beef heifers were also more responsive to addition of serum and growth factors. These findings demonstrate that multiple factors interact to control mammary growth. Mammary fat pad of ruminants, unlike rodents, appears to inhibit epithelial growth.

Keywords: Heifers, mammary, growth, breed.

INTRODUCTION

Genetic selection for milk production has resulted in dramatic increases in average milk yield of dairy cows, but despite these advances the physiological mechanisms that mediate superior milk production are poorly understood. The potential for studies on diverse genetic groups to contribute to understanding of mammary function and improvement of selection criteria has been emphasized by Tucker (1981). In previous work, we demonstrated breed differences in the ability of mammary tissue from immature cattle to produce milk proteins *in vitro* (McFadden *et al.*, 1988, 1989). However, potential differences in proliferative ability of mammary tissue were not addressed.

Because number of mammary secretory cells in the udder constitutes a basic limitation to milk production (Knight and Peaker, 1982), and is highly correlated with milk yield, differences in regulation of mammary growth may be a primary source of genetic variation in milk yield. Growth of mammary epithelium is subject to regulation by endocrine signals, local interaction with surrounding mesenchyme, and intrinsic ability of the epithelium to respond to mitogenic cues (Forsyth, 1989). Each of these factors might contribute to genetic differences in potential for mammary development.

Thus, the present study was designed to 1) determine the roles and interactions of these factors in regulation of mammary growth and, 2) identify potential breed differences in regulation of mammogenesis.

MATERIALS AND METHODS

Five beef and five dairy heifers were purchased for use in this experiment. Heifers were matched as nearly as possible for age and bodyweight. Beef heifers were cross-breds while dairy heifers were of the Holstein breed. All heifers were bled twice during the week prior to the first slaughter. Sera were harvested and 10 ml from each sample were pooled within breed and filter-sterilized to provide "beef" and "dairy" serum pools for use in culture experiments. Where appropriate, pooled sera or foetal calf serum (FCS) were added to culture media to achieve a final concentration of 5%.

Heifers were slaughtered in pairs at 7 to 9 months of age. Mammary epithelial organoids were isolated by enzymatic digestion and filtration, then cultured within rat-tail collagen gels in 24-well culture plates as described by Yang *et al.*, (1979), but modified for bovine mammary tissue (McGrath, 1987; Shamay *et al.*, 1988). Briefly, mammary parenchyma was dissected from the surrounding stroma under sterile conditions, parenchyma was minced finely, and epithelial cells were isolated by digestion in medium containing collagenase and hyaluronidase. Epithelial organoids were isolated by sequential filtration through 250 then 50 um mesh Nitex filters. Organoids retained on the 50 um filter were seeded in collagen gels at a density of approximately 2 X 10^5 cells/well.

Organoids were cultured 8 days at 37C in an atmosphere of 5% CO_2-95% air. Basal medium consisted of medium 199 supplemented with .25% BSA, 5 ug/ml transferrin, 1 ng/ml

[1]Animal & Veterinary Science Department, University of Idaho, Moscow, ID, 83843, USA.

selenic acid, 100 U/ml soybean trypsin inhibitor, 1 ug/ml reduced glutathione, and .2% antibiotic-antimycotic solution. Other additives are described below. Cultures were incubated in 1 ml of medium which was renewed every 48 hours.

Co-cultures of mammary epithelium with mammary fat pad were conducted by placing a sterile, stainless steel grid atop the collagen gel. Explants of mammary fat pad (~2mm^3) were prepared during tissue processing and incubated in 37C medium until the initiation of culture. Three to four explants per well were placed on the grid such that they were physically separated from the gel, and were suspended at the medium:atmosphere interface.

Culture treatments were designed as a 2x2x3x4 factorial. Factors included: 1) breed of epithelial cell-donors, either beef or dairy; 2) culture medium, either basal or supplemented with 10 ng/ml insulin-like growth factor-I (IGF-I) and 50 ng/ml epidermal growth factor (EGF); 3) breed of fat pad explant-donors, i.e., beef, dairy or none; and 4) source of serum added to media (beef, dairy, FBS or none). All treatments were run in triplicate wells.

Cultures were pulsed with 1 uCi [^{3}H]-thymidine 24 hours prior to termination. Cells were released from the gels by incubation in acetic acid, washed 4X, and recovered by centrifugation. Cell growth response was determined by measuring tritiated thymidine incorporation into a TCA-insoluble fraction and total DNA content (Labarca and Paigen, 1980) per culture.

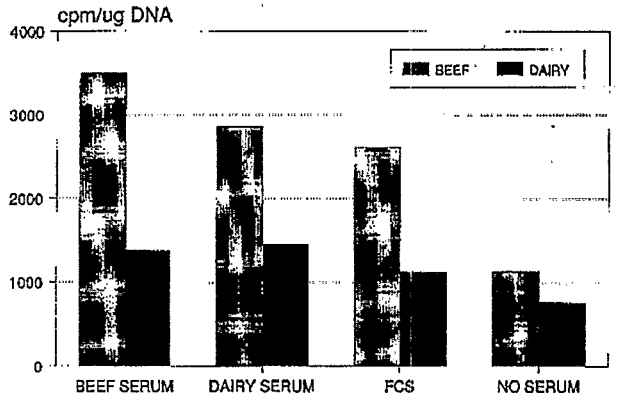
Data were analyzed using the general linear models procedure of SAS (1985) to determine significance of main effects and interactions. Where indicated by significant F-tests, treatment means were compared by LSD.

RESULTS AND DISCUSSION

Organoid growth within collagen gels was characterized by branching, tubular morphogenesis similar to mammary ducts *in vivo* and typical of mammary epithelia cultured within collagen (McGrath *et al.*, 1987). Overall, growth of mammary organoids from beef heifers was more than double that of cells from dairy heifers (2518 vs 1192 cpm/ug; P<0.01). The overall effect of adding growth factors to media was to elicit a 50% increase in growth (2265 vs 1445 cpm/ug; P<.01). In addition, a significant interaction between breed source of cells and presence of growth factors (P=.06) indicated that beef cells were also more responsive to growth factors, as seen in Figure 1.

The stimulatory effect of IGF-I and EGF is consistent with other reports on ruminant mammary tissue *in vitro* (McGrath *et al.*, 1987; Shamay *et al.*, 1988; Winder *et al.*, 1989) and *in vivo* (Collier and McGrath, 1988). In contrast, the unexpectedly higher growth and responsiveness to growth factors observed in the cells from beef heifers is difficult to reconcile with the established differences in mammary development and milk production between beef and dairy breeds. However, we found similarly unexpected results in a previous study in which mammary explants from prepubertal beef bulls secreted more milk proteins and were more responsive to hormones *in vitro* than those from dairy bulls (McFadden *et al.*, 1988). Unlike explants from bulls, similar

FIGURE 1: Effect of IGF-1 and EGF on incorporation of [^{3}H]-thymidine into DNA in mammary epithelial organoids from beef and dairy heifers. Pooled SE = 156.1.

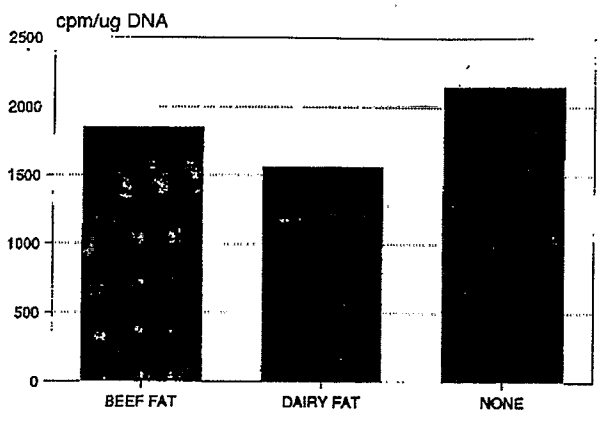


studies in heifers demonstrated the expected breed differences in favor of explants derived from dairy heifers (McFadden *et al.*, 1989). Others have reported markedly greater mammary development and function in mammary tissue from dairy vs beef cattle during gestation and lactation (Keys *et al.*, 1989), consistent with expected breed differences. In the present study, it is possible that heifers of the two breeds were in different physiological states, despite our attempts to pair according to age and weight. If the beef heifers were physiologically closer to puberty, it may have affected the ability of the mammary epithelium to grow and respond to mitogens *in vitro*. Thus, the greater growth of organoids from beef heifers may reflect a difference in physiological state at the time of tissue removal, rather than a difference in genetic potential for growth.

Source of sera added to cultures had a highly-significant effect on mammary growth (P<.001). Overall, growth in the presence of FBS or dairy heifer serum was about double that in the absence of serum (P<.05) whereas beef heifer serum elicited a further increase in growth (P<.05). However, the effect of serum was dependent on breed-source of cells as revealed by a significant interaction between serum-type and cell-type (P<.01; Figure 2). In general, epithelia from beef heifers were more responsive to all sera than dairy-derived cells, consistent with their greater sensitivity to mitogenic growth factors (Figure 1). Furthermore, while serum source had no marked effect on dairy cells, growth of beef cells was maximal in the presence of beef serum (P<.05). While this result must be interpreted cautiously due to the "snapshot" nature of both serum and epithelium samples, it is also suggestive of a difference in physiological state of heifers of the two breeds, as proposed above to account for the unexpected direction of breed differences.

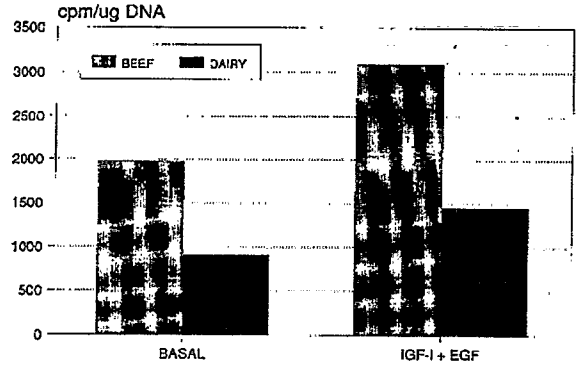
Growth of epithelial organoids was significantly affected by co-culture with explants of mammary fat pad (P<.01; Figure 3). Although explants from both beef and dairy donors reduced epithelial growth compared to absence of fat pad, the difference was significant only for dairy fat pad (P<.01). The general negative effect of fat pad on organoid growth suggests that ruminant fat pad may be capable of exerting an inhibitory influence on mammary epithelial growth. This is in contrast to the situation in rodents, wherein

FIGURE 2: Effect of source of serum on incorporation of [^{3}H]-thymidine into DNA in mammary epithelial organoids from beef and dairy heifers. Where present, sera were added at 5% final concentration. FCS = foetal calf serum. Pooled SE = 246.9.



co-culture of mammary cells with fat pad explants led to increased epithelial growth (Carrington and Hosick, 1985). Indeed, a fat pad is necessary to support growth of transplanted mammary epithelia in rodents (reviewed in Forsyth, 1989).

FIGURE 3: Effect of co-culture in the presence of mammary fat pad explants on incorporation of [^{3}H]-thymidine into DNA in mammary epithelial organoids from heifers. Pooled SE = 135.1.



It is possible that the apparent difference in the effects of ruminant and rodent fat pad on mammary epithelial growth is related to the difference in fatty acid composition of lipid stored in the fat pad. Ruminant depot fat is characterized by a high degree of saturation due to biohydrogenation of lipids in the rumen. In contrast, stored lipid in rodents generally reflects the fatty acid composition of the diet, and is typically much higher in polyunsaturated fats. The potential role of fat pad-derived fatty acids is supported by our previous findings that increasing unsaturated fatty acids in mammary fat pad of ewe lambs was associated with enhanced epithelial development (McFadden *et al.*, 1990) and growth of a bovine mammary epithelial cell line is stimulated by unsaturated fatty acids (Cockrell *et al.*, 1992). Others (Beck and Hosick, 1988) suggested that the positive effect of mouse mammary fat pad-conditioned media on mammary epithelial growth was mediated by unsaturated fatty acids. However, it is equally possible that the fat pad explants may have released paracrine inhibitors of epithelial growth such as transforming growth factor-B (Plaut, 1992). Whether the effect of fat pad was exerted by one of these, or by yet other means, remains unclear.

Taken together, the results of the present study clearly demonstrate the complex, integrated nature of mammary growth regulation. While breed differences were observed, it seems probable that these reflect a difference in physiological state at the time of sampling (supported by increased epithelial growth and responsivity to GFs and sera, as well as less-inhibitory effect of fat pad from beef heifers) rather than a true difference in genetic potential for mammary development. In addition, the negative influence of mammary fat pad on epithelial proliferation implies a further level of local regulation of mammogenesis. Specific mechanisms that mediate these regulatory influences remain to be elucidated.

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