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BOVINE MAMMARY EXPLANT VERSUS PRIMARY CELL CULTURES: EFFECT OF BOVINE SOMATOTROPIN AND INSULINLIKE GROWTH FACTOR-I ON DNA CONTENT AND PROTEIN SYNTHESIS

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SUMMARY

Cellular DNA, milk protein content, and protein secretion by bovine mammary explants were compared to cultures of confluent and growing primary bovine mammary secretory cells over 4 d. Explants were obtained at slaughter from eight Holstein cows (120 ± 35 d lactation). Primary cells were grown to confluence, cryopreserved, thawed, and cultured through five passages. Explants and cells were cocultured with liver and adipose tissue in the presence of somatotropin, insulinlike growth factor-I, and somatotropin + insulinlike growth factor-I. Cellular DNA and milk proteins were assayed using fluorescent probes and flow cytometry. Media proteins were assayed by densitometer scanning of electrophoresis gel bands. DNA content of explant, confluent, and growing primary cells increased similarly through the 96 h incubation. DNA content in G_0G_1 phase was increased by: (a) insulinlike growth factor-I in explant cells; (b) somatotropin, insulinlike growth factor-I, and their combination in confluent primary cells; and (c) the combination of somatotropin and insulinlike growth factor in growing primary cells. Approximately 65% of explant and confluent primary cells were in the G_0G_1 or differentiated phase compared to 47% for the growing primary cells. Whey protein content and secretion were similar among cell types. Explant cells contained and secreted more β-casein than primary cells but secretion trends for β-casein and k-casein were similar after 48 h for both cell types. Results suggest that primary cell cultures are comparable to explant cultures when used to study mechanisms of DNA and milk protein synthesis and secretion.

Key words: bST; IGF-I; lactalbumin; casein; mammary cells.

INTRODUCTION

Casein and whey proteins account for 96% of total milk proteins (8). Milk from dairy cows contains approximately 30 g casein and 2.5 to 7.2 g whey proteins/l (17,22,34). In addition to their value in whole milk, milk proteins are used in a large number of formulated foods (24). Early research on mechanisms of milk protein synthesis and secretion involved *in vivo* studies. More recent studies have employed various tissue culture methods.

Early mammary tissue culture studies relied on tissue slices (2), tissue explants (9,11,13,15,18), and isolated acini (3,25). Current studies tend to use either established cell lines (16) or primary cell cultures (5,6,28,31,32).

There are advantages to both tissue and cell culture models. During the first 24 h in culture explants are more representative of the events occurring *in vivo*. However: (a) explant preparation destroys small ducts that may inhibit the passage of lipids and proteins from the alveoli to the culture medium; (b) explants are functional for less than 96 h (19); and (c) explants are usually collected at slaughter and thus, experiments cannot be repeated with tissue from the same animal. Conversely: (a) cell cultures secrete lipids and proteins (6,10,16,31,32) directly into the medium; (b) cells can be cultured for extensive periods of time; and (c) cultured cells can be cryopreserved and used for numerous experiments.

The objective of this study was to compare DNA and milk protein content in the cell and milk protein secretion by explant and primary cell culture models. Cell growth and differentiation were also examined over a 4 d incubation period.

MATERIALS AND METHODS

Tissue explant cultures. Mammary, liver, and adipose tissues were obtained at slaughter from eight multiparous Holstein cows at 120 ± 34.9 d into lactation. Milk production averaged 28.4 ± 4.1 kg milk/day. The tissues were transported to the laboratory and explants prepared as described (9). Mammary explants were cocultured with adipose and liver tissue in tissue culture inserts (9,18) for 24, 48, 72, or 96 h in 95% air and 5% CO_2 at 39° C. Mammary explants and primary cells were cultured with adipose and liver tissue because adipose and liver tissue are required for bovine somatotropin (bST) to affect lipid synthesis (9) and for insulinlike growth factor-I (IGF-I) to affect DNA synthesis (21).

Primary cell cultures. Primary bovine mammary secretory epithelial cells were prepared as described (5,6). Primary cells were plated (2×10^5 cells/well) on collagen and grown to confluence for 8 d prior to the day of slaughter (Day 0) in medium described in (5,6). On Day 0, adipose and liver tissues were placed on inserts and cocultured with the confluent primary cells. Also on Day 0, additional primary cells (growing primary cells) were plated (2×10^5 cells/well) on collagen and cocultured with adipose and liver tissues on tissue culture inserts for 24, 48, 72, or 96 h in 95% air and 5% CO_2 at 39° C.

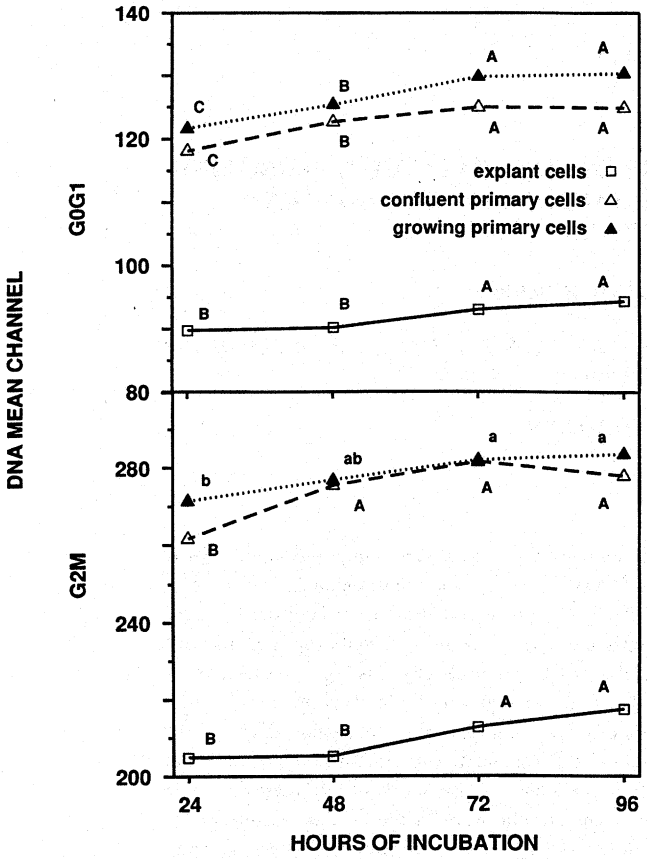

FIG. 1. Effect of incubation time on the DNA content of explant, primary confluent and primary growing cells in the G_0G_1 and G_2M phases of the mitotic cycle. DNA content is expressed as the mean electronic channel of fluorescent cells in the G_0G_1 or G_2M peaks—the greater the fluorescence, the higher the mean channel. Points within lines indicated by different letters are different (*A,B,C*: $P < 0.01$ and *a,b,c*: $P < 0.02$).

Culture media and hormone treatments. On Day 0, basal medium [medium 199, sodium bicarbonate (26.15 m*M*), HEPES (15 m*M*), penicillin, 100 U/ml; Fungizone, 0.25 μg/ml; streptomycin, 100 μg/ml, 5 μg cortisol/ml (13) and 3% fetal bovine serum (FBS)] was added to explant, confluent primary, and growing primary cell cultures. Medium was changed daily. Four hormone treatments were superimposed: (a) control medium (C); (b) 1 μg pituitary derived bST/ml medium (0.81 IU/mg, NIH-B-18, Bethesda, MD); (c) 1 μg recombinant derived human IGF-I/ml medium (BioSource International, Camarillo, CA); and (d) bST + IGF-I.

After incubation, primary cells were suspended as described (5), centrifuged and the cell pellet stored in 70% ethyl alcohol at −20° C. The explants were minced four times with a McIlwain (Mickle Laboratory Engineering Co., Ltd., Gomshall, Surrey, England) tissue chopper (21). Epithelial cells were isolated from the minced tissue, fixed in 70% ethyl alcohol and stored at −20° C as described (21).

Casein and whey protein content of culture medium. After incubation, culture medium was cooled to remove fat and decanted into glass tubes. Milk proteins were precipitated with trichloroacetic acid, washed with ethyl ether, and treated with Laemili buffer, and applied to a 15% acrylamide gel for electrophoretic assay as described (21). The gels were dried and the β-casein, k-casein, and α-lactalbumin bands scanned with a densitometer (Shimadzu, Columbia, MD).

DNA, β-casein, and whey protein content of cultured cells. DNA content of explant and primary mammary epithelial cells was measured by labeling the DNA with propidium iodide as previously described (21). Whey proteins and β-casein content of explant and primary cells were measured by labeling cells with rabbit antisera specific for bovine whey proteins and β-casein (donated by Dr. J. Spies, Eastern Utilization, ARS, USDA) as described (20). Fluorescein (FITC) labeled goat anti-rabbit IgG was used as the second antibody (Kirkegaard and Perry, Gaithersburg, MD).

Flow cytometer assay. The number of cells fluorescing and the intensity of fluorescence were measured using an EPICS Profile Flow Cytometer (Coulter Electronics, Hialeah, FL) equipped with a 488 nm argon ion laser. Laser power was set at 15 mW. The sheath pressure was 7.5 psi. The degree of fluorescence in individual cells was measured through a 525 nm band pass filter in electronic channels from 0 to 1023. The DNA and protein concentration of the cells was expressed as the average or mean channel of all the cells in a given assay. The higher the channel number, the greater the cell DNA or protein content. Single mammary epithelial cells were separated from cellular debris, doublets, and other possible cell types on a forward scatter versus log side scatter histogram and gated to a second histogram to measure log fluorescence. As shown in Keys et al. (20), gating is accomplished by drawing a circle around a specific cell type on the first histogram through an interactive process. Only fluorescence emanating from cells within the circle is used to calculate the mean channel of fluorescence. Mammary epithelial cells from healthy, nonmastitic udders as used in this study are relatively but not entirely free of blood cells such as lymphocytes and neutrophils that are more common in mastitis-infected quarters. Primary mammary epithelial cells as used in this study were obtained from colonies arising from single cells and should be free of contamination by other cell types as demonstrated in (20). The forward scatter gain was set at 1.5 while the photomultiplier tube (PMT) used to detect side scatter was set at 200 v. The PMTs for fluorescence were set at 600 v and 850 v, respectively, for milk protein and DNA determinations. Mean channel of peak fluorescence and the percentage of cells in the G_0G_1, S, and G_2M phases were determined using Cytologic (Coulter).

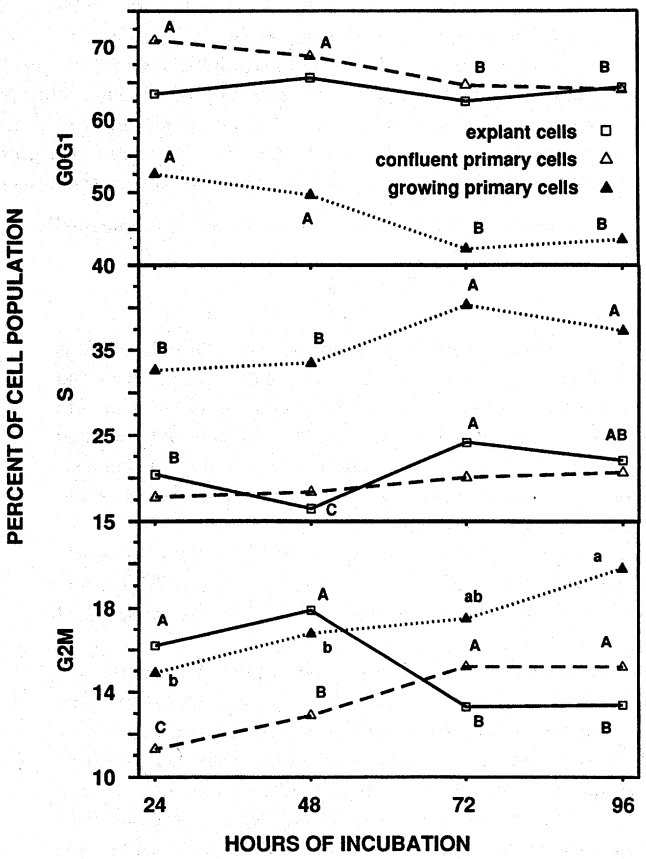

FIG. 2. Effect of incubation time on the percentage of explant, primary confluent, and primary growing cells in the G_0G_1, S, and G_2M phases of the mitotic cycle. Points within lines indicated by different letters are different (*A,B,C*: $P < 0.01$ and *a,b,c*: $P < 0.05$).

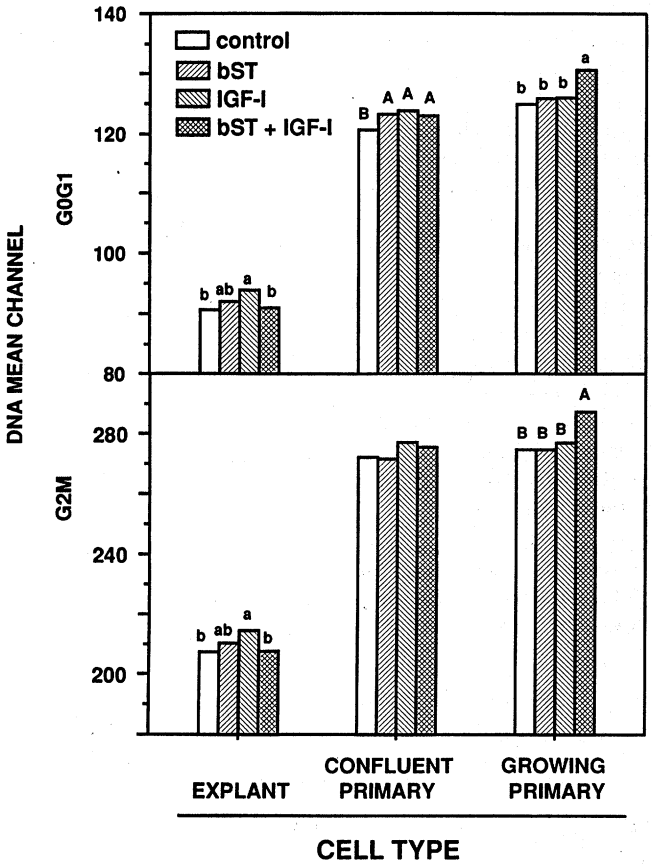

FIG. 3. Effect of bovine somatotropin (bST), insulinlike growth factor-I (IGF-I), and bST + IGF-I on the DNA content of explant, primary confluent, and primary growing cells in the G_0G_1 and G_2M phases of the mitotic cycle. DNA content is expressed as the mean electronic channel of fluorescent cells in the G_0G_1 or G_2M peaks—the greater the fluorescence, the higher the mean channel. *Bars* within clusters indicated by different letters are different (*A,B,C*: $P < 0.01$ and *a,b,c*: $P < 0.06$).

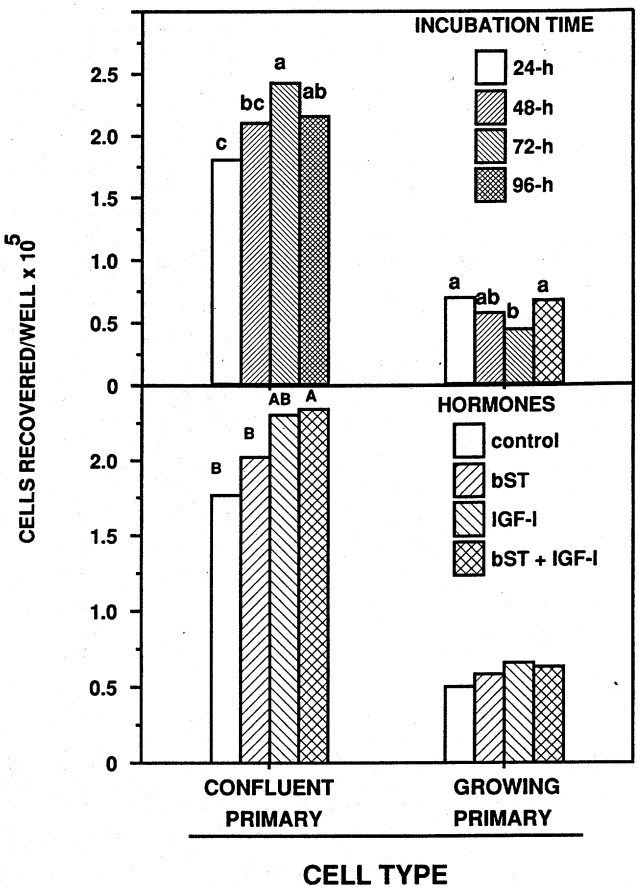

FIG. 4. The effect of incubation time and hormones [bovine somatotropin (bST) and insulinlike growth factor-I (IGF-I)] on the number of primary confluent and primary growing cells recovered per well. *Bars* within clusters indicated by different letters are different (*A,B,C*: $P < 0.01$ and *a,b,c*: $P < 0.05$).

Statistical analysis. The mixed model procedure of SAS (12) was used to determine the significance of cell type, hormone treatment, and incubation time. Cell type was tested with a model that included cow, hormone treatment, incubation time, and the two-way interactions between cell type, hormone treatment, and incubation time. Each cell type was then tested individually with a model that included cow, hormone treatment, incubation time, and hormone treatment x incubation time interaction. Error terms were determined by the mixed model program. Mean comparisons were performed between cell type, hormone treatment, incubation time, and interaction means if those effects were significant at $P < 0.10$. Data are presented as least-square means.

RESULTS AND DISCUSSION

The DNA content of mammary explant cells in the G_0G_1 and G_2M phases of the mitotic cycle was lower ($P < 0.0001$) than that of confluent or growing primary cells (Fig. 1). This was attributed in part to variation in the number of propidium iodide binding sites on the DNA of the individual donor cows. DNA content increased ($P < 0.02$) by 5–6% in all three cell types over the 96 h incubation. The effect ($P < 0.01$) of incubation time on DNA content was linear for all cell types (Fig. 1). In addition to nuclear DNA, cells also contain mitochondrial DNA. It is unlikely, however, that mitochondrial DNA,

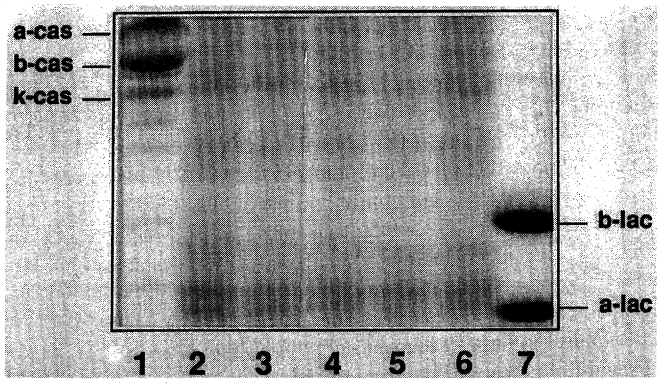

FIG. 5. Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis of milk proteins secreted into the medium. *Lanes 1–7* contain: 1, mixed casein standard; *2–6*, medium samples; and *7*, whey protein standard.

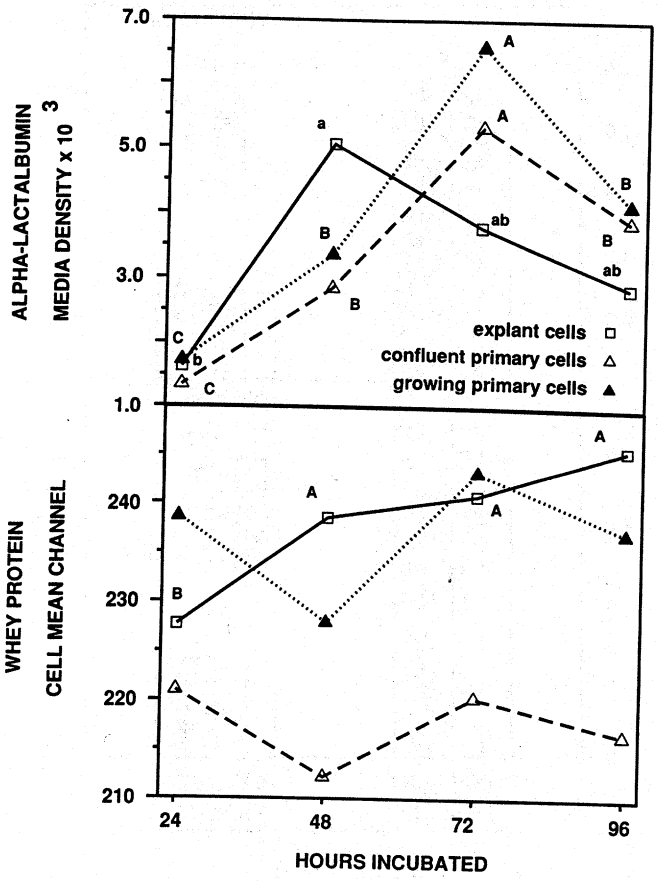

FIG. 6. The effect of incubation time on the relative concentration of whey protein in explant, primary confluent, and primary growing cells and the secretion of α-lactalbumin into the medium. Protein content is expressed as the mean of all electronic channels containing fluorescent cells—the greater the fluorescence, the higher the mean channel. Points within lines indicated by different letters are different (*A,B,C*: $P < 0.01$ and *a,b,c*: $P < 0.09$).

which consists of less than 18 000 base pairs (7), was the source of the 5–6% change in DNA concentration.

The majority of the explants (63.3 ± 0.8%) and confluent primary cells (68.1 ± 0.8%) were in the G_0G_1 or differentiated phase of mitosis (Fig. 2). The growing primary cells did not reach confluence by 96 h and only ($P < 0.0001$) 46.9 ± 1.1% were in G_0G_1. Conversely, 36.1 ± 1.2% of the growing primary cells were in S-phase compared to only 21.0 ± 0.8% of the explant cells and 18.2 ± 0.9% of the confluent primary cells ($P < 0.0001$). Differences ($P < 0.0001$) in the G_2M phase between the explant (15.6 ± 0.4%), confluent primary (13.6 ± 0.5%), and growing primary cells (17.4 ± 0.6%) were not as dramatic. The percentage (63%) of G_0G_1 cells observed in these early lactation cows (120 ± 3.4 d) was smaller than the 90% figure observed in midlactation cows (226 ± 26 d) in a previous experiment (21). This suggests that bovine mammary tissue continues to increase in cell number during early lactation.

Others demonstrated that IGF-I can stimulate DNA synthesis in undifferentiated mammary epithelial cells from calves (26) and mammary explants from lactating cows (4). In this study IGF-I increased ($P < 0.07$) DNA content of explant cells while bST + IGF-I increased ($P < 0.03$) the DNA content of growing primary cells (Fig. 3). All three hormone treatments increased ($P < 0.01$) the DNA content of confluent primary cells in the G_0G_1 phase cells. Growing primary cells were not affected by bST or IGF-I alone as were the confluent primary cells. The failure of growing primary cells to reach confluence by 96 h could account for their failure to respond to the individual hormones (Fig. 4). In a previous study (21) with mid- to late-lactation (266 ± 26 d) cattle, IGF-I depressed the DNA content of explant cells. Hormones had no effect on the ratio of cells in the G_0G_1, S, or G_2M phases.

The effect of incubation period and hormone treatment on the number of primary cells at the end of incubation is shown in Fig. 4. Confluent primary cells increased ($P < 0.02$) through 72 h, then decreased. Conversely, growing primary cells decreased ($P < 0.05$) through 72 h and then increased. The addition of IGF-I alone or in combination with bST increased ($P < 0.01$) the number of confluent primary cells. Hormones had no effect ($P > 0.39$) on the number of growing primary cells.

The electrophoretic pattern of proteins secreted into the medium was similar for explant and primary cells (Fig. 5). A band was present for α-lactalbumin but not for β-lactoglobulin. A very weak band appeared in the area of β-casein and a denser band in the area of k-

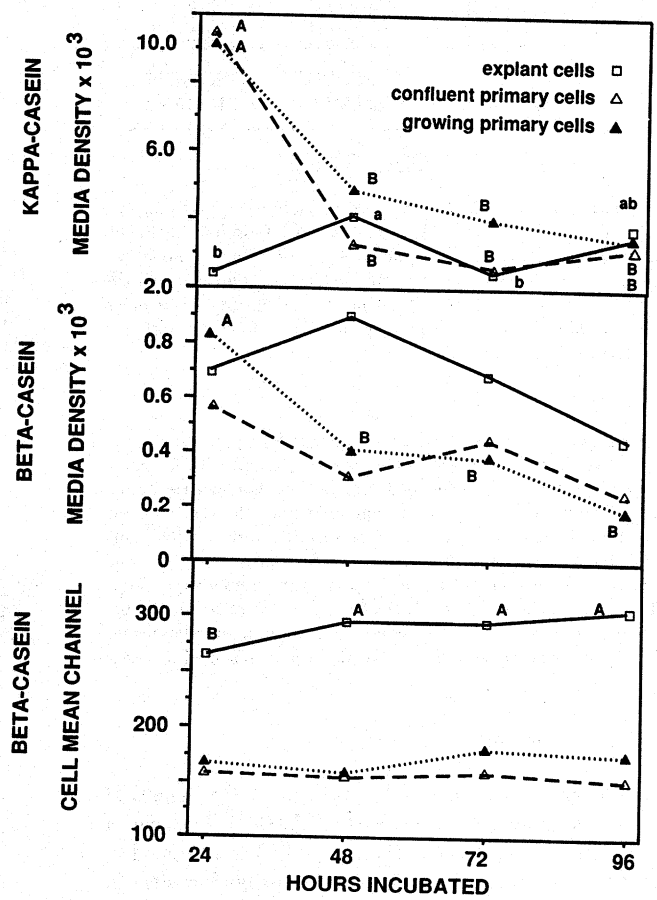

FIG. 7. Effect of incubation time on the relative concentration of β-casein in explant, primary confluent, and primary growing cells and the secretion of β-casein and k-casein into the medium. Casein content is expressed as the mean of all electronic channels containing fluorescent cells—the greater the fluorescence, the higher the mean channel. Points within lines indicated by different letters are different (*A,B*: $P < 0.01$ and *a,b*: $P < 0.03$)

casein. The weak β-casein band could have been due to the 3% FBS in the medium. Plasmins, which are present in serum, hydrolyze α_s-casein and β-casein but not k-casein (27). k-Casein holds the casein micelles containing α_s-casein and β-casein in colloidal suspension and protects them from precipitation by calcium ions (23,33). The appearance of k-casein in the medium suggests that the mechanism of micelle formation was functioning in both explant and primary cells.

Whey protein content of explant cells increased ($P < 0.006$) through 96 h while secretion of α-lactalbumin into the medium decreased ($P < 0.09$) after 48 h (Fig. 6). This suggests a breakdown in the secretion mechanism of explant cells. The primary cells maintained ($P > 0.10$) their cellular whey protein concentration but continued to increase ($P < 0.0001$) secretion through 72 h. The β-casein content (Fig. 7) of primary cells remained constant ($P > 0.10$) over the 96 h period while that of explants increased ($P < 0.0001$). The increase in whey and β-casein concentration within explant cells occurred simultaneously with the increases in DNA content discussed above (Fig. 1). Others (1,29,30) have observed an association of cellular DNA concentration with changes in protein synthesis. They suggested (1) that this association was due to amplification of specific genes.

Confluent primary cells contained 8.7% less ($P < 0.0001$) whey protein and secreted the same ($P > 0.40$) amount of α-lactalbumin compared to explant cells (Fig. 6). However, confluent primary cells contained 46.3% less ($P < 0.0001$) β-casein and secreted 42.8% less ($P < 0.004$) β-casein than explant cells (Fig. 7). Explant cells secreted less ($P < 0.03$) k-casein than confluent or growing primary cells particularly so during the first 24 h of incubation (Fig. 7).

Whey protein content of explants was depressed ($P < 0.04$) by bST, IGF-I, and bST + IGF-I compared to control (234, 238, 234, and 247 mean channel, respectively). A similar effect with bST and IGF-I on total milk protein synthesis was observed in an earlier experiment using explants from mid- to late-lactation cattle (21). In this experiment, hormones had no effect ($P > 0.10$) on protein content or release by primary cells, but others (14) reported an enhancement of protein synthesis by primary bovine mammary cells with IGF-I.

These results show that primary bovine mammary epithelial cells, grown to confluence on collagen, are similar to mammary tissue explants with respect to changes in DNA content with time, hormonal treatment, and the number of differentiated cells in the total cell population. Differences in whey protein content and secretion were small. Explant cells contained and secreted more β-casein than primary cells but secretion trends for β-casein and k-casein were similar after 48 h for both cell types. These results suggest that primary cell cultures are comparable to explant cultures when used to study mechanisms of DNA and milk protein synthesis and secretion.

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