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Sialomucin complex (SMC) is a heterodimeric glycoprotein complex consisting of a mucin subunit ASGP-1 (ascites sialoglycoprotein-1) and a transmembrane subunit ASGP-2, which can act as a ligand for the receptor tyrosine kinase ErbB2. SMC is a highly expressed protein on the surface of ascites 13762 rat mammary adenocarcinoma cells, approximately 10^2 times the level in lactating mammary gland and 10^4 times that in virgin mammary gland. SMC is sharply increased at mid-pregnancy in a manner similar to β-casein. Unlike β-casein, SMC appears to be regulated posttranscriptionally, as its transcript is present in both virgin and pregnant mammary tissue and SMC synthesis is induced rapidly in cultured primary mammary epithelial cells from either normal pregnant or virgin rats. SMC protein, but not transcript levels, are significantly reduced when mammary cells are cultured in Matrigel, a reconstituted basement membrane which stimulates casein expression. SMC precursor is synthesized in Matrigel at a 10-fold lower rate. Matrigel has no effect on either the level of SMC or its transcript in cultured 13762 mammary tumor cells. These results indicate that SMC is a novel product of normal mammary gland and milk, which is post-transcriptionally regulated in normal mammary gland, but not in 13762 mammary adenocarcinoma cells.

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Table of Contents

Introduction

Sialomucin complex (SMC) was recently identified as one of the milk membrane mucins (1). It was originally discovered as the major glycoprotein complex on the surface of highly malignant, metastatic 13762 rat ascites mammary adenocarcinoma cells (2-4). The complex consists of a peripheral, O-glycosylated mucin subunit ASGP-1 (2, 3, 5), and an N-glycosylated integral membrane glycoprotein ASGP-2 to which ASGP-1 is tightly, but non-covalently, bound (4, 6). SMC is transcribed from a single gene as a 9 kb transcript (7, 8), which is translated into a single polypeptide that is proteolytically cleaved early in its transit to the cell surface (9). Mature glycosylated ASGP-1 has a molecular weight of >500 kDa (2, 3), with a polypeptide Mr of ≈220 kDa (8), and is comprised of three domains: an N-terminal unique sequence, a large tandem repeat region rich in serine and threonine residues that can be O-glycosylated and a C-terminal unique sequence (8). ASGP-2 is a 120-140 kDa protein that consists of seven domains: two hydrophilic N-glycosylated regions, two EGF-like domains, a cysteine rich domain, a transmembrane domain, and a small cytoplasmic domain (7).

Sialomucin complex is proposed to have dual functions in tumor cells. 1) ASGP-1 is thought to provide anti-recognition and anti-adhesive properties to tumor cells (5, 10, 11). In A375 melanoma cells stably transfected with SMC DNA linked to a tetracycline-regulated promoter, expression of SMC abolishes cell-matrix adhesion and cell-cell interactions and reduces killing by natural killer cells (12, 13). This anti-recognition property may be important to the high metastatic capacity of the 13762 ascites cells (2, 3, 14). 2) The two EGF-like domains of ASGP-2 have all of the consensus residues present in active members of the EGF growth factor family (7), and ASGP-2 has been shown to bind to and modulate phosphorylation of the receptor ErbB-2. Thus, the transmembrane subunit ASGP-2 is proposed to modulate signaling through the EGF family of receptors via its interaction with ErbB-2 for which no ligand has been described to date (15). This interaction may play a role in the constitutive phosphorylation of ErbB-2 in the 13762 ascites cells and the high proliferative activity of these cells.

In addition to mammary gland sialomucin complex is expressed in a number of normal secretory epithelial tissues in the adult rat, including small and large intestine, trachea, and uterus (1, 11). SMC is constitutively expressed in the normal adult rat intestine in Paneth cells at the bases of the crypts of Lieberkuhn of the small intestine and in the goblet cells of the colon (1). In the trachea and uterus SMC is expressed on the apical surface of the cells lining the lumen (11, 16). SMC is expressed constitutively in the trachea, while in the uterus its expression is regulated by the ovarian hormones estrogen and progesterone at the mRNA level (16). These results suggest that SMC has multiple and complex regulatory mechanisms in the normal adult rat.

In an effort to understand the regulation of SMC in normal mammary gland, we have examined both mammary tissue and cultures of primary mammary epithelial cells. *In vivo* SMC expression is superficially similar to that of β-casein. SMC is present at a low level in virgin gland and increases sharply during mid-pregnancy and appears to be developmentally regulated. However, unlike β-casein, SMC message is expressed at all times in the normal developing mammary gland. Thus, in the normal mammary gland, SMC appears to be regulated post-transcriptionally. *In vitro*, SMC expression is quite different from that of β-casein. Unlike β-casein, high levels of SMC can be induced within hours in cultured virgin mammary epithelial cells. In addition, SMC levels are significantly reduced when MEC are cultured in the presence of Matrigel, a reconstituted extracellular matrix preparation in contrast to β-casein, which requires ECM for expression.

However, culture of MEC in Matrigel does not affect SMC message levels, suggesting that this cell culture system can be used to mimic SMC expression *in vivo*. Interestingly, both SMC protein and message levels in the 13762 ascites tumor cells are unaffected by Matrigel, indicating that the post-transcriptional regulation of SMC is lost in these cells. These data suggest that SMC is a novel post-transcriptionally-regulated product of the mammary gland and milk and that this regulation is disrupted in the 13762 tumor cells.

Results

SMC Expression in Normal Developing Mammary Gland

β-casein has been used extensively as a marker for mammary epithelial differentiation, and its expression pattern has been described in detail both *in vivo* and *in vitro* in response to various hormones, extracellular matrix components, and growth factors. Thus, as a control we have chosen to compare SMC expression to it in some of our studies. To study the expression pattern of SMC in normal developing mammary gland and to compare its expression pattern to that of β-casein, mammary homogenates from virgin, pregnant, lactating, and involuting rats were analyzed by anti-ASGP-2 and anti-β-casein immunoblots. In all tissues studied to date, including mammary gland (1, 11), ASGP-1 and ASGP-2 are present as a complex, allowing us to use analysis of ASGP-2 as a marker for the presence of SMC. As previously reported (1), SMC is minimal in virgin mammary gland. Its level increases sharply (approximately 40-fold) during mid-pregnancy, reaches a maximum in late pregnancy and during lactation, and decreases during involution (Fig. 1). The expression pattern of β-casein appears to be similar, but it reaches maximal levels about 1 to 2 days earlier than SMC. β-casein is maintained throughout lactation and decreases more rapidly than SMC during involution. These data suggest that, like β-casein, SMC is developmentally regulated in normal mammary gland, but that it may have a different regulatory mechanism.

Characterization of isolated rat mammary epithelial cells

The mammary gland is made up of several cell types, and SMC, like β-casein, is expressed in the secretory luminal epithelial cells (1). In order to study specifically the effect of exogenous factors on SMC expression, it is necessary to work with isolated mammary epithelial cells. Thus, for all studies, mammary epithelial cells (MEC) were isolated using the following method adapted from previously described protocols (17-20). Briefly, mammary glands were excised from virgin or pregnant female Fischer 344 rats. The tissue was minced finely, resuspended in digestion media (1 mg/ml collagenase type II and 100 U/ml penicillin/100 mg/ml streptomycin in Ham's F-12 medium) and incubated at 37°C with shaking for 45 minutes. Fully and partially digested epithelial cell clusters were pelleted and incubated a second time in digestion buffer. Released epithelial cell clusters were pelleted, resuspended in PBS, and passed through a 520 mm cell sieve to remove undigested material. Mammary epithelial cell clusters in the resulting filtrate were captured on a 70 mm nylon membrane. Cell clusters were collected by rinsing the membrane with PBS and were subsequently washed three times in PBS prior to plating. Incubating freshly isolated cells on a plastic tissue culture plate for 1 hour permitted attachment and removal of fibroblasts.

Initially, isolated epithelial cells were analyzed to ascertain whether the isolated cells were mammary epithelial cells and whether the apparent increase in SMC in developing mammary gland is due to a real increase in SMC production or due to the increase in the proportion of epithelial cells relative to other cell types during pregnancy. Mammary epithelial cells were isolated as described

from virgin and 11 and 18 day pregnant rats. Cells were lysed in 1% SDS in water, protein concentration was determined by Lowry assay, and 5 μg total protein was subjected to immunoblot analysis with anti-ASGP-2 and anti-β-casein antibodies. To test whether cell surface SMC is destroyed by collagenase, MAT-B1 ascites cells, which express high levels of SMC, were treated with collagenase under the same conditions. Immunoblots of collagenase-treated ascites cells showed no difference in the level of SMC compared to that of untreated cells (data not shown). As seen in whole tissue, SMC is low in virgin mammary epithelial cells and increases sharply in MEC from mid- and late- pregnant animals (Fig. 1B). β-Casein expression is also minimal in virgin MEC and is detectable at maximum levels in MEC from both mid- and late- pregnant animals. As seen in whole tissue, β-casein reaches maximum levels earlier than SMC. Thus, the isolated cells reflect the mammary tissue and can be used as a model for the study of regulatory mechanisms.

To determine whether the expression pattern of the SMC transcript is similar to that for the protein, total RNA was prepared from freshly isolated mammary epithelial cells from virgin and pregnant rats, and Northern blot analysis was performed using probe A2G2-#9, a 1.7 kb fragment spanning the 5' unique region and four tandem repeats of SMC cDNA. Surprisingly, SMC transcript is present at equivalent levels in MEC at all time points tested (Fig. 2). This expression pattern for SMC transcript is different from that for the protein, which is minimal in virgin gland and increases sharply as pregnancy proceeds. Furthermore, this pattern is also different from that of β-casein message, which is undetectable in MEC until mid-pregnancy (21). These results indicate that SMC has a different regulatory mechanism than β-casein and that regulation of SMC in normal rat mammary epithelial cells *in vivo* occurs post-transcriptionally.

Expression of SMC in rat primary mammary epithelial cell cultures

We have chosen primary rat mammary epithelial cell culture to study the regulation of SMC in normal rat mammary tissue for a number of reasons: 1) primary mammary epithelial cell culture has been used to elucidate the regulatory mechanisms of several milk proteins, including β-casein, 2) the level of differentiation/functionality of the MEC can be maintained or manipulated, and 3) we have the reagents necessary to do experiments with rat MEC. For these studies mammary epithelial cells were cultured using the following protocol. Following isolation, mammary epithelial cell clusters were resuspended and plated in equal aliquots in Ham's F-12 medium containing either 10% FCS and 100 U/ml penicillin/100 mg/ml streptomysin or 5 mg/ml insulin, 10 mg/ml transferrin, 0.3 mg/ml sodium selenite, and 100U/ml penicillin/100 mg/ml streptomysin. Where indicated, media were supplemented with lactogenic hormones (5 mg/ml insulin, 1 mg/ml hydrocortisone, and 3 mg/ml prolactin). For plating with embedding in Matrigel, cells were resuspended in 1.5 ml ice cold Matrigel diluted 1:3 with serum-free Ham's F-12 medium, plated at 1.5 ml/mm^2 of tissue culture plastic and allowed to solidify at 37°C for 30 min. The solidified Matrigel was then overlaid with two ml of either serum-free or serum-containing medium. Cells were cultured at 37°C in 5% CO_2 for 48 hours prior to harvest. Cells were collected from culture on plastic dishes by scraping cells off the dish. Cells were harvested from Matrigel cultures using the recommended protocol for Matrisperse, an enzyme-free Matrigel dissociation buffer. For all samples, harvested cells were pelleted, washed with PBS, and lysed in 100 ml 1% SDS in water. Protein concentration of the cell lysates was measured by Lowry assay, and 5 μg total protein was loaded for immunoblot analysis.

The *in vivo* expression patterns of SMC and β-casein are different, suggesting that SMC is regulated differently than β-casein or other "early" milk protein genes.In the presence of a reconstituted basement membrane (Matrigel), mammary epithelial cells from pregnant mice and

virgin rats form alveolar structures and can be induced to express β-casein. Thus, culture of MEC in Matrigel can be used to mimic the in vivo state. To compare the expression pattern of SMC to that of β-casein in these culture conditions, mammary epithelial cells were isolated from mid-pregnant rats as described. Cells were cultured either on plastic or embedded in Matrigel in the presence or absence of fetal calf serum. After 48 hours, cleared cell lysates were prepared for anti-ASGP-2 and anti-β-casein immunoblots, and total RNA was prepared for Northern blot analysis with probe A2G2-#9. In freshly isolated mid-pregnant mammary epithelial cells the level of SMC is minimal, while that of β-casein is high (Fig. 3A). However, when the MEC are cultured on plastic with 10% FCS, SMC is detected at a high level while β-casein, as expected, is reduced significantly. In the absence of serum on plastic SMC is expressed at a lower level, and β-casein is undetectable. In the presence of Matrigel and serum the level of SMC is low and that of β-casein is high. In Matrigel without serum the level of SMC is minimal and β-casein is unaffected. These data suggest that SMC levels are significantly enhanced by a factor in fetal calf serum and that SMC is greatly reduced by a factor present in the reconstituted basement membrane. Interestingly, although the protein level of SMC is negatively affected by the presence of Matrigel, the SMC transcript level is not decreased by culture on plastic or in Matrigel (Fig 3B). The negative effect of Matrigel on SMC protein levels is very different from its effect on β-casein, which is regulated by the ECM at the transcript level. These results suggest that SMC, under these conditions, is regulated post-transcriptionally, as it appears to be *in vivo*. Thus, this culture system is useful for mimicking the *in vivo* situation and for determining factors that affect SMC expression in normal mammary gland.

Expression of SMC in MEC from virgin animals

β-Casein expression requires pregnancy or MEC culture under specific conditions that mimic the pregnant state (20, 22). Because the SMC transcript is present in abundance in MEC from virgin animals, these cells may already be ready for SMC production and awaiting the appropriate signal to synthesize the protein. Mammary epithelial cells were isolated from virgin rats and cultured on plastic with 10% serum to determine if priming by pregnancy is necessary for the induction of SMC protein production or if a change in the environment is sufficient for SMC biosynthesis. After 24 to 72 hours in culture, the cells were harvested and subjected to immunoblot analysis with anti-ASGP-2 antibodies. In cultured virgin mammary epithelial cells, SMC reaches a maximal level in 24 hours, and the level is maintained for at least 48 hours (Fig. 4A). Thus, pregnancy is not required for the production of SMC. Induction of SMC biosynthesis appears to require only release of mammary epithelial cells from the extracellular matrix (environment), suggesting that in the virgin gland there is an inhibition of SMC synthesis which is released by removal of MEC or by the changes induced by pregnancy.

To determine if extracellular matrix affects SMC in MEC from virgin animals similarly to that for MEC from pregnant animals, MEC from virgin rats were isolated and cultured in the presence of 10% serum either on plastic or embedded in Matrigel as described above. After 48 hours in culture, cells were harvested and subjected to immunoblot analysis with anti-ASGP-2 antibodies. In freshly isolated mammary epithelial cells, SMC is undetectable, as expected. When cultured on plastic, the level of SMC is high, and as in the cultured MEC from pregnant rats, the presence of ECM greatly inhibits the production of SMC (Fig. 4B). This inhibition occurs both when the cells are embedded in the Matrigel and when they are plated on top of it (data not shown), suggesting that one or more factors present in the Matrigel is responsible for the reduction of SMC levels in cultured mammary epithelial cells.

To study the effect of ECM on SMC in normal MEC already expressing the protein, MEC from virgin rats were prepared and cultured on plastic with 10% serum to induce high levels of SMC production. After 48 hours, the cells were removed from the plate by non-enzymatic cell dissociation buffer and replated either on plastic or embedded in Matrigel for 48 hours. Cleared cell lysates were subjected to immunoblot analysis with anti-ASGP-2 antibodies. After 48 hours on plastic, SMC was present in abundance (Fig. 5). When cells were replated on plastic, the level of SMC remained high but at a slightly lower level than that of the first plating. The level of SMC in the cells embedded in Matrigel was significantly reduced. Thus, the level of SMC in normal MEC which already express the protein can be modulated by ECM.

Effect of extracellular matrix components on SMC expression in cultured MEC

Extracellular matrix components are important in mammary gland development. For example, β-casein expression is upregulated by both laminin and collagen which are major components of the mammary gland extracellular matrix and Matrigel (23, 24). Thus, one of the ECM components in Matrigel may be responsible for the post-transcriptional regulation of SMC in the cultured MEC. To determine if any individual extracellular matrix component affects SMC expression in normal mammary gland, mammary epithelial cells were isolated from virgin rats and plated in Ham's F-12 media supplemented with ITS on tissue culture plates coated with collagen IV, collagen I, fibronectin, or laminin. After 48 hours, cells were harvested, and 5 mg total protein were subjected to SDS-PAGE and immunoblot analysis with mAb 4F12. SMC levels were slightly higher in each of the samples that were cultured on an extracellular matrix protein compared with those cultured on plastic (Figure 6). This could be due to the cells being able to attach better and be more "healthy" and thus, the extracellular matrix protein coated plates mimic the in vivo state better than culturing on plastic alone. However, the effect from each individual ECM component was not significant, suggesting that some other factor is largely responsible for regulating SMC levels in these cells.

Effect of estrogen and progesterone on SMC levels in cultured MEC

In the uterus, ASGP-2 expression appears to be regulated by estrogen and progesterone (16). To test the effect of estrogen and progesterone on ASGP-2 expression in mammary tissue,virgin mammary epithelial cells were prepared. MEC were cultured for 48 hours on plastic in phenol red free Ham's F-12 media supplemented with insulin, transferrin, and sodium selenite (ITS) in the presence or absence 27 ng/ml estrogen or 1 mg/ml progesterone. Neither estrogen nor progesterone had any effect on the expression of SMC under these culture conditions (Fig. 7). These data are consistent with those for MUC1, which in the uterus is regulated by estrogen like SMC but is unaffected by estrogen in mammary epithelial cells (Carson, D., personal communication). Estrogen is thought to "prime" the mature mammary gland for response to other factors that modulate mammary development and milk protein synthesis during pregnancy (25). Thus, SMC like other developmentally regulated mammary proteins such as β-casein, is responsive to factors present during pregnancy after estrogen has played its role.

The SMC promoter (5' upstream sequence) contains a number of putative estrogen response elements (unpublished data). This suggests that the SMC gene may be responsive to estrogen. We have shown that the SMC transcript in normal mammary tissue is present in abundance even when the protein level is minimal. Estrogen may be a factor necessary for transcription but not translation of SMC. The level of SMC protein was unaffected by estrogen in culture. This may be because the

SMC gene is not responsive to estrogen or because the effect is indirect or mediated by the presence of other cell types. To test the possibility that SMC message levels may be affected by estrogen, ovariectomized rats will be injected with estrogen for two days. Total RNA will then be isolated and Northern blots will be performed with probe A2G2-#9. This analysis should indicate whether the SMC gene is estrogen responsive.

Effect of growth factors on SMC levels in cultured MEC

Growth factors also play an important role in mammary gland development. For example, β-casein expression is inhibited by the growth factors TGFβ and EGF, and their downregulation during pregnancy is thought to allow upregulation of β-casein expression (26-30). In addition, EGF and NDF have both been shown to affect mammary epithelial cell morphology and β-casein expression (31, 32). To determine how various growth factors affect SMC in mammary tissue, mammary epithelial cells were isolated from virgin rats. Virgin rats were used because I demonstrated that high levels of SMC can be induced in virgin mammary epithelial cells and SMC levels can be modulated by Matrigel similarly to the day 11 pregnant mammary epithelial cells. MEC were plated on plastic in Ham's F-12 supplemented with ITS (5 mg/ml insulin, 10 mg/ml transferrin, 0.3 mg/ml sodium selenite) in the presence or absence of 25 nM EGF or 1X NDF (from a 20X stock of conditioned media). After 40 hours, cells were harvested and 5 mg total protein were subjected to SDS-PAGE and immunoblot analysis with mAb 4F12. Neither EGF nor NDF had any effect on SMC levels under these conditions (Figure 8). These data indicate that either these growth factors no not affect SMC expression in the mammary epithelial cells under these conditions or that they are part of a more complicated pathway and must act synergistically with other factors to have an effect.

The growth factors I am testing with this system were chosen because they have been shown to affect expression of other milk proteins or because they are present in Matrigel. TGFα, TGFβ, and HGF also affect expression of other milk proteins. In addition TGFβ is present in Matrigel in a physiologically significant concentration, so it may play a role in the downregulation of SMC by Matrigel. Thus, I will test the effect of these growth factors on the expression of SMC in cultured MEC using the conditions described in this report.

Effect of Matrigel on SMC levels in MAT-B1 tumor cells

Matrigel contains a number of components, including proteases. Thus, a protease present in the Matrigel, which could digest cell surface proteins, could be responsible for the decreased levels of SMC in MEC cultured in it. To rule out this possibility, MAT-B1 ascites tumor cells, which express SMC abundantly on the cell surface, were cultured on plastic or embedded in Matrigel for 48 hours. Cleared cell lysates and total RNA were prepared and subjected to immunoblot analysis with anti-ASGP-2 antibodies and Northern blot analysis with probe A2G2-#9, respectively. Whether cultured on plastic or in Matrigel, the level of SMC was unchanged in the cultured MAT-B1 tumor cells (Fig. 9A), suggesting that proteolysis does not cause the decreased level of SMC in Matrigel. This finding is consistent with other results. For example, the level of ErbB-2 in MEC cultured in Matrigel is unaffected (data not shown). Moreover, culture of rat tracheal epithelial cells (16) in Matrigel does not reduce their level of SMC, as would be expected from a proteolytic effect at the cell surface. The level of SMC message in the ascites tumor cells was also unaffected by the presence or absence of ECM (Fig. 9B). These data suggest that cell surface SMC is not degraded by proteases in Matrigel, and that regulation of SMC by ECM has apparently been

changed significantly in these tumor cells.

SMC synthesis in virgin MEC

Regulation of SMC by ECM appears to be post-transcriptional in cultured MEC. This type of regulation may be by modification or degradation of SMC message, modulation of SMC message translation or degradation of SMC protein. The steady state levels of SMC message are unaffected by different culture conditions, suggesting that SMC message degradation is not the mechanism by which SMC is regulated in this system. To study how ECM affects SMC message translation, mammary epithelial cells were cultured either on plastic or embedded in Matrigel. After 24 hours cells were washed twice with PBS. Cells were starved for 30 minutes in Cys/Met free DMEM supplemented with 100 U/ml penicillin/100 mg/ml streptomycin, 2 mM glutamine, and 10 mM Hepes, and incubated in 1 ml labeling medium (starvation medium + 230 mCi [^{35}S]-Cys + [^{35}S]-Met per ml) for times ranging from 5 to 180 minutes. Labeled cells were washed twice with PBS and lysed in 400 ml of 2% SDS in H_2O. Lysed cells were boiled for 1 minute, sonicated for 10 minutes in a bath sonicator, and diluted in 2 ml Triton immunoprecipitation buffer (2.5% Triton-X100, 190 mM NaCl, 60 mM Tris HCl, 6 mM EDTA, pH 7.4). Diluted cell lysates were centrifuged at 175,000 x g for 40 min at 4°C. Cell lysates were immunoprecipitated with polyclonal anti-ASGP-2 antiserum and protein A agarose beads overnight at 4°C with rotation. Immunoprecipitates were washed with RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS, 50 mM Tris base, pH 8.0) six times for 15 minutes each at 4°C with rotation. A fraction of immunoprecipitation supernatant was collected for analysis of total labeled protein. Washed immunoprecipitates were resuspended in 20 μl SDS sample buffer and immunoprecipitate supernatant was diluted 1:1 in SDS sample buffer. Diluted samples were analyzed by SDS PAGE and fluorography with Fluoro-Hance autoradiography enhancer. After labeling, the cells were washed, lysed, and immunoprecipitated with polyclonal anti-ASGP-2 antibody, which recognizes the SMC precursor as well as mature ASGP-2. It was necessary to immunoprecipitate the precursor because this product is intracellular and would not be affected by an extracellular protease. Immunoprecipitated SMC precursor was subjected to SDS-PAGE and fluorography, as was an aliquot of immunoprecipitated supernatant. To normalize for total protein, the bands for the SMC precursor and total protein were quantified by densitometry. The value for the SMC precursor was compared to that obtained for total protein. The SMC precursor band from cells cultured on plastic was easily visible by 20 minutes of labeling while that from cells cultured in Matrigel was not visible until 90 minutes of labeling (Fig. 10). When the ratio of SMC precursor to total labeled protein was calculated, the cells cultured on plastic synthesized SMC about eight times faster than those cultured in Matrigel. Thus, a factor in Matrigel reduces the mammary epithelial cells' ability to synthesize SMC, further suggesting that SMC is regulated post-transcriptionally in this system.

Conclusions

Sialomucin complex is developmentally regulated in normal rat mammary gland. *In vivo*, the protein is expressed at a basal level in virgin gland and increases sharply during mid-pregnancy, similarly to β-casein. However, SMC reaches maximum levels about two days later than β-casein, suggesting that it has a different regulatory mechanism. Interestingly, the SMC transcript is present at equal levels in virgin and pregnant mammary gland. This is in stark contrast to the expression pattern of β-casein, where the presence of transcript becomes detectable around day 6 of pregnancy

(21). The expression pattern of SMC *in vivo*, where the level of protein does not directly correspond to the level of transcript suggests a post-transcriptional mechanism of regulation for this protein.

By establishing rat mammary epithelial cell primary cultures, we have shown that the expression patterns of SMC and β-casein are different, suggesting that SMC is regulated differently than β-casein or other early milk protein genes. Expression of β-casein in isolated mammary epithelial cells is dependent on the presence of extracellular matrix components (or basement membrane). In the presence of a reconstituted basement membrane (from EHS or Matrigel), mammary epithelial cells from pregnant mice and virgin rats can be induced to express β-casein (20, 30). There are several major differences in the behavior of β-casein and SMC in primary MEC cultures. 1) SMC is found at the highest levels when pregnant rat MEC are cultured on plastic and at very low levels when the cells are embedded in Matrigel. β-casein, on the other hand, is found at maximal levels when pregnant rat MEC are embedded in Matrigel and only at minimal levels when the cells are cultured on plastic. 2) Individual extracellular matrix components such as laminin, fibronectin, collagen I, and collagen IV do not have a significant effect on the level of SMC in these cell cultures. However, β-casein expression has been shown to be upregulated by laminin and collagen IV (21). 3) SMC transcript levels are not affected by MEC culture on plastic or in Matrigel, while β-casein transcript levels have been shown to be low (or non-existent) when MEC are cultured on plastic and high when MEC are embedded in Matrigel (35). 4) SMC protein production can be induced in MEC from virgin rats within hours of culture on plastic, suggesting that MEC do not need to be primed by pregnancy to be capable of synthesizing SMC. β-Casein expression, on the other hand, requires MEC priming by pregnancy or long term culture under specific conditions for expression (20, 22). Primary culture of normal rat MEC does seem to mimic the *in vivo* state with respect to SMC expression (as well as β-casein expression, as shown by others). Furthermore, these data suggest that SMC is regulated by a post-transcriptional mechanism.

There are also several differences in SMC expression in normal MEC and the 13762 rat mammary adenocarcinoma, in which SMC was first identified. The 13762 rat mammary adenocarcinoma ascites cells express SMC at a level at least 100-fold higher than that in normal lactating mammary gland (1). 1) Unlike normal MEC, which express SMC at high levels upon removal from the animal, the MAT-B1 tumor cells significantly reduce SMC expression within 48 hours during culture after removal from the animal. 2) Matrigel does not seem to affect SMC levels in MAT-B1 tumor cells, either at the level of protein or transcript. In normal MEC, however, Matrigel reduces SMC levels in newly cultured normal MEC and in MEC that already express SMC. It appears that regulation of SMC in MAT-B1 tumor cells is different (has been disrupted) from that of normal MEC. In the MAT-B1 cells, the transcript and protein are both synthesized equally well on plastic or in Matrigel, while in normal MEC the transcript is made but the protein synthesis is reduced significantly. We have no evidence to suggest that SMC from the tumor cells is different from SMC from normal cells. Thus, the maintenance of SMC in the presence of Matrigel confirms that the reduction of SMC seen in normal MEC is not due to the presence of a protease in the Matrigel.

From the data presented here, SMC, a product of differentiated mammary tissue, appears to be regulated post-transcriptionally in normal mammary tissue. There are several ways protein production can be regulated post-transcriptionally. These include stability of the transcript, changes in the rate of message translation, and changes in the rate of protein degradation or turnover. The steady state levels of SMC transcript were unchanged either by developmental state *in vivo* or by culture conditions *in vitro*, suggesting that SMC transcript stability in these conditions is unchanged.

Because the level of SMC protein is significantly reduced in Matrigel, it appears that SMC protein is turning over in the cultured cells. Moreover, when cells cultured on plastic or Matrigel are metabolically labeled and SMC precursor is immunoprecipitated and quantified relative to total labeled protein, SMC is translated at ~8-fold higher rate on plastic than in Matrigel. Changes in SMC protein stability in these culture conditions are being investigated. However, taken together these results suggest that SMC biosynthesis is post-transcriptionally regulated in normal MEC by some factor in Matrigel. Thus, SMC is an unusual post-transcriptionally regulated milk protein. Elucidation of its regulatory mechanism in normal developing mammary gland and its disruption in the 13762 tumor cells will give further insight into both normal developmental processes and tumor progression.

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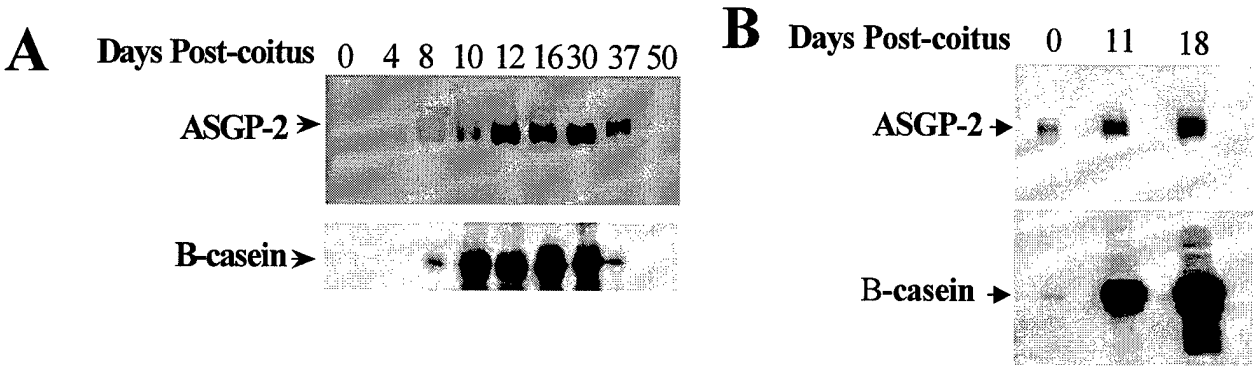

Figure 1. **Expression of SMC and β-casein in the mammary gland.** SDS-solubilized samples of virgin (day 0), pregnant (days 4, 8, 10, 12, and 16), lactating (day 30), and involuting (days 37 and 50) mammary tissue were loaded (50 ug/lane wet weight) for SDS-PAGE as indicated at the top of each blot. A) Immunoblots with anti-ASGP-2 or anti-β-casein antibodies are as indicated at the left of the figure. B) SDS-solubilized samples of isolated mammary epithelial cells from virgin (day 0) and pregnant (days 11 and 18) rats were loaded (5 ug/lane total protein) for SDS-PAGE as indicated at the top of each blot. Immunoblots were performed with anti-ASGP-2 or anti-β-casein antibodies as indicated at the left of the figure.

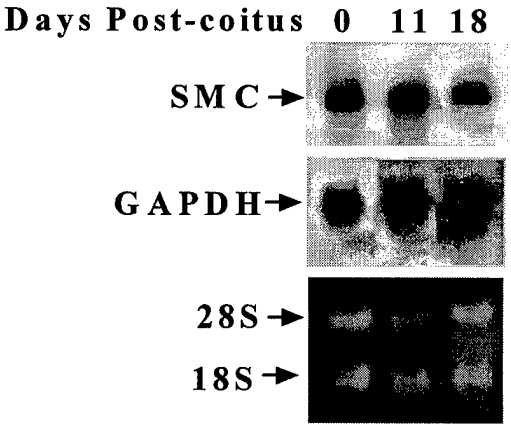

Figure 2. **Northern blot analysis of SMC mRNA in normal rat mammary epithelial cells.** Total RNA was isolated from virgin (day 0) and pregnant (days 11 and 18) mammary epithelial cells and 25 ug/lane were loaded for Northern blot analysis. Northern blots were probed with A2G2-#9 or GAPDH as indicated at the left of the figure.

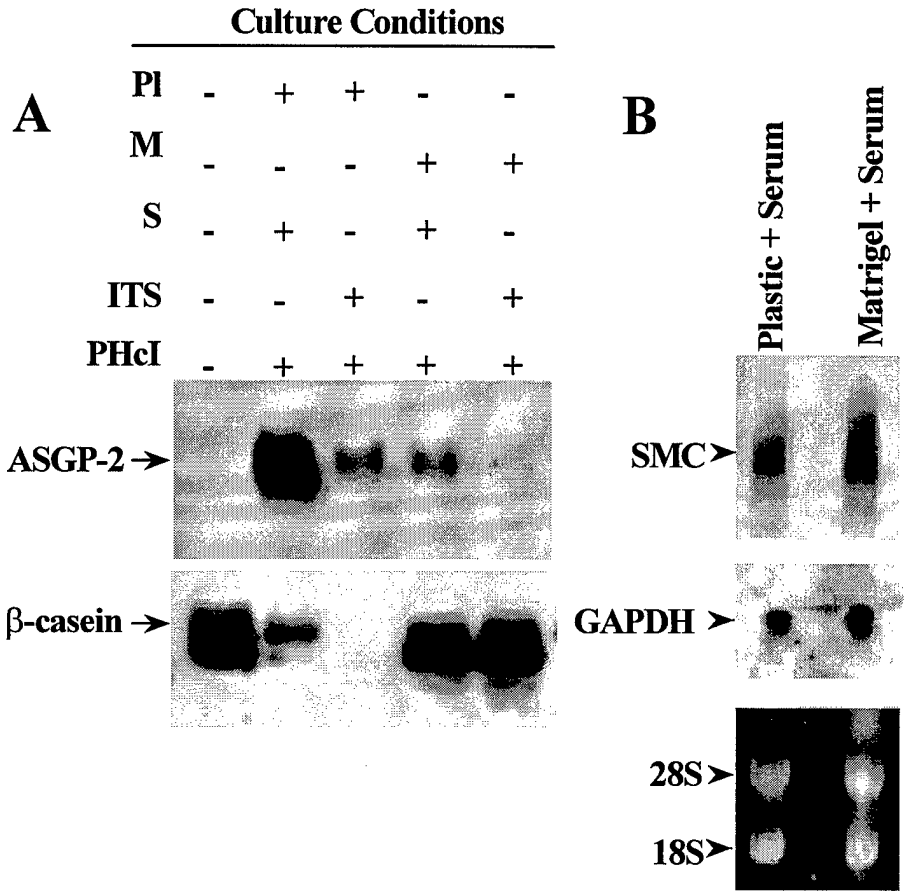

Figure 3. **Effect of Matrigel on SMC expression in pregnant rat mammary epithelial cells.** Normal rat mammary epithelial cells were collected by collagenase digestion of day 11 pregnant rat mammary tissue followed by differential centrifugation. MEC were plated in Ham's F-12 media either on plastic or embedded in Matrigel in the presence or absence of 10% fetal calf serum as indicated at the top of the figure. A) Immunoblots of cultured pregnant mammary epithelial cells. SDS-solubilized cell lysates were loaded (5 μg/lane) for SDS PAGE, and immunoblots were performed with monoclonal antibodies against ASGP-2 and β-casein as indicated at the left of the figure. B) Northern blot of cultured pregnant mammary epithelial cells. Total RNA was isolated from cultured epithelial cells and 25 μg/lane was loaded for Northern blot analysis. Northern blots were probed with A2G2-#9. Pl, plastic; M, Matrigel; S, serum; ITS, insulin, transferrin, and selenium; PHcI, lactogenic hormones prolactin, hydrocortisone, and insulin.

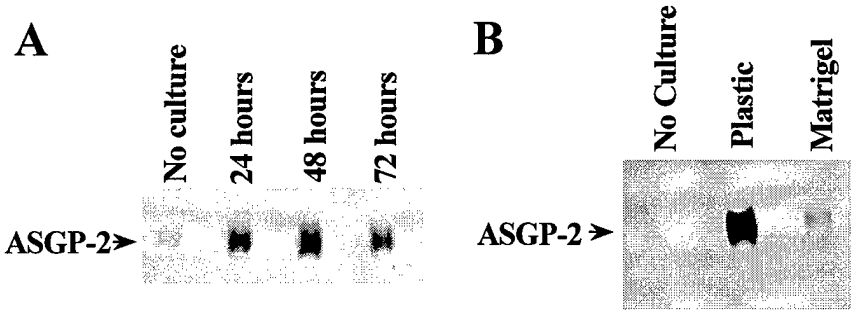

Figure 4. **Expression of SMC in mammary epithelial cells from virgin rats.** Normal rat mammary epithelial cells were collected by collagenase digestion of virgin rat mammary tissue. MEC were plated in Ham's F-12 media either on plastic or embedded in Matrigel in the presence of 10% fetal calf serum. Immunoblots were performed with anti-ASGP-2 antibodies as indicated at the left of the figure. A) Induction of SMC expression in cultured virgin mammary epithelial cells. B) Effect of Matrigel on SMC expression in cultured mammary epithelial cells from virgin rats.

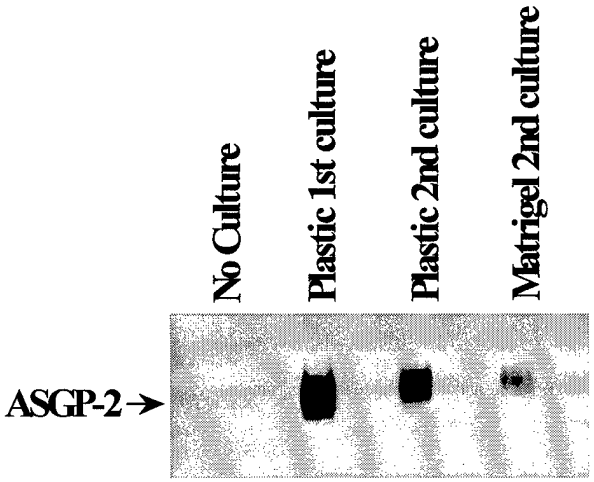

Figure 5. **Effect of Matrigel on SMC levels in normal mammary epithelial cells already expressing SMC.** Virgin MEC were isolated and cultured on plastic in Ham's F-12 medium supplemented with 10% FCS. After 48 hours, cells were removed from the plate with a non-enzymatic cell dissociation buffer. Half the cells were then replated in Ham's F-12 medium supplemented with 10% FCS either on plastic or embedded in Matrigel. After an additional 48 hours in culture, cells were harvested and 5 μg total protein was subjected to immunoblot analysis with anti-ASGP-2 antibodies.

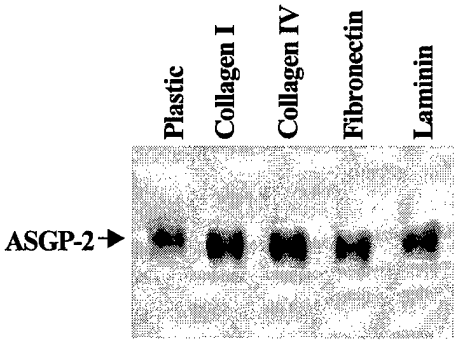

Figure 6. **Effect of extracellular matrix proteins on SMC expression in normal rat mammary epithelial cells.** Mammary epithelial cells were isolated from virgin rats and cultured on plastic or matrix protein-coated plates as indicated in Ham's F-12 medium supplemented with insulin, transferrin, and sodium selenite for 48 hours. Cells were harvested and subjected to immunoblot analysis with mAb 4F12.

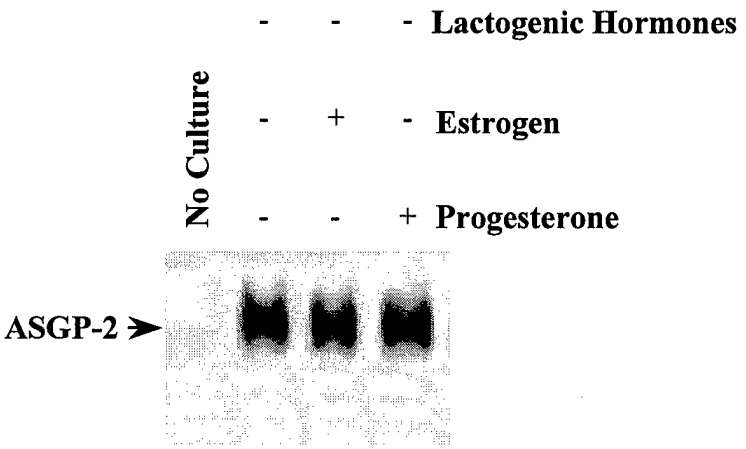

Figure 7. **Effect of estrogen and progesterone on SMC expression in normal mammary epithelial cells.** Normal mammary epithelial cells were isolated from day 11 pregnant rats and cultured on plastic in phenol red free Ham's F-12 medium supplemented with insulin, transferrin, and sodium selenite in the presence or absence or estrogen or progesterone. After 48 hours cells were harvested and subjected to immunoblot analysis with mAb 4F12.

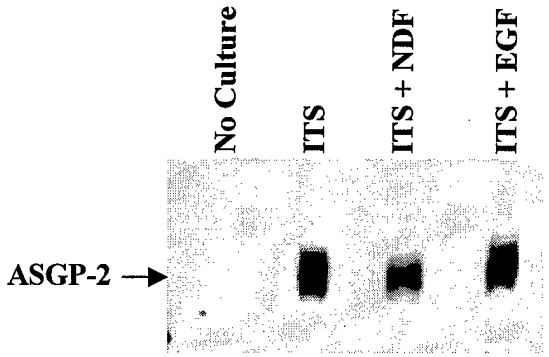

Figure 8. **Effect of growth factors on SMC expression in normal mammary epithelial cells.** Mammary epithelial cells were isolated from virgin rats and cultured on plastic in Ham's F-12 medium supplemented with insulin, transferrin, and sodium selenite (ITS) in the presence or absence of EGF or NDF as indicated at the top of the figure. After 48 hours cells were harvested and subjected to immunoblot analysis with mAb 4F12.

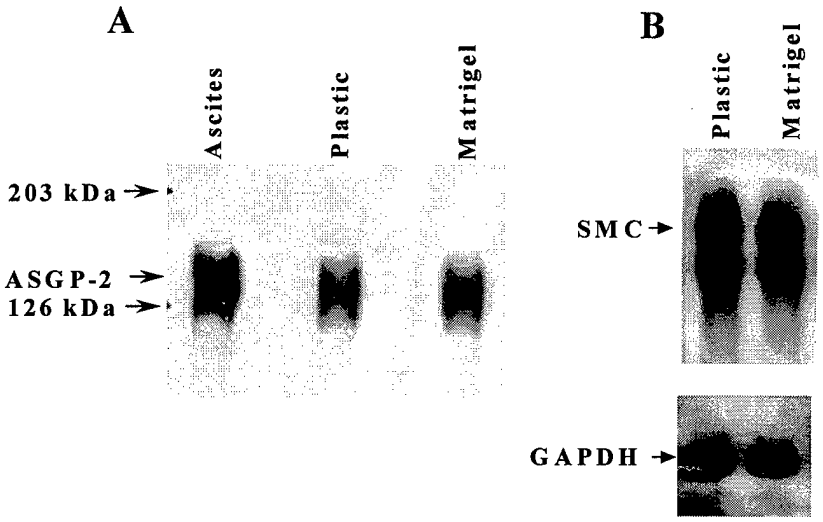

Figure 9. **Effect of Matrigel on expression of SMC in 13762 MAT-B1 tumor cells.** Ascites MAT-B1 mammary adenocarcinoma cells werer collected and plated in Ham's F-12 media either on plastic or embedded in Matrigel in the presence of 10% fetal calf serum. A) Immunoblot with anti-ASGP-2 antibody. B) Northern blot of SMC message using a probe spanning the 5' 1.7 kb of ASGP-2 message.

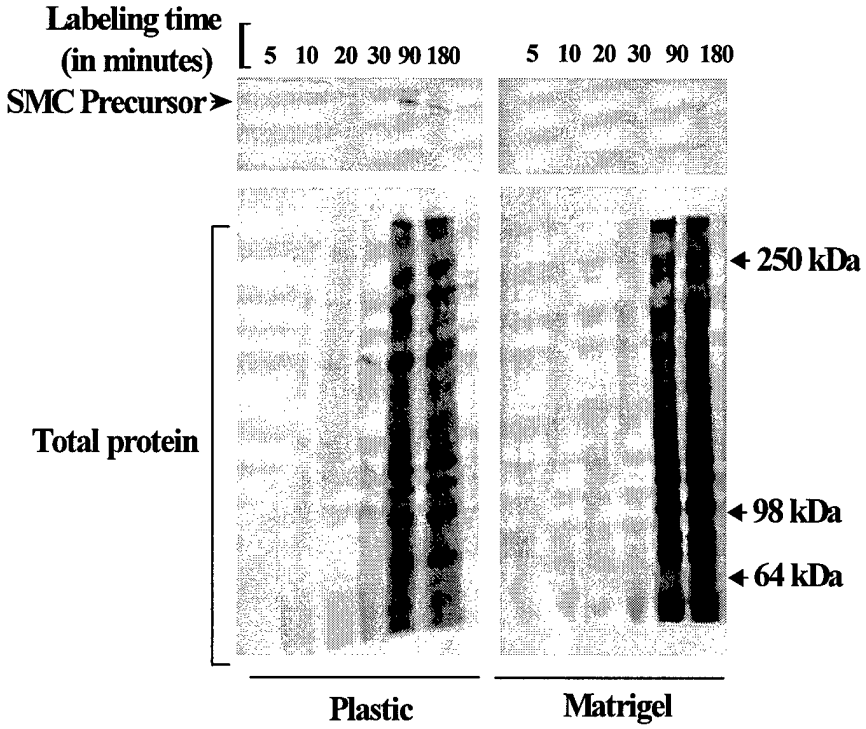

Figure 10. **Effect of Matrigel on SMC precursor synthesis in normal rat mammary epithelial cells.** Rat mammary epithelial cells were collected by collagenase digestion of virgin rat mammary tissue. Epithelial cells were plated in Ham's F-12 media either on plastic or embedded in Matrigel in the presence of 10% fetal calf serum. After 24 hours, cells were labeled for the times indicated above with [^{35}S]Met + [^{35}S]Cys. Cells were lysed in 2% SDS, diluted into a Triton X-100 buffer, and immunoprecipitated with anti-ASGP-2 polyclonal antibody and protein A agarose. Immunoprecipitates were subjected to SDS-PAGE and fluorography.