Extracellular Matrix Regulates Whey Acidic Protein Gene Expression by Suppression of TGF-α in Mouse Mammary Epithelial Cells: Studies in Culture and in Transgenic Mice

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Abstract. Whey acidic protein (WAP) is an abundant rodent milk protein. Its expression in mouse mammary epithelial cell cultures was previously found to require the formation of an extracellular matrix (ECM)-induced three-dimensional alveolar structure. In the absence of such structures, cells were shown to secrete diffusible factors leading to suppression of WAP expression. We demonstrate here that (*a*) TGF-α production and secretion by mammary cells is downregulated by the basement membrane–dependent alveolar structure, and (*b*) compared with β-casein, WAP expression is preferentially inhibited both in culture and in transgenic mice when TGF-α is added or overexpressed. Thus, (*c*) the enhanced TGF-α production when cells are not in three-dimensional structures largely accounts for the WAP-inhibitory activity found in the conditioned medium. Since this activity can be abolished by incubating the conditioned medium with a function blocking antibody to TGF-α. The data suggest that ECM upregulates WAP by downregulating TGF-α production. We also propose that changes in TGF-α activity during mouse gestation and lactation could contribute to the pattern of temporal expression of WAP in the gland. These results provide a clear example of cooperation among lactogenic hormones, ECM, and locally acting growth factors in regulation of tissue-specific gene expression.

WHEY acidic protein (WAP)[1] is a highly abundant, major whey protein in rodent milk (Piletz et al., 1981; Hennighausen et al., 1982). The expression of the protein is used as a molecular marker for mammary gland differentiation, and its promoter has been used widely to permit tissue-specific expression of heterologous genes in the mammary gland of the rodents. In mouse mammary gland, WAP mRNA can be detected by Northern analysis beginning from day 14 of pregnancy, reaching the highest level during lactation (Pittius et al., 1988a,b). This temporal expression differs from the other better-studied milk proteins such as β-casein, whose expression in the gland begins on day 8 of gestation or even earlier. Despite its abundance and the wide usage of its promoter, little is known about the regulation of WAP and the reason behind the remarkable difference in the time course of WAP and β-casein expression.

Previous work performed in our laboratory showed a novel regulation of WAP gene expression in cultures of primary mouse mammary epithelial cells by extracellular matrix (ECM; Chen and Bissell, 1989). It was found that WAP expression had an absolute requirement for the formation of three-dimensional alveolar structures, achieved by culturing cells on a reconstituted-basement membrane (Li et al., 1987; Barcellos-Hoff et al., 1989). When formation of alveolar structures was prevented by culturing the cells on plastic, type I collagen gel, floating collagen gel, or fixed Engelbreth-Holm-Swarm (EHS) matrices (the latter two allow β-casein expression), WAP was not expressed. Diffusible factors in the conditioned medium of these cultures were shown to be responsible for inhibition of WAP expression. We postulated that WAP is negatively regulated by one or more secreted factors. It followed that a three dimensional structure with a central lumen, enclosed by a polarized epithelium layer and surrounded by basement membrane, suppressed either the expression of the inhibitor(s) or the access to the putative receptors, or both.

Work previously performed in several laboratories has indicated that TGF-β and EGF, when added to mammary epithelial cell cultures, inhibit β-casein synthesis (Taketani and Oka, 1983a,b; Mieth et al., 1990; Chen and Bissell, 1989 and unpublished data). Such inhibitory activity of TGF-β was also demonstrated in mouse mammary explant cultures (Robinson et al., 1993), as well as in transgenic mice (Jhappan et al., 1993). In collaboration with Derynck's labora-

1. *Abbreviations used in this paper*: ECM, extracellular matrix; EHS, Engelbreth-Holm-Swarm; PMME, primary mouse mammary epithelial; WAP, whey acidic protein.

tory, we found previously that extracellular matrix regulates the transcription of TGF-β1 mRNA and protein in mammary epithelial cells (Streuli et al., 1993). It was found that when a mammary epithelial cell strain, CID-9, was cultured either on EHS or floating collagen I gel, TGF-β1 promoter activity was dramatically suppressed. In addition, β-casein was expressed in floating collagen and EHS cultures, but not in attached collagen gel or plastic cultures where TGF-β1 activity was relatively high. We therefore proposed that TGF-β1 is an ECM-modulated β-casein inhibitor (Streuli et al., 1993). As discussed above, however, WAP is not expressed on floating collagen gel (Chen and Bissell, 1989). Thus a downregulation of TGF-β1 activity, and the presence of an endogenous basement membrane in floating collagen cultures (Streuli and Bissell, 1990), is not sufficient to allow WAP expression. Since EGF and TGF-α share the same receptor (Derynck, 1988), and TGF-α has been shown to be produced at high levels in human mammary cells (Zajchowski et al., 1988), we examined the possible inhibitory role of TGF-α for WAP expression in mouse mammary epithelial cells.

Here, we identify TGF-α as the major mediator of ECM-regulation of WAP expression. We show that production of TGF-α by mouse mammary epithelial cells is indeed culture substratum-dependent. We further show that TGF-α accounts for most, if not all, of the WAP inhibitory activities present in the conditioned medium of the cells cultured on plastic dishes. The significance of these findings in cell culture is strengthened by demonstrating that WAP gene expression is largely inhibited in the mammary gland of MMTV-TGF-α transgenic mice. We therefore propose that ECM up regulates WAP expression via downregulation of TGF-α in culture, and that temporal WAP expression in vivo may be directly related to the level of activity of this growth factor and the availability of its receptor.

Materials and Methods

Substrata, Growth Factors, and Antibodies

EHS matrix was prepared by urea extraction at 4°C from EHS tumors passaged in C57BL mice (Kleinman et al., 1982). This material was stored for up to 4 wk at 0°C. Each preparation contained 5–10 mg protein per ml, and was routinely assayed for activity and absence of protein degradation on 5% reducing SDS-PAGE followed by silver staining. One milliliter or one hundred fifty microliter of EHS was plated on a 96-mm or 35-mm tissue culture dish (Falcon, Lincoln Park, NJ), respectively. In [³H]thymidine-labeling experiments, coverslips were placed in 35-mm culture dishes before EHS was carefully spread onto both the dish and the coverslips. Synthetic mouse EGF was purchased from Sigma Chem. Co. (St. Louis, MO). Recombinant human TGF-α and monoclonal anti–human TGF-α antibody were generous gifts from Rik Derynck (Department of Growth and Development, UCSF). Polyclonal sheep anti–rat TGF-α antibody was prepared and characterized as previously described (Russell et al., 1993).

Cell Culture

Primary mouse mammary epithelial (PMME) cells were isolated from 15-d timed-pregnant CD-1 mice (Charles Rivers Laboratories, Wilmington, MA), as previously described (Emerman et al., 1977; Lee et al., 1984; Barcellos-Hoff et al., 1989). They were cultured on various culture substrata in F12 medium (GIBCO BRL, Gaithersburg, MD) containing lactogenic hormones (5 μg/ml of insulin [Sigma], 1 μg/ml of hydrocortisone (Sigma) and 3 μg/ml of prolactin [NIH]). For the first 40 h after plating, 1 mg/ml fetuin (Sigma Chem. Co.) and 10% FCS (Sigma) were also included in the medium. The initial plating density was 2 × 10⁷ cells/100-mm dish and 2 × 10⁶/35-mm dish, with a plating efficiency of ∼50%. The initial serum-containing medium was replaced after 40 h by a differentiation

medium, which was F12 plus only the three lactogenic hormones. Thereafter, the medium was changed every other day. For inhibition studies, cells were cultivated in differentiation medium on EHS substratum for at least 24 h after the removal of serum. They were then incubated with either the conditioned medium from plastic or the growth factors for 48 h before RNA was extracted.

The CID-9 cell line (Schmidhauser et al., 1990) was derived by an enrichment of the epithelial population from COMMA-1-D, a nontransformed mouse mammary cell strain (Danielson et al., 1984). This enrichment process increased by more than fivefold the percentage of cells that could express β-casein as the result of ECM and lactogenic hormone treatment. CID-9 cells were maintained in growth medium (DMEM/F12 from Gibco, 5% FCS, 5 μg/ml of insulin, and 10 μg/ml gentamycin [UCSF]). At the onset of experiments, cells were plated on various substrata in differentiation medium (DMEM/F12, lactogenic hormones with 2% FCS for the first day of plating, and then in 0% serum for the following 6 d).

[³H]Thymidine-labeling of PMME Cells and Autoradiography

PMME cells were isolated from day-15-pregnant CD-1 mice as described previously (Emerman et al., 1977; Lee et al., 1984). Cells (2 × 10⁶ cells/35-mm dish) were plated on EHS-precoated coverslips which were placed in 35-mm tissue culture dishes. The cells were cultured on the coverslips in the presence of lactogenic hormones. On day 4 of culturing, two dishes with identical morphologies were selected. One was then treated with 40 ng/ml of TGF-α included in the differentiation medium, and the other was fed only with the differentiation medium for 48 h. During the last 24 h, [³H]thymidine (10 μCi/ml, 67 Ci/mmol) was added to both dishes. At the end of 6 d, the cells were fixed with methanol-acetone (1 vol:1 vol), stained with di-amino-phenyl-indole (DAPI, Sigma), and air dried before they were coated with Kodak NTB2 emulsion. They were developed overnight and thymidine-labeled nuclei were visualized and photographed under bright field microscopy.

RNA Preparation and Northern Analysis

Total RNA from cultured cells was prepared as described by Chomczynski and Sacchi (1987), and RNA extraction from the gland was done according to the method described by Talhouk et al. (1992). Three micrograms of each sample was resolved by electrophoresis on a 1% agarose gel. RNA was then transferred onto nylon filters (Hybond-N). ³²P-labeled WAP or β-casein cDNAs were used to probe the Northern blots. WAP cDNA insert (a gift from Lother Hennighausen, NIH) was excised with restriction enzymes and gel-purified from a plasmid. β-casein cDNA insert was a gift from Jeffrey Rosen (Baylor College of Medicine, Houston, TX). Hybridization of the filters with a probe for 28-s ribosomal RNA was often used as a control for RNA quality and even loading. Where quantification is indicated, bands with intensities falling within the linear range of the autoradiography were scanned and analyzed by densitometry.

Metabolic ³⁵S-Labeling

PMME cells which had been cultured for 5 d were methionine-starved for 1 h before ³⁵S-labeled methionine (ICN Biomedicals, Costa Mesa, CA) was added at 350 μCi/ml (specific activity; 1,152 Ci/mmol). One milliliter of F12 differentiation medium (see description in cell culture) was added to each 35-mm dish and the cells were labeled for 16 h.

For labeling the gland, the inguinal mammary glands were dissected out from timed-pregnant CD-1 mice at different stages of gestation. One or two glands from each animal were cut with scissors into small pieces of ∼2 mm in diameter before they were incubated in 4 ml of F12 medium that contained 250 μCi/ml of [³⁵S]methionine (ICN Biochemicals, Costa Mesa, CA) and lactogenic hormones. The mixture was then incubated in a humidified incubator with gentle rocking for 5 h.

Lysate Preparation and Quantification

Conditioned media were collected from cells in culture. After removal of cell debris by centrifugation, they were stored at −70°C. Cells were then lysed in radioimmune precipitation buffer (RIPA, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM Na₂EDTA, 2 mM PMSF, 5 μg/ml aprotinin, 0.1% SDS, 0.2% NP-40, and 0.5% sodium deoxycholate). After homogenization by sonication and removal of cell debris by centrifugation, the lysates were stored at −70°C.

For labeled glands, small pieces of tissue which had been incubated with [^{35}S]methionine were added on top of a 0.45-μm filter unit to separate them from the labeling medium. The organoids were then rinsed with cold F12 medium before being frozen in liquid nitrogen. RIPA buffer was added to the frozen tissue pieces before they were subjected to homogenization. The homogenate was spun three times in the microfuge to get rid of tissue debris. Subsequently, the supernatant was stored at $-70°C$. Total incorporation of radioactivity was calculated from the TCA-precipitable counts contained in the aliquots taken from each lysate. Various amount of TCA-precipitable counts were used for immunoprecipitation with different antibodies.

Immunoprecipitations

7.1. with WAP Antiserum. (A generous gift from Floyd Schanbacher, OSU.) At least 2×10^7 TCA-precipitable counts from each lysate were used to incubate with rabbit anti–mouse WAP antiserum for 2 h on ice. Thirty five microliter of Protein A–Sepharose (Sigma, 10% wt/vol in RIPA) was added to each sample, which was diluted with RIPA in a final volume of 500 μl. After 45 min of gentle shaking in 4°C, the antibody-antigen complex was precipitated by centrifugation. The Sepharose beads were then washed three times with ice cold RIPA buffer and once with 50 mM TrisCl (pH 7.4), and stored at $-70°C$.

7.2. with TGF-α Antiserum. From each cell lysate, at least 5×10^6 TCA-precipitable counts were used to incubate with polyclonal sheep anti–rat TGF-α antibody for 2 h on ice. Secondary antibody against sheep IgGs (Zymed Labs., South San Francisco, CA) was then added followed by 0.1 vol of 20% (wt/vol) protein A–Sepharose beads (Sigma). The antigen-antibody complex was pelleted and the Sepharose beads were washed as described above.

For TGF-α immunoprecipitation of the conditioned medium, at least 10^6 TCA-precipitable counts were used for each sample. To confirm that the immunoprecipitated proteins were indeed TGF-α–related, a second set of the same samples were treated with TGF-α antiserum which had been preincubated with excessive amounts (1 μg/ml) of synthetic rat TGF-α (Peninsula Laboratories Inc., Belmont, CA). The precipitation procedure, after the addition of the secondary antibody, was carried out as previously described (Cheng et al., 1993).

Gel Electrophoresis and Autoradiography

All the immunoprecipitated proteins were separated on a denaturing polyacrylamide gel (20% acrylamide/0.5% bis-acrylamide) containing 10% (vol/vol) glycerol (Giulian et al., 1985). After electrophoresis, gels were fixed in a solution with 50% methanol and 10% acetic acid, washed in ddH$_2$O for 30 min, and treated with gel amplifier (Amersham Corp., Arlington Heights, IL). They were then vacuum-dried before being exposed to X-ray films (Kodak) for autoradiography.

TGF-α Radioimmunoassay

A sensitive radioimmunoassay for rat TGF-α (Russell et al., 1993) was used to measure levels of immunoreactive TGF-α in conditioned media as well as cell lysates from CID-9 cells, primary mammary epithelial cell cultures and glands. Cell and gland lysates were prepared as previously described (Sympson et al., 1994), and gland lysates were quantified by Bradford assay. A polyclonal sheep antiserum was raised against synthetic rat TGF-α (Peninsula Laboratories); the latter was ^{125}I-labeled and used as the standard tracer for the assay. In previous dose-response curves, it was determined that mouse-derived TGF-α immunoreactivity was parallel to that of the rat TGF-α standard in the RIA (Russell et al., 1993). Levels of mouse TGF-α were therefore expressed in equivalent units of rat TGF-α standard.

Results

ECM and a Three-Dimensional Structure Regulate TGF-α Production and Secretion in Mammary Epithelial Cells

TGF-α has been shown to be expressed in mouse mammary gland during certain developmental stages, and the mitogenic activity of this growth factor has been implicated in mammary gland ductal morphogenesis (Vonderhaar, 1987;

Snedeker et al., 1991). We examined the possibility that TGF-α regulation may be connected to WAP regulation in culture. Primary mammary epithelial cells isolated from 15-d pregnant mouse mammary glands were cultured either on plastic or on EHS in the presence of lactogenic hormones. Conditioned media and the cell lysates were subjected to TGF-α radioimmunoassay (see Materials and Methods). It was found that TGF-α levels in the lysate, as well as in the conditioned medium of cells cultured on plastic, were substantially higher than those of cells cultured on EHS matrix (Fig. 1 *A*). We therefore examined the regulation of TGF-α in CID-9 cells cultured on various substrata including EHS, floating collagen gel, attached collagen gel and plastic, using radioimmunoassay. It was found that the level of TGF-α was suppressed only when cells were cultured on EHS, and only when three-dimensional spheres were formed (Fig. 1 *B*). In all other culture conditions, plastic, attached collagen, and even floating collagen, high levels of TGF-α were found, with the level on plastic being the highest. Thus downregulation of TGF-α by ECM, indeed parallels WAP expression in culture, and is dependent on formation of three-dimensional structures.

To examine the molecular forms of TGF-α protein, we immunoprecipitated the conditioned medium of primary cells cultured either on EHS or on plastic with a polyclonal sheep antiserum against synthetic rat TGF-α. Two major proteins with apparent molecular mass of 20 kD and 43 kD were precipitated from plastic-conditioned medium but not EHS-conditioned medium (Fig. 1 *C*). Since the most commonly secreted form of TGF-α is a 5.6-kD peptide, we verified the specificity of these proteins by their ability to compete with recombinant rat TGF-α for binding to the antibody. We found that the two proteins were no longer immunoprecipitated when the antibody had been preabsorbed with excessive amount of recombinant rat TGF-α, indicating the presence of a TGF-α specific epitope on the two proteins.

Exogenous TGF-α Inhibits WAP Expression in Cells Cultured on EHS

The demonstration that TGF-α level in PMME cells is inversely proportional to the WAP expression suggested that TGF-α may be a potential WAP inhibitor. Recombinant TGF-α was added to cultures where PMME cells had already formed alveolar structures on EHS. Under these culture conditions, the growth factors present in the EHS preparations had no measurable effect on WAP expression since "factor-free" EHS, (Taub et al., 1990) did not induce a higher level of WAP expression in cells on EHS (data not shown). While both β-casein and WAP mRNA levels were found to be reduced by the presence of the exogenous growth factor, inhibition of WAP expression was much more dramatic (Fig. 2 *A*). When a TGF-α–binding antibody (Bringman et al., 1987) was incubated with the recombinant growth factor before it was added into the culture medium, WAP mRNA level was somewhat restored. A monoclonal antibody against human growth hormone, treated as above, did not restore WAP mRNA levels, confirming that the inhibition of WAP expression was specifically due to TGF-α. The effect of different concentrations of TGF-α on the levels of WAP protein was subsequently examined by immunoprecipitation of WAP protein in the cell lysates (Fig. 2 *B*). WAP protein (a 14-kD ma-
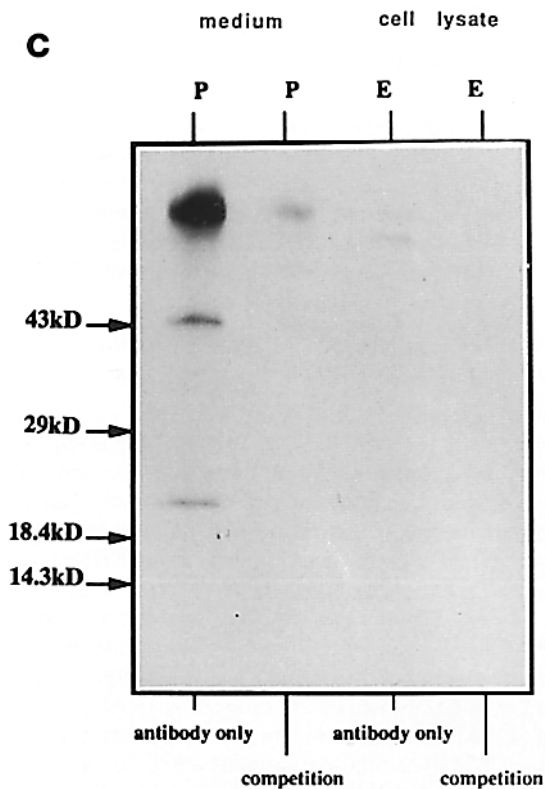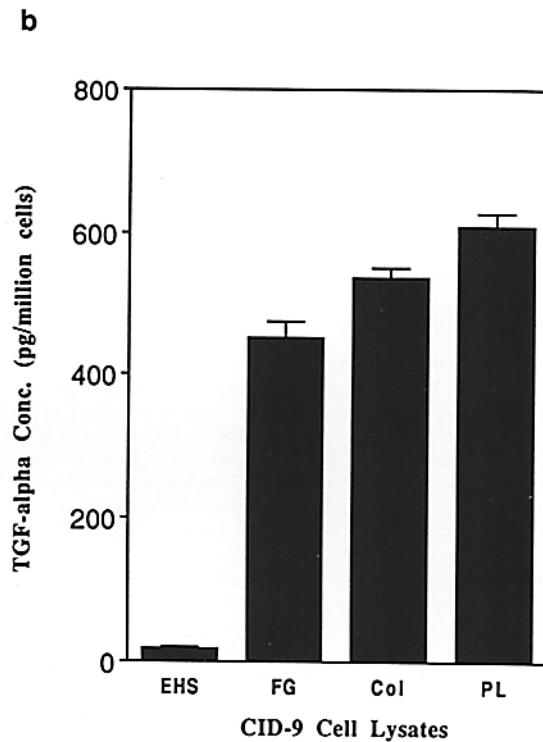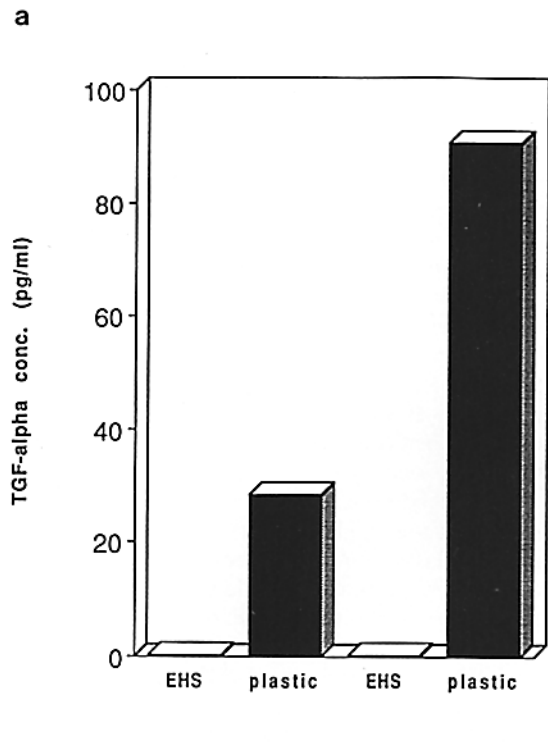
a

b

CID-9 Cell Lysates

c

medium   cell lysate

P   P   E   E

43kD →

29kD →

18.4kD →
14.3kD →

antibody only   antibody only

competition   competition

Figure 1. TGF-α activity in the lysates and conditioned media of mammary epithelial cells are modulated by culture substrata. (*A*) TGF-α radioimmuno-assay of lysates and conditioned media from primary cultures. Cells were cultured in differentiation medium. 48-h-conditioned media were collected, and cells were lysed. TGF-α radioimmunoassay was performed as described in Materials and Methods. TGF-α concentrations in the lysates were expressed as the amount of purified rat TGF-α binding that was competed by the lysates. Contributions from the culture medium, lysis buffer, and EHS substratum were subtracted from the corresponding values. For primary cultures, the cell numbers at the end of the culturing on EHS and plastic conditions were approximately the same. Therefore, TGF-α concentrations in pg/ml could be directly compared among different conditions. This bar graph is an average of duplicate plates from a typical experiment. (*B*) TGF-α radioimmunoassay of cell lysates from CID-9 cells cultured for 6 d on various culture substrata: EHS, floating collagen gel (FG), attached type I collagen gel (Col), and plastic (PL). Each bar represents the average plus SD for TGF-α concentration from three separate cultures with identical conditions. Unlike primary cells, CID-9 cells continue to grow in the differentiation medium at various rates on different culture substrata during the 6-d culture period. Thus, one extra dish for each condition was used to obtain cell numbers at the time of lysate preparation. The values depicted in the graph have been normalized to cell number. (*C*) TGF-α immunoprecipitation from the conditioned media. Primary cells were cultured either on plastic (*P*) or EHS (*E*) for 5 d before they were labeled with [³⁵S]methionine for 15 h. Conditioned media were then collected, cleared of cell debris, and TGF-α immunoprecipitation was performed with a polyclonal sheep antiserum against rat TGF-α (see Materials and Methods). Immunological specificity of the precipitated proteins was confirmed by their ability to compete with purified rat TGF-α for binding of the antibody competition.
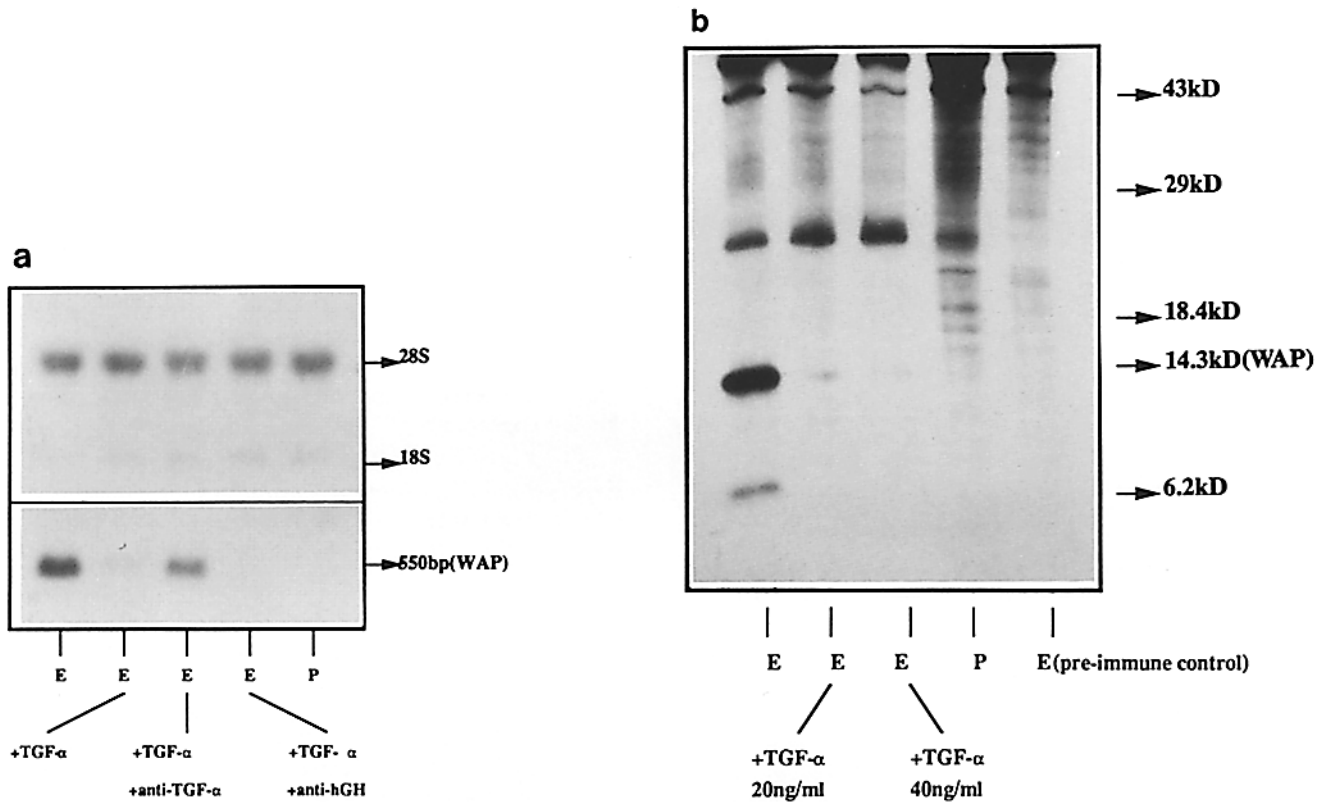
Figure 2. Expression and synthesis of WAP can be inhibited by exogenous TGF-α. (*A*) Northern analysis for WAP mRNA. Total RNA was obtained from primary cells cultured on EHS (lanes marked with *E*) or plastic (*P*) for 6 d. For the last two days of culturing, 20 ng/ml of recombinant human TGF-α was added to cells on EHS (*E*+TGF-α). A monoclonal antibody against either TGF-α (30 μg/ml; *E*+TGF-α+anti-TGF-α) or human growth hormone (*E*+TGF-α+anti-hGH) was incubated with the recombinant TGF-α before they were added to the cells. Positions for WAP mRNA (*lower panel*) and ribosomal RNAs (*upper panel*) are indicated. (*B*) Immunoprecipitation with WAP antibody. Cells were cultured on either EHS (*E*) or plastic (*P*) for 5 d before they were labeled for 15 h with [³⁵S]methionine and immunoprecipitated with a rabbit polyclonal anti–mouse WAP. WAP protein level was reduced upon TGF-α treatment (*E*+TGF-α 20 ng/ml and *E*+TGF-α 40 ng/ml). As a control for the antibody, the same amount of lysate from an EHS culture was incubated with preimmune rabbit serum (*E*, preimmune control) in place of the WAP antiserum.

jor form and a 6-kD minor form) levels in PMME cells cultured on EHS were decreased as a function of increasing amounts of TGF-α added to the medium. The maximum inhibition was achieved with addition of 20 ng/ml of TGF-α. The band at 25 kD was due to cross-reactivity of the antiserum to another protein, since it was not present in the precipitate brought down by preimmune serum. The size of this protein suggested it could be α-casein. In a separate experiment testing whether other TGF-α–like growth factors could inhibit WAP expression in our three-dimensional culture, EGF (20 ng/ml) was found to also inhibit WAP expression in cells cultured on EHS (data not shown), indicating that EGF receptor was present and most probably involved in the WAP inhibition pathway.

TGF-α Neutralizing Antibody Reduces the WAP Inhibitory Activity Found in the Plastic-Conditioned Medium

Previous work in our laboratory had demonstrated that conditioned media from all monolayer PMME cultures (plastic, attached collagen, floating collagen, air dried EHS, etc.) contained diffusible factors that could inhibit WAP expression in cells cultured on EHS (Chen and Bissell, 1989). We showed above that exogenous TGF-α, as well as EGF, inhibited WAP expression, and that TGF-α synthesis and secretion were upregulated in cells cultured on plastic. We therefore investigated the possibility that TGF-α, or proteins related to TGF-α, may be the putative WAP inhibitor(s) identified previously. Primary cells which had already formed alveolar structures on EHS substratum were treated with plastic-conditioned medium with or without preincubation with anti–rat TGF-α antibody (see Materials and Methods). While plastic-conditioned medium reduced WAP mRNA levels in cells cultured on EHS (Fig. 3, lane 2), incubation with a TGF-α neutralizing antibody prevented such an inhibition (lane 3), indicating that TGF-α in the conditioned medium was most probably responsible for the inhibition of WAP. Due to the nature of our assay for WAP expression, we can conclude only qualitatively that the WAP mRNA level in cells treated with plastic-conditioned medium preincubated with antiserum against TGF-α (Fig. 3, lane 3) is similar to that in cells on EHS (Fig. 3, lane 1). Therefore, we cannot rule out the possibility that other minor WAP inhibiting factors may also be produced by these cells when they are cultured on a plastic substratum.
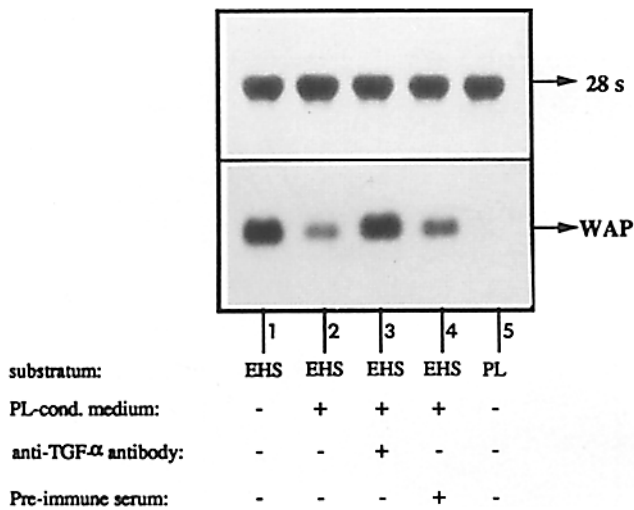
substratum:	1	2	3	4	5
	EHS	EHS	EHS	EHS	PL
PL-cond. medium:	-	+	+	+	-
anti-TGF-α antibody:	-	-	+	-	-
Pre-immune serum:	-	-	-	+	-

Figure 3. WAP inhibitory activity in the PMME-plastic–conditioned medium can be reduced by a neutralizing antibody against TGF-α. RNA was extracted from cells cultured for 6 d on the indicated substrata. On day 4, cells were either fed with fresh medium (lane *1* and lane *5*), or with a 48-h conditioned medium from plastic cultures (lanes *2–4*). In lanes *3* and *4*, a polyclonal antibody against rat TGF-α and preimmune serum, respectively, were incubated with the conditioned medium.

TGF-α Can Disrupt the Alveolar Sphere Formation and Induce DNA Synthesis in PMME Cells Cultured on EHS

Since an intact three-dimensional structure is known to be crucial for WAP expression and TGF-α appeared to inhibit WAP expression, we asked whether such an inhibition was via disruption of the alveolar structures formed on EHS. TGF-α (40 ng/ml) was added to the cells once the 3D structures were formed (Fig. 4, *A–D*). After 48 h of incubation with TGF-α, ~35% of the structures had collapsed and were visibly disintegrated to differing degrees. In addition, cells which migrated out had incorporated thymidine, whereas cells still remaining in the spherical structures or their peripheries were not. It is worth noting that in the condition where no growth factor was added exogenously, even small cell clusters (with three or four cells) were negative for thymidine incorporation (*arrow* in 4 *A*). This indicates that TGF-α initially disrupted cell–cell interactions necessary for the alveolar structures, and then stimulated DNA synthesis in those cells which were no longer associated with these structures.

TGF-α Protein Levels and Molecular Forms Are Regulated during Pregnancy

We now have confirmed that the WAP promoter is active in virgin and early pregnant mammary glands (Sympson et al., 1994), yet the WAP messenger RNA is detected only after 15 d of pregnancy. Since we have demonstrated that TGF-α and its related proteins inhibit WAP expression in culture, the possibility of TGF-α being responsible for the delay in WAP temporal expression was examined. TGF-α radioimmunoassay indicated a drop (~50%) in TGF-α immunoreactivity in late pregnancy (57 pg/mg protein lysate; calculated

from the average of two experiments) compared to virgin gland and early pregnancy (96 and 104 pg/mg protein lysate, respectively). TGF-α immunoprecipitation using lysates from metabolically labeled 8-d or 15-d glands from pregnant animals were more dramatic (Fig. 5). A 14.3-kD TGF-α protein was precipitated from the gland of 8-d pregnant mice, but the band disappeared in the 15-d pregnant animal. The size of the precipitated protein suggested that it might be a different glycosylation form of the 5.6-kD TGF-α protein (Dart et al., 1985; Ignotz et al., 1986) as previously suggested by Bringman et al. (1987). We therefore suggest that the loss of the 14.3-kD TGF-α protein and the reduction in TGF-α level, measured by the radioimmunoassay in the pregnant animal (day 15 or later) could provide at least part of the explanation for the appearance of WAP in late pregnancy.

Expression of WAP and β-Casein in Mammary Glands from MMTV-TGF-α Transgenic Mice Are Downregulated during Pregnancy and Lactation

If TGF-α inhibits WAP expression in vivo, then TGF-α transgenic mice should show a preferential reduction in WAP levels compared to β-casein. We took advantage of the MMTV-TGF-α transgenic mice, which had been constructed previously (Matsui et al., 1990). It had been shown that overexpression of TGF-α, targeted to the mammary gland by MMTV promoter, caused alveolar and terminal ductal hyperplasia in female mice. Northern analysis of total RNA isolated from transgenic mouse glands of various developmental stages (Fig. 6) showed a significant reduction of WAP mRNA level in the mid-pregnant transgenic mammary gland compared with the normal, nontransgenic gland (~5% of the normal levels based on densitometry readings of the autoradiographs). An even more dramatic reduction was found in the glands of the lactating transgenic animals (as little as 2% of the normal levels). The inhibition of β-casein expression by TGF-α overexpression, while substantial, was less profound; β-casein mRNA was reduced to 50% in the gland of the pregnant mice, but more severely in the gland of the lactating mice (7% of normal). The inhibition of milk protein production in the mammary glands of TGF-α transgenic mice was confirmed further by immunohistochemistry of the tissue sections from the gland, using antibodies against WAP or β-casein. We found that while both WAP and β-casein proteins were much less prevalent in the glands from mid-pregnant transgenic mouse than the normal mouse of the same stage; the reduction of WAP level was more dramatic than that of β-casein (data not shown). In the transgenic line from which we obtained the tissue sections, less than 15% of the alveoli in a gland from a pregnant animal were stained positive for WAP, compared with more than 80% in a gland from a normal animal, and the level of staining was down to the margin of detection in the gland of the transgenic mice. Additionally, it was difficult to visualize the staining because of the disrupted morphology of the gland: compared to the normal gland, the alveoli were in general much smaller with shrunken central lumina surrounded by multi-layers of epithelial cells (picture not shown). Even the low amount of milk proteins produced were not properly secreted into the lumen as a result of the disorganized gland structure.
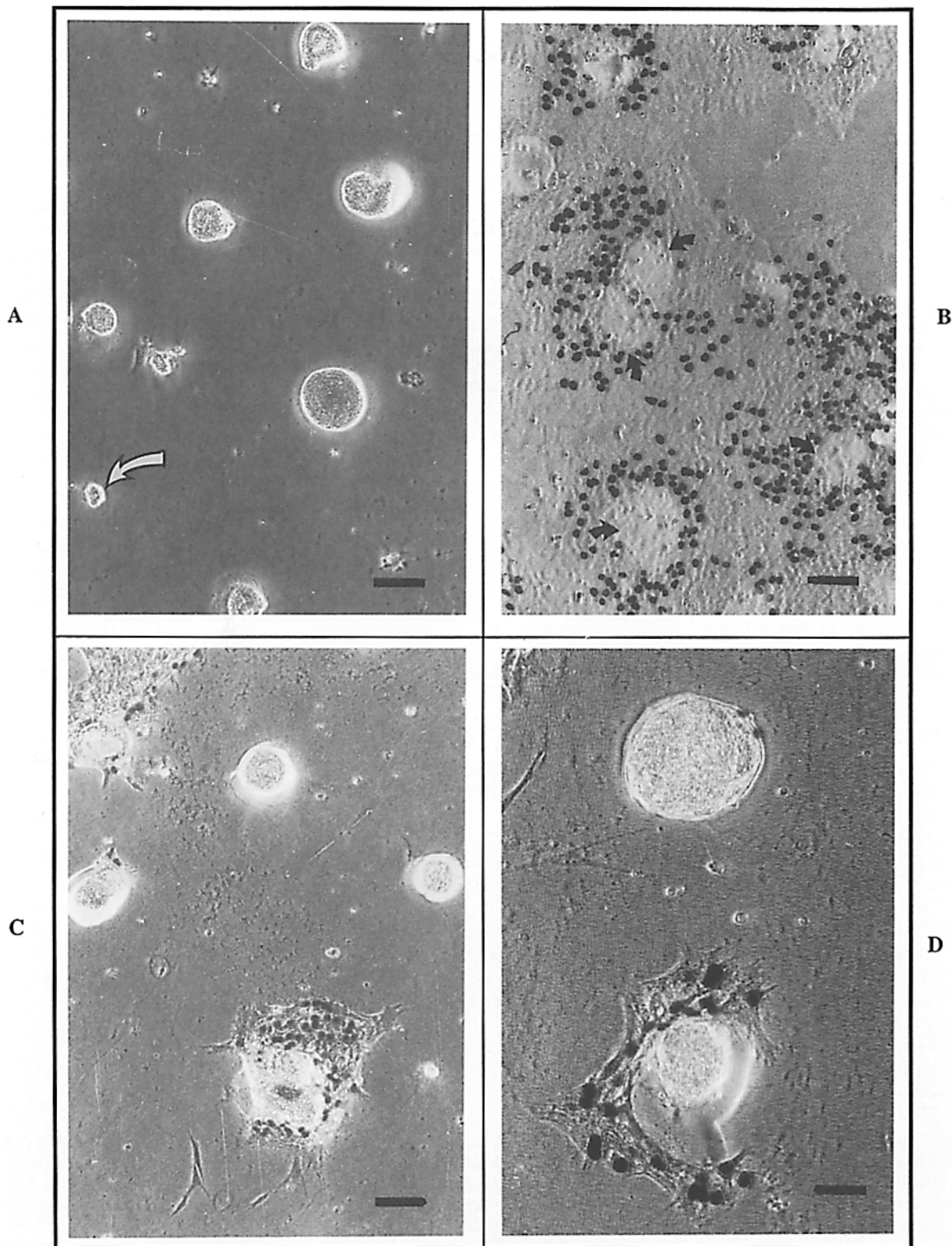
Since we found that inhibition of β-casein expression by

Figure 4. Disruption of the spherical structures by the addition of TGF-α. (*A*) Mammary epithelial cells were cultured on EHS for 6 d. [³H]Thymidine was included in the medium during the last 24 h of culturing. Notice that the alveolar structures are uniformly intact and no thymidine incorporation is observed even in small clusters (*arrow*). (*B–D*). After 4 d of culturing on EHS, 40 ng/ml of TGF-α was added to the cell for another 2 d. In addition, [³H]thymidine was included in the culture medium for the last 24 h. Notice that alveolar structures were partially destroyed and DNA synthesis was triggered. (*B*) An area where the effect of TGF-α was severe. The arrows show the ghosts of alveolar structures. (*C* and *D*) Two different areas representative of a culture after TGF-α treatment. The cluster on the top part of (*D*) is a sphere that is beginning to be affected by TGF-α. Notice the two cells on the periphery of the sphere which are positively labeled for thymidine incorporation. Bars: (*A*) 0.1 mm; (*B*) 0.1 mm; (*C*) 0.1 mm; (*D*) 0.05 mm.
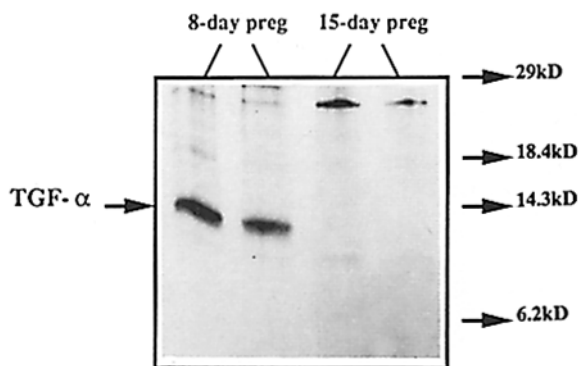
Figure 5. TGF-α immunoprecipitation in early and late-pregnant glands. Mammary glands from two different sets of 8-d, or 15-d pregnant CD-1 mice were labeled with [³⁵S]methionine for 5 h before the tissues were homogenized and immunoprecipitated with sheep polyclonal anti–rat TGF-α antibody. The positions of the molecular mass markers and the 14.3-kD TGF-α band are indicated.

TGF-α in tissue culture was not nearly as pronounced as WAP, the strong inhibition of β-casein in the glands of the transgenic mouse appeared surprising. It is quite likely that abnormally high levels of TGF-α in the transgenic mice, especially during lactation, could induce the synthesis of factors such as TGF-β1, which in turn could inhibit β-casein.

Discussion

The establishment of culture conditions which allow mammary epithelial cells to form three-dimensional structures with close resemblance to alveoli in lactating glands has made it possible to address the important question of how tissue structure may influence functional differentiation. Since whey acidic protein is not expressed by monolayer cultures (Chen and Bissell, 1989), the nature of the regulation by the alveolar structures must be considered in studying the regulation of this protein. Our studies reported here demonstrate an intricate interaction between tissue structure, ECM, and growth factors. We show that ECM plays a dominant role in the regulation of mammary epithelial cell morphology and differentiation by downregulation of growth factor activities. However, the dominant effect of ECM can be reversed if growth factor concentration is increased inappropriately. Thus, a full functional differentiation is dependent on a crucial balance between an intact basement membrane, a low level of growth factor activity and appropriate hormones. The importance of interactions between the ECM and growth factors in regulating tissue structure and function has been studied in a number of systems: basic fibroblast growth factor (bFGF), for example, can either stimulate endothelial cell growth or promote capillary differentiation depending on the ECM molecules used to coat the culture dish (Ingber and Folkman, 1989); bFGF signaling in Swiss 3T3 cells and MM14 myoblasts were shown to be modulated by heparan sulfate proteoglycan (Rapraeger et al., 1991). Growth factors were shown to be actively involved in the ECM regulation of morphogenic differentiation of a sym-

pathetic neuronal cell line (Rogelj et al., 1989), and of cultured baby mouse kidney cells (Taub et al., 1990). Synthesis of ECM proteins were shown to be modulated by growth factors, such as TGF-βs (Roberts et al., 1990; Massague, 1990), which in turn are regulated by an organized basement membrane (Streuli et al., 1993). Since a change in growth factor levels could be a much more rapid process than reorganization of the basement membrane, the dynamic interactions between ECM and growth factors could be an important fine tuning mechanism for tissue function in vivo. Nevertheless, a report by Ferguson et al. (1992) has demonstrated surprisingly, cyclical changes in the composition of BM in human breast throughout the menstrual cycle. It is therefore likely that the hormonal changes during the menstrual cycle are accompanied with changes in both growth factor activities and the composition of basement membrane which in turn would result in changes in the functional status of the gland.

TGF-α and Proteins Immunologically Related to TGF-α Produced by Mammary Epithelial Cells

Expression of TGF-α in adult animals has been documented in several tissues such as pituitary gland (Kobrin et al., 1986), brain (Wilcox and Derynck, 1988), keratinocytes
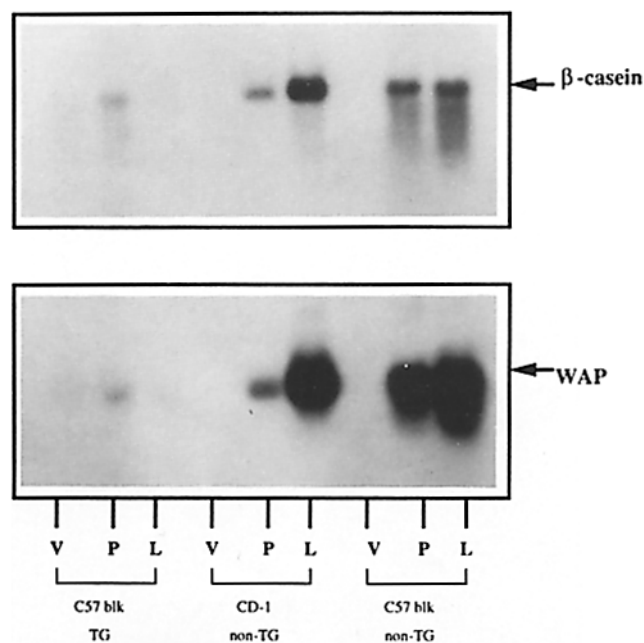

Figure 6. Milk protein gene expression is downregulated in MMTV-TGF-α transgenic mice during pregnancy and lactation. Northern analyses were performed with equal amounts of total RNA, (3 µg per lane; equal loading confirmed by ethidium bromide staining of the 18s and 28s ribosomal RNAs in the gel before transferring to the nylon filter), isolated from mammary glands of normal (non-TG) or MMTV-TGF-α transgenic mice (TG) at various developmental stages: (V, virgin; P, 18-d pregnancy; L, 2-d lactation). Two different strains of normal mice (C57 black and CD-1) were compared with the transgenic mice made in a C57 black background. Positions of β-casein and WAP mRNAs are indicated. We selected autoradiograms where the intensity of WAP and β-casein bands in the TG lanes are comparable. Thus the degree of inhibition can be directly visualized by comparison to the control lanes.

(Coffey et al., 1987), virgin mouse mammary gland, and 14-d pregnant mouse mammary gland (Snedeker et al., 1991). The molecular form and localization of the TGF-α gene product, however, have been overlooked in the past. It is known that TGF-α is synthesized as a 59–amino acid precursor transmembrane protein, processed by proteolytic cleavage into secreted proteins (for review, see Derynck, 1988). Size heterogeneity in both the precursor form and the secreted form is an important feature of this polypeptide since the precursor protein has a number of N- and O-glycosylation sites, and the sites for proteolytic cleavage can vary. Secreted proteins with molecular masses ranging from 5–20 kD have been reported (Derynck, 1988). In addition, various forms of TGF-α protein, including the unprocessed pro-form, have been found to be biologically active (De-Larco and Todaro, 1978; Todaro et al., 1980; Brachmann et al., 1989; Wong et al., 1989; Luetteke and Lee, 1990). In one report, a 14.3-kD protein, together with the common 5.6-kD secreted form of TGF-α, was precipitated by a TGF-α antiserum in mouse macrophages (Luetteke et al., 1993). Another report by Alexander et al. (1993) showed that immunoprecipitation with an antibody against TGF-α, precipitated three proteins with molecular masses of 5.5 kD, 14.2 kD, and 20 kD from conditioned media of rat mammary adenocarcinoma cell cultures. We now have demonstrated that a protein ∼14 kD is produced in the mammary gland of 8-d pregnant mice, but no longer produced in the late-pregnant (15 D) glands. In addition, we have identified two proteins secreted by primary mammary epithelial cells cultured on plastic; both can compete with purified rat TGF-α for the binding of a TGF-α antibody. One of these two proteins has an apparent molecular mass of 20 kD, which could be the unprocessed precursor form of TGF-α; the other protein has a much larger molecular mass (∼43 kD), and therefore is unlikely to be a TGF-α precursor. Conceivably, the 43-kD protein could have an epitope recognizable by the anti–TGF-α antibody. The exact identity of this protein remains to be determined. The difference between the size of TGF-α immunoprecipitated in culture and in the gland could be due to differences in posttranslational processing, such as glycosylation of the precursor in vivo and in culture.

ECM Regulates WAP Expression Via Modulating TGF-α Activity

We have demonstrated that TGF-α, or proteins that are immunologically related to TGF-α, are major components of the postulated WAP inhibitors (Chen and Bissell, 1989). These results, together with the results on the role of TGF-β1 in β-casein gene expression (Streuli et al., 1993; also see our results on TGF-α production in culture), demonstrate a sequential regulation of growth and differentiation by ECM-induced modulation of growth factor activities. For β-casein expression, the presence of a basement membrane on floating collagen gel (Streuli and Bissell, 1990) is sufficient to downregulate TGF-β1 activity leading to induction of β-casein expression. Downregulation of TGF-α activity, however, requires not only the presence of a basement membrane, but also a proper three-dimensional structure. Since TGF-α and TGF-β have been found to have very different properties particularly in their signaling pathways (Shum et al., 1994; Wrana et al., 1992) and their mitogenic effects on

epithelial cells (Vonderhaar, 1987; Coffey et al., 1988; Mead and Fausto, 1989; Ryan et al., 1994), it is not surprising that the two factors have distinct functions and are regulated differently in mammary epithelial cells.

WAP expression in culture depends more on the presence of hydrocortisone than on prolactin, another important difference between WAP and β-casein expression (Pittius et al., 1988b). Hydrocortisone has been shown to induce the tight junction formation in a murine mammary cell line of ductal origin (Zettl et al, 1992). A rat mammary adenocarcinoma cell line has been shown to lose the ability to form tight junctions in the presence of glucocorticoid when it is transfected with a TGF-α expression vector (Firestone and Buse, 1994). It is therefore possible that the hydrocortisone requirement for WAP expression reflects a need for tight junctions which are necessary for maintaining alveolar structures with polarized lumina. We propose that TGF-α may interfere with cell–cell interaction and consequently three-dimensional structures, by affecting the integrity of the junctional complexes.

Possible Link between the Change in TGF-α Activity during Pregnancy and the Differential Temporal Regulation of Milk Protein Expression

TGF-α inhibits both β-casein and WAP; however, the latter is much more affected by this growth factor both in culture and in transgenic mice. This differential regulation may account for the long-standing observation that WAP is expressed in pregnant mice later in gestation than is β-casein (Pittius, 1988a,b). Snedeker et al. (1991) have shown that TGF-α mRNA is detectable in mature virgin or 14-d pregnant gland but undetectable in premature virgin, or 10-d lactating gland. Since TGF-α is known to undergo posttranscriptional modifications (DeLarco and Todaro, 1978; Todaro et al, 1980; Massague, 1990), both the level of the protein and its receptor binding need to be considered in addition to mRNA levels. We now have shown that the level of TGF-α, measured by radioimmunoassay, as well as the profile of TGF-α immunoprecipitable proteins, change as the pregnancy proceeds. There is a correlation between the decrease in total TGF-α immunoreactivity and the disappearance of a 14.3-kD TGF-α immunoprecipitable protein as the gland enters late pregnancy. Therefore, the drop in TGF-α activity coincides with WAP expression in the gland. Since TGF-α is known to bind to EGF receptor in order to exert its biological function, the level of EGF receptor also needs to be considered. EGF receptor level has been shown previously to increase in the beginning of pregnancy (Edery et al., 1985), reaching the highest level by day 10. Taking into account changes of both protein and receptor levels from early (before day-10) to late- (after day-15) pregnancy, we propose that TGF-α is a physiologically relevant regulator of WAP gene expression. It is possible that WAP expression in late pregnancy and lactating stages is not only due to downregulation of TGF-α production, but also to decreased levels of the receptor for TGF-α. EGF has previously been demonstrated to be a mitogen for mammary epithelial cells (Vonderhaar, 1987). However, both EGF mRNA and the EGF precursor protein have been shown to be present in all stages of mammary gland development including lactation (Brown et al., 1989; Snedeker et al., 1991). This indicates that EGF has a very different function in the lactating mammary gland

from that of TGF-α. Brown et al. (1989) have proposed a role for milk EGF in the development of the gastrointestinal tract and perhaps other organs of the neonate.

A Possible Mechanism for Downregulation of TGF-α as a Result of Formation of Three-Dimensional Alveolar Structures

TGF-α has been shown to be an autocrine growth factor involved in the control of normal and malignant cell growth in various systems including human keratinocytes (Coffey et al., 1988; Vassar and Fuchs, 1991), hepatocytes (Mead and Fausto, 1989), colorectal epithelial cells (Untawale et al., 1993), mammary epithelial cells (see Ciardiello et al., 1991 for review), and rat tracheal epithelial cells (Ferriola et al., 1992). In addition, autoinduction of TGF-α expression has been documented as a novel mechanism of autoregulation of cell growth (Coffey et al., 1987, 1992; Untawale et al., 1993). We have shown here that TGF-α expression is suppressed in mouse mammary epithelial cultures only when both a basement membrane and an alveolar structure are present. Since cells cultivated on EHS respond to exogenous TGF-α, there must be receptors on the basolateral surface of the cells in the alveolar structures. The preferential basolateral localization of EGF receptor in epithelial cells have previously been demonstrated in other epithelial cell systems such as human gastric mucosa (Orsini et al., 1993), and human breast cancer cells (Monaghan et al., 1990; for review see Fukuyama and Shimizu, 1991). Snedecker et al. (1991) have immunolocalized EGF to the luminal surface of ductal epithelium during mammary gland ductal morphogenesis (Snedecker et al., 1991). This group has demonstrated that the differentiated alveolar epithelium in the lactating mammary gland can synthesize EGF precursor and have found that abundant levels of EGF are detected in milk (Brown et al., 1989). Thus EGF poses no threat to differentiation or WAP expression in pregnant and lactating mammary gland presumably because it is secreted apically and does not interact with the basolateral EGF receptor. In contrast, TGF-α was localized to the basal cap-cell layer in the developing rat mammary gland (Snedecker et al., 1991) and one of our laboratories has demonstrated lateral TGF-α staining within the ductal mammary epithelium of MMTV-TGF-α transgenic mice (Matsui et al., 1990; Dempsey, P. J., and R. J. Coffey, unpublished results). In addition, we have shown recently that pro-TGF-α is targeted preferentially to the basolateral surface of polarized epithelial cells and can act in an autocrine manner (Dempsey and Coffey, 1994). Furthermore, we have found in vitro differences in growth factor production, consumption and response as well as EGF receptor recycling in epithelial cells when grown as a polarized monolayer on transwell filters as compared to cells grown on plastic (Dempsey, P. J., H. S. Wiley, and R. J. Coffey, unpublished results). These findings suggest that the polarized state and the three dimensional architecture of the mammary epithelium observed in vitro and in vivo may influence the production and routing of TGF-α and consequent signaling through the EGF receptor. Additional complexity ensues from the secretory nature of the mammary epithelium and warrants further investigation.

Components in the microenvironment of a cell normally include the neighboring cells, the ECM, the tissue- and developmental stage-specific hormones, and the locally acting growth factors. The fate of a cell within a tissue is determined by the net result of interactions among these components, and the structure and function of a tissue therefore is ultimately dependent on its immediate microenvironment (Stoker et al., 1990). These studies provide a striking example of how the tissue structure and functional differentiation in mammary epithelium are controlled by the concerted action of ECM, neighboring cells, hormones, and growth factors in the microenvironment of the breast tissue.

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