Extracellular Matrix Regulates Expression of the TGF-β1 Gene

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Abstract. Transforming growth factor-β (TGF-β) is a potent regulator of cell proliferation and modulates the interactions of cells with their extracellular matrix (ECM), in part by inducing the synthesis of various ECM proteins. Three different isoforms of TGF-β are synthesized in a defined pattern in specific cell populations in vivo. In the specific case of TGF-β1, this well-defined and limited expression stands in sharp contrast to its synthesis by virtually all cells in culture.

Using mammary epithelial cells as a model system, we evaluated the substratum dependence of the expression of TGF-β1. The level of TGF-β1 expression is high in cells on plastic, but is strongly downregulated when cells are cultured on a reconstituted basement membrane matrix. In contrast, TGF-β2 mRNA levels in cells on either substratum remain unchanged. Using the chloramphenicol acetyl transferase gene as reporter gene under the control of the TGF-β1 promoter, we show that transcription from this promoter is suppressed when the cells are in contact with either endogenously synthesized or exogenously administered basement membrane. TGF-β1 promoter activity is strongly induced by the absence of basement membrane, i.e., by direct contact of the cells with plastic. This modulation of transcription from the TGF-β1 promoter occurs in the absence of lactogenic hormones which allow full differentiation. Our results thus indicate that basement membrane is an important regulator of TGF-β1 synthesis, and explain why most cells in culture on plastic express TGF-β1 in contrast with the more restricted TGF-β1 synthesis in vivo. We propose that there is a feedback loop whereby TGF-β1-induced synthesis of basement membrane components is repressed once a functional basement membrane is present. Finally, these results together with our current knowledge of regulation of TGF-β1 and TGF-β2 synthesis, suggest that, in vivo, TGF-β1 may play a major role in regulating the ECM synthesis and the cell–ECM interactions, whereas TGF-β2 may be more important in morphogenetic processes.

MUCH attention has been focused in recent years on the biology of transforming growth factor-β (TGF-β),[1] a potent regulator of cell proliferation. TGF-β can exert a variety of effects, depending on the nature of the target cell and the physiological conditions. It stimulates the proliferation of many cell types of mesenchymal origin, yet is growth inhibitory for many other cell types. A major activity of TGF-β is its ability to stimulate the synthesis and deposition of various extracellular matrix (ECM) proteins and to increase the expression of integrins, receptors that mediate cellular interactions with ECM proteins. This is frequently accompanied by an increased synthesis of protease inhibitors and a repression of the synthesis of ECM-degrading proteases (for review see Roberts and Sporn, 1990). Taken together, these activities can result in increased cell–ECM interactions and adhesiveness. In comparison with other secreted factors, TGF-β can be considered as the most potent regulator known to date of ECM formation and cell–ECM interactions.

Our current knowledge of the localization and biological activities of TGF-β suggests that it exerts its activities in the context of organ and tissue remodeling (Heine et al., 1987; Thompson et al., 1989; Fitzpatrick et al., 1990; Gatherer et al., 1990; Millan et al., 1990; Pelton et al., 1990a,b, 1991; Schmid et al., 1990). This implies that TGF-β may function as a physiological regulator of woundhealing, developmental tissue differentiation and morphogenesis, and tumor development. Since most cells have the capacity to express and secrete TGF-β and have cell surface TGF-β receptors (Roberts and Sporn, 1990), it is generally believed that TGF-β exerts its role by autocrine and paracrine mechanisms. In addition, since TGF-β binds to several ECM proteins and since ECM contains TGF-β, it can be assumed that the ECM and especially the basement membranes represent a reservoir from which TGF-β can be retrieved so that it can exert its various activities on the surrounding cells.

1. *Abbreviations used in this paper:* CAT, chloramphenicol acetyltransferase; ECM, extracellular matrix; EHS, Engelbreth-Holm-Swarm; TGF-β, transforming growth factor-β.

Several species of TGF-β have been identified and characterized by cDNA cloning. Three different TGF-β isoforms, each encoded by separate genes, are expressed in mammalian cell systems. These TGF-β species have a high degree of structural identity in their mature polypeptide sequences and have similar biological activities in culture. Their cellular synthesis is subject to differential regulation and they are differentially localized in vivo. In situ hybridization and isoform-specific immunohistochemistry of the TGF-β1, -β2, and -β3 mRNAs and proteins have documented that the three TGF-β species have a well-defined and characteristic expression pattern during murine development (Heine et al., 1987; Thompson et al., 1989; Fitzpatrick et al., 1990; Gatherer et al., 1990; Millan et al., 1990; Pelton et al., 1990a,b, 1991; Schmid et al., 1990). Their cell and tissue distribution is partially overlapping, frequently resulting in the expression of more than a single TGF-β form in specific cells and tissues. This localized expression pattern of the individual TGF-β forms is also reflected to some degree in cells in culture. TGF-β2 and -β3 expression by cells in culture is apparent only in select cell lines and primary cultures, which are, as far as can be assessed, in accordance with cell types normally expressing these TGF-β species in vivo. In contrast, the expression pattern of TGF-β1 in cultured cells clearly contradicts the localized TGF-β1 expression in vivo. For example, during mouse development TGF-β1 expression occurs mainly in cell types of hematopoietic origin, endothelial cells and epithelia, especially in skin. Yet, cell culture experiments indicate that virtually all cells synthesize TGF-β1 in culture, in disagreement with the findings in vivo.

In this study, we sought to understand the basis of the discrepancy between the virtually universal expression of TGF-β1 in cultured cells and its localized expression in vivo. In particular, we have paid attention to the influence of the substratum on the expression of TGF-β1 by mammary epithelial cells. It is indeed conceivable that removal of the cells from their natural environment and especially the absence of ECM might contribute to the discrepancy between their behavior in culture and in vivo. Thus, we hypothesized that ECM itself might be able to regulate TGF-β1 expression, and that the absence of a physiological substratum when cells are cultured on plastic dishes, which resembles a "wounded" environment, might induce the otherwise suppressed production of TGF-β1. Therefore, it is likely that increased TGF-β1 synthesis would subsequently induce the secretion and deposition of several ECM proteins in an autocrine manner, contributing to an establishment of more physiological cell–ECM interactions.

We have evaluated our hypothesis using epithelial cells derived from the mouse mammary gland. These cells have been used previously to explore the influence of ECM on the regulation of gene expression and differentiation (Lee et al., 1984, 1985; Li et al., 1987; Chen and Bissell, 1989; Barcellos-Hoff et al., 1989; Aggeler et al., 1991; Schmidhauser et al., 1990, 1992; Streuli and Bissell, 1990; Streuli et al., 1991). Our findings document that the absence of a basement membrane has a profound and specific effect on the expression of TGF-β1, resulting in an upregulation of TGF-β1 expression when cells are maintained on plastic as substratum. Using the TGF-β1 promoter linked to a reporter gene, we show that this effect is due to an enhanced transcription from the TGF-β1 promoter. The induction of TGF-β1 ex-

pression in the absence of ECM and its suppression by the presence of a basement membrane may provide a feedback loop resulting in a balance between TGF-β1 expression and basement membrane formation.

Materials and Methods

Substrata and Cell Culture

EHS matrix was prepared from EHS tumors passaged in C57BL mice (Kleinman et al., 1986) and used as described (Barcellos-Hoff et al., 1989; Streuli et al., 1991). Collagen I was prepared from rat tails (Lee et al., 1984) and stored at a concentration of 2–3 mg/ml. Thick collagen gels were made with 150 μl collagen I per cm² and either left attached to the dish or released into the medium 24 h after plating (Streuli and Bissell, 1990).

Primary mammary epithelial cells were prepared from 14.5-d-pregnant CD-1 mice and cultured on physiological substrata in the presence of insulin (5 μg/ml) and lactogenic hormones (1 μg/ml hydrocortisone, 3 μg/ml prolactin) as described elsewhere (Lee et al., 1985; Emerman et al., 1988; Streuli et al., 1991). The mouse mammary epithelial cell line CID-9 and its transfected derivatives were cultured routinely in DME/F12 medium containing 5% FCS and 5 μg/ml insulin (Schmidhauser et al., 1990). For CAT assays and differentiation assays, these cells were plated onto relevant substrata and cultured with 2% serum for 24 h. The medium was then changed to serum-free DME/F12 containing insulin and lactogenic hormones as above, and the cells were fed every 2 d.

mRNA Analysis

RNA was extracted from tissues and cultured cells by the guanidinium thiocyanate method as described (Sambrook et al., 1989). Polyadenylated RNA was isolated by adsorption to oligo d(T)-cellulose (Sambrook et al., 1989). Northern analysis for the presence of specific mRNAs was done as follows. Total RNA was electrophoresed in 1% agarose, 5% formaldehyde gels, followed by electrotransfer to nylon membranes (Genescreen or Hybond-N) and UV cross-linking. Equal loading was confirmed by comparing intensities of ethidium bromide-stained ribosomal RNAs. Membranes were probed with TGF-β1 (Derynck et al., 1986) or -β2 (Miller et al., 1989) specific cDNA fragments ^{32}P-radiolabeled by the random priming method (Boehringer Mannheim Corp., Indianapolis, IN) according to the manufacturer's directions. All hybridizations were performed under high stringency conditions (Derynck et al., 1988). Unhybridized probe was removed by washing membranes with 0.1× SSC, 0.2% SDS at 60°C.

Quantitation of TGF-β Secretion

The conditioned medium of cultured cells was collected and assayed for TGF-β-induced inhibition of proliferation of CCL64 mink lung cells with or without acid activation, as previously described (Arrick et al., 1990; Meager, 1991).

TGF-β1 Promoter-CAT Expression Plasmid

The 1,373-bp BamHI-SacII restriction fragment containing the TGF-β1 promoter and the first 11 bp of the long 5′ untranslated region (Kim et al., 1989) was isolated from the recombinant λ-phage βMA1, which carries the first exon of the human TGF-β1 precursor gene (Derynck et al., 1987). This restriction fragment was placed upstream of the sequence coding for the CAT coding sequence in such a way that the ATG initiator codon followed the SacII recognition site by only four basepairs: CCGCGG CAAA ATG. The sequence immediately preceding the ATG was designed to achieve an optimal translation efficiency (Kozak, 1989). This sequence context was introduced by site-directed mutagenesis on the template incorporated into M13 mp18 as described (Zoller and Smith, 1984). All sequences were determined using the dideoxy sequencing method on single-stranded templates (Sambrook et al., 1989). The final plasmid pβ1P-CAT incorporated the transcription unit linking the TGF-β1 promoter to the CAT coding sequence, in the same plasmid background as the plasmid S/A-CAT (Jakobovits et al., 1988).

Transformations and Selection

Rapidly growing CID-9 cells were transfected with pβ1P-CAT by the calcium phosphate procedure (Sambrook et al., 1989). 40 μg plasmid DNA

(and 4 μg pSV2neo DNA) were applied per 85-mm culture dish and stable transfected cells were selected using 400 μg G418/ml medium. After 4 wk, surviving clones were pooled and expanded. Cells were plated at equal density (6.2 × 10⁴ per cm²) on plastic culture dishes, on Engelbreth-Holm-Swarm (EHS) matrix, or on collagen I gels for various assays as described previously (Schmidhauser et al., 1990).

CAT Assays

To measure the CAT enzyme levels, as an indication of promoter activity, expressed from the TGF-β1 promoter in transfected cells cultured under different conditions, cells were harvested with dispase (40–60 min, 37°C), washed to remove dispase, lysed in 0.5% NP-40 and further solubilized by freeze thawing. To normalize the extracts, cell protein was quantified in a micro Bradford assay; excess EHS matrix which would interfere with the protein assay was removed efficiently by the dispase treatment. Aliquots representing equal amounts of total cell protein (10 μg) were incubated with ¹⁴C-chloramphenicol (0.1 μCi) and acetyl coenzyme A (70 μg) for 6 h at 37°C, extracted with ethyl acetate and separated by TLC. The levels of acetylated chloramphenicol were quantitated by scraping the radioactive spots from the TLC plate and counting in a scintillation counter (Sambrook et al., 1989). All measurements were done in duplicate and the difference between the duplicate values was <5%.
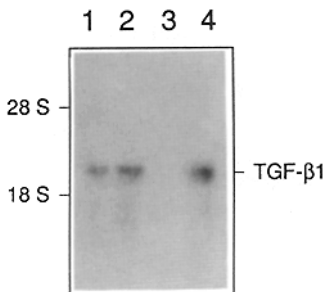
Protein Assays

To confirm that the cell lysates had been normalized correctly, 3 μg of the cell protein from CAT assay lysates were separated on reducing SDS-polyacrylamide gels and then stained with silver. Western blotting was performed by separating 1 μg of cell protein on reducing SDS-polyacrylamide gel, transferring the protein onto Immobilon-P transfer paper, and then probing the blot with an antibody that recognizes a spectrum of mouse caseins (Lee et al., 1985).
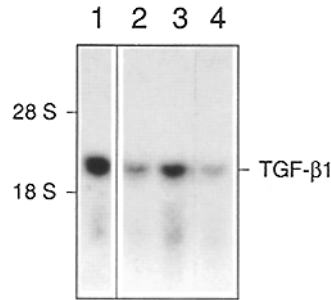
Results

ECM-dependent Regulation of TGF-β1 mRNA Levels

To assess whether TGF-β1 was expressed by mammary gland and whether its expression was developmentally regulated, the steady-state levels of mRNA from glands of CD-1 mice at different stages of the pregnancy cycle were compared by Northern analysis (Fig. 1). The presence of TGF-β1 mRNA was apparent in the mammary tissue of nonpregnant mice (Fig. 1, lane 1). During two periods of active tissue remodeling, i.e., during pregnancy (Fig. 1, lane 2) and gland involution after lactation (Fig. 1, lane 4), the level of TGF-β1 mRNA in the mammary gland was more abundant. In contrast, TGF-β1 mRNA levels were downregulated during lactation when milk is being synthesized; at this stage no TGF-β1 transcripts were detected in Northern blots of un-

Figure 2. TGF-β1 mRNA is upregulated in cells cultured on plastic dishes. RNA was prepared from midpregnant mammary gland (lane *1*), and from uncultured epithelial cells isolated from the gland (lane *2*). These epithelial cells were also plated onto plastic dishes (lane *3*) or EHS matrix (lane *4*) and cultured for 6 d before extracting total RNA. A Northern blot of these RNAs (10 μg) was then hybridized with a cDNA for human TGF-β1.

fractionated mRNA (Fig. 1, lane *3*). Such temporal variations during development of the mammary gland also occur with the expression of TGF-β2 and -β3 mRNA (Robinson et al., 1991).

The presence of TGF-β1 transcripts in mammary gland prompted us to ask whether any of the TGF-β species were expressed by primary epithelial cells in culture. The epithelial cell component of the mammary glands of mid-pregnant mice were separated from adipose and stromal tissue by collagenase digestion, followed by differential centrifugation (Lee et al., 1985; Emerman and Bissell, 1988; Streuli et al., 1991). TGF-β1 mRNA was clearly present in these uncultured epithelial cells (Fig. 2, lane *2*). The higher level of TGF-β1 mRNA from undigested gland tissue (Fig. 2, lane *2*) presumably reflects a significant contribution from the stromal tissue, which was removed during the epithelial cell isolation procedure. Experiments with epithelium-free mammary fat pads have confirmed that, indeed, the mammary stroma does express significant quantities of TGF-β1 during pregnancy (Robinson et al., 1991).

Polyadenylated mRNA was then prepared from cultured cells and was shown to contain TGF-β1 and TGF-β2 transcripts (Fig. 3). TGF-β3 was barely detectable (not shown). Because considerable phenotypic changes occur in mammary epithelial cells cultured on different substrata (Aggeler

Figure 1. The levels of TGF-β1 mRNA are modulated during the pregnancy cycle. Total RNA was extracted from the mammary glands of 8-wk-old virgin mice (lane *1*), and from the glands of 14.5-d-pregnant (lane *2*), 2-d-lactating (lane *3*), and 5-d-involuting mice (lane *4*). 10 μg RNA, separated in 1% agarose-formaldehyde gels, was transferred to nylon membranes and the resulting Northern blot was hybridized with a cDNA for human TGF-β1.

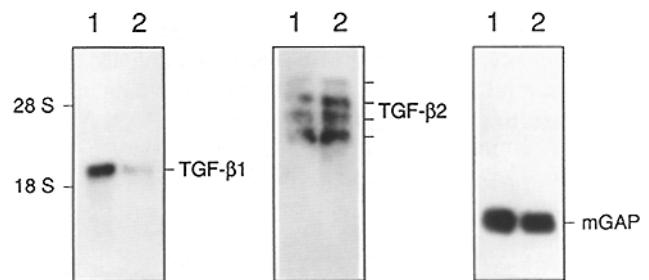*Figure 3.* Expression of TGF-β1, but not TGF-β2, is substratum dependent. Northern blots of polyadenylated mRNAs (10 μg) isolated from primary mouse mammary epithelial cells that had been cultured for 6 d either on plastic dishes (lanes *1*) or on EHS matrix (lanes *2*), were probed in parallel with radiolabeled cDNAs coding for human TGF-β1 and human TGF-β2. The four previously identified TGF-β2 transcripts were evident in mammary epithelial cells. A mouse glyceraldehyde phosphate dehydrogenase cDNA (mGAP) provided a suitable control for confirming equal loading of the two different mRNAs.

et al., 1991; Streuli and Bissell, 1990), we compared the steady-state levels of TGF-β mRNAs in cells plated on plastic dishes (Fig. 3, lanes 1) and on a basement membrane-rich matrix derived from EHS tumour (EHS matrix, or "Matrigel") (Fig. 3, lanes 2). When these cells were cultured on this matrix, TGF-β1 mRNA was drastically reduced in comparison with cells on plastic (Fig. 3). The level of TGF-β2 mRNA, on the other hand, was unchanged.

Measurement of TGF-β protein levels by a sensitive bioassay was frequently impossible due to the presence of an inhibitor of the assay in the conditioned medium (data not shown). In addition, antibody-based TGF-β assays did not provide sufficient sensitivity to measure TGF-β levels under our conditions. However, when only low levels of this inhibitor were apparent, it was clear that the levels of total TGF-β (thus including both TGF-β1 and -β2) was significantly higher when the cells were cultured on plastic, compared with cells cultured on ECM. As an example, after 3 d the concentration of secreted TGF-β in the conditioned medium was 2.1 ng/ml, when grown on ECM, versus 3.1 ng/ml from cells on plastic. Between d 3 and 5, the cells on ECM secreted 1.3 ng/ml TGF-β, whereas cells on plastic produced 5.7 ng/ml TGF-β. In yet another experiment, the level of TGF-β expressed by the cells on plastic was 2.1-fold higher than the TGF-β secreted by the cells on EHS matrix. Thus, although it was not possible to discriminate between both TGF-β isoform protein levels, our mRNA and protein results do demonstrate that these different substrata have dramatic and specific effects on the expression of TGF-β1, but not TGF-β2 mRNAs. We know from previous studies (Li et al., 1987; Barcellos-Hoff et al., 1989), which are also confirmed here (see below), that milk protein expression is induced on the basement membrane matrix and inhibited on plastic. Functional differentiation into polarized milk secreting cells and TGF-β1 expression are therefore regulated in opposite directions.

To ask whether culturing the cells on plastic induced TGF-β1 expression or whether ECM inhibited expression, we compared TGF-β1 mRNA levels in isolated mammary epithelial cells before cell culture with those cultured on either plastic or on EHS matrix (Fig. 2, lanes 3 and 4, respectively). The TGF-β1 mRNA level in the cells cultured on EHS matrix was similar to that in uncultured cells and was not inhibited. However, the much higher TGF-β1 mRNA level in cells cultured on plastic stands in sharp contrast with that in uncultured cells. Thus the absence of a suitable ECM as substratum resulted in an increased TGF-β1 expression, whereas culture of the epithelial cells on the basement membrane-rich EHS matrix maintained the TGF-β1 expression at similar levels to those seen in vivo.

ECM Regulates TGF-β1 Expression at the Transcriptional Level

To evaluate whether this substratum dependence was a result of transcriptional regulation, we measured the expression of an easily assayable reporter gene under the control of the TGF-β1 promoter. We isolated a restriction fragment containing the TGF-β1 promoter (Kim et al., 1989) from a recombinant phage containing the first exon of the human TGF-β1 gene (Derynck et al., 1987). Previous studies have shown a high degree of sequence conservation between the human and mouse TGF-β1 promoters and especially their
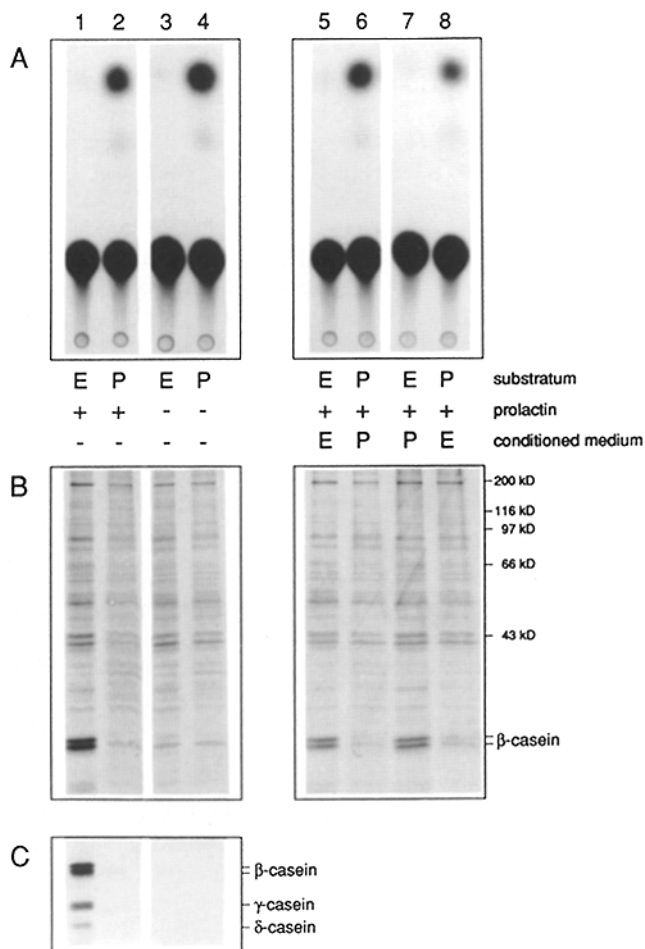
putative regulatory sequences (Geiser et al., 1991). The 1,373-bp BamHI-SacII fragment containing the promoter region and the first 11 bp of the 5' untranslated region was linked upstream from the sequence encoding chloramphenicol acetyltransferase (CAT). The resulting expression plasmid TGF-β1P-CAT containing the TGF-β1 promoter and 5' untranslated sequence fused to the CAT coding sequence was then transfected into the mammary epithelial cell line CID-9, which has been shown to behave and differentiate very similarly to freshly isolated primary mammary epithelial cells (Schmidhauser et al., 1990). In addition, these cells also displayed a downregulated TGF-β1 mRNA expression when cultured on basement membrane (data not shown). Cotransfection of the TGF-β1 promoter plasmid with a neomycin-resistance encoding plasmid pSV2-Neo allowed the selection and generation of stable transfected cell clones, that contained integrated pTGF-β1P-CAT sequences.

The stable transfected cell lines were then cultured on plastic culture dishes for 6 d and compared with those on EHS matrix. The levels of accumulated CAT enzyme, expressed from the TGF-β1 promoter, were quantitated using the standard assay for ^{14}C-chloramphenicol conversion (Fig. 4, lanes 1 and 2). There was a considerable difference in the CAT enzyme levels (10–13 times more CAT enzyme in cells cultured on plastic), and thus in the TGF-β1 promoter activity, in the cells cultured on the two types of substrata. The lack of extracellular matrix therefore results in a dramatic increase of transcription from the TGF-β1 promoter, and the difference in TGF-β1 mRNA levels between cells cultured on plastic and ECM is due largely to differences in transcriptional activity.

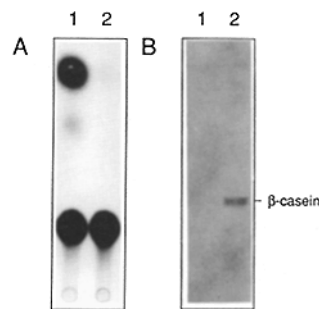## Basement Membrane Itself Provides Signals to Regulate TGF-β1 Expression

The pTGF-β1P-CAT plasmid transfected CID-9 cells used above had been cultured so far in the presence of lactogenic hormones, prolactin and hydrocortisone. However, the combined presence of both these components and EHS matrix results in the acquisition of a differentiated phenotype characteristic of mammary epithelial cells in vivo. A marker of this differentiated phenotype is the expression of caseins which constitute the major proteins in milk. To examine whether the low expression from the TGF-β1 promoter is related to functional differentiation or is solely the result of cultivation on ECM, the transfected cells were cultured on plastic or on ECM in the absence of prolactin and hydrocortisone (Fig. 4, lanes 3 and 4). There was no functional differentiation under these conditions, as assessed by the absence of caseins. However, CAT enzyme accumulated to similar levels in either the presence or absence of the lactogenic hormones. Thus, the difference in transcription from the TGF-β1 promoter is not linked to the acquisition of differentiation-specific functions per se, but is solely a response to the nature of the substratum.

An explanation for the effect of the substrata might be that the mammary epithelial cells on ECM or plastic secrete a factor that influences the level of TGF-β1 promoter activity. It is for example known that conditioned medium from mammary cells cultured on plastic suppresses the synthesis of one of the milk proteins, whey acidic protein (Chen and Bissell, 1989). A simple experiment was therefore performed to see whether secreted factors were responsible for the observed

Figure 5. TGF-β promoter activity is downregulated on floating collagen I gels. pβ1P-CAT-transfected cells were cultured on thick collagen I gels for 6 d. The gels either were left attached to the culture dish (lanes *1*) or were floated into the medium after 2 d (lanes *2*). Total cell protein was then separated for (*A*) CAT assays of TGF-β promoter activity, or (*B*) Western blotting with a casein-specific antibody. In the experiment in *A*, the levels of acetylated chloramphenicol expressed as cpm per μg cell protein per min enzymatic reaction time (total reaction time was 6 h) were as follows: (lane *1*) 7.76; (lane *2*) 0.28.

Figure 4. ECM controls the activity of the TGF-β1 promoter. Pooled clones of CID-9 cells, stably transfected with pβ1P-CAT, were plated at equal density (6.2 × 10⁴ per cm²) on plastic culture dishes (lanes *2, 4, 6,* and *8*) or on EHS matrix (lanes *1, 3, 5,* and *7*). The cells were then cultured either with (lanes *1, 2,* and *5-8*) or without (lanes *3* and *4*) prolactin. In one experiment (lanes *5-8*), medium conditioned from cells cultured on plastic dishes or on EHS matrix was added for 4 d; this medium was either added back to the homologous dishes (lanes *5* and *6*) or to the converse culture conditions (lanes *7* and *8*). (*A*) CAT assays of TGF-β1 promoter activity in the cells cultured under these different conditions. In this experiment the levels of acetylated chloramphenicol expressed as cpm per μg cell protein per min enzymatic reaction time (total reaction time was 6 h) were as follows: (lane *1*) 0.21; (lane *2*) 2.09; (lane *3*) 0.26; (lane *4*) 3.27; (lane *5*) 0.24; (lane *6*) 2.20; (lane *7*) 0.16; and (lane *8*) 1.54. (*B*) Cell proteins separated by polyacrylamide gel electrophoresis and stained with silver confirmed that the cell lysates indeed had been normalized correctly, and showed that only on the EHS matrix and only in the presence of prolactin (lanes *1, 5,* and *7*) were substantial amounts of β-casein synthesized. (*C*) That these bands were actually casein was shown by Western blotting 1 μg of cell protein onto Immobilon-P transfer paper, and then probing the blot with an antibody that recognizes a spectrum of mouse caseins.

modulation of TGF-β1 expression. The transfected CID-9 cells were cultured in the presence of conditioned medium from cells on either plastic dishes or the basement membrane EHS matrix in the presence of lactogenic hormones (Fig. 4, lanes *5-8*). CAT assays and parallel casein assays

showed that the TGF-β1 promoter activity was not affected by these different conditioned media, and, thus, did not depend on the presence of secreted soluble factors.

Formation of an Endogenous Basement Membrane Downregulates TGF-β1 Promoter Activity

We have shown previously that the differentiation of mammary cells that occurs as a result of cultivation on a floating type I collagen substratum, correlates with the formation of an endogenous basement membrane (Streuli and Bissell, 1990). We therefore evaluated the influence of collagen type I matrix on TGF-β1 promoter activity in the presence of lactogenic hormones. If downregulation of TGF-β1 expression on EHS matrix is due to the presence of basement membrane components, transcription from the TGF-β1 promoter should be reduced after flotation of the collagen I gel. On attached collagen I gels, CID-9 cells behave similarly to those on plastic; in addition, they do not differentiate and do not synthesize a basement membrane (Streuli and Bissell, 1990). This is in contrast to their behavior on floating collagen I gels, where the cells contract the gel and synthesize a basement membrane, and, as a result of these changes, differentiate and secrete milk proteins. Under these conditions, the formation of a basement membrane can be demonstrated by immunostaining. The transfected CID-9 cells were therefore cultured on attached or floating collagen I gels and the CAT activities directed from the TGF-β1 promoter were measured. The cells on attached collagen gels displayed a high level of promoter activity, analogous to cells on plastic, whereas on the floating gels, the cells exhibited a greatly suppressed promoter activity as seen with cells on EHS matrix (Fig. 5). Thus, as with an exogenous basement membrane, the presence of an endogenous basement membrane suppresses the transcription from the TGF-β1 promoter. Since we have already shown that such suppression of the promoter is not dependent on the differentiated phenotype, we can conclude that the basement membrane itself provides signals to regulate TGF-β1 transcription.

Discussion

In this study, we have established that expression of the TGF-β1 gene is regulated negatively by extracellular matrix. Transcription from the TGF-β1 promoter is high in the absence

of ECM and is considerably lower in the presence of EHS matrix or an endogenously synthesized basement membrane. The much lower TGF-β1 promoter activity in the presence of basement membrane is not a result of differentiation per se, since inhibition of functional differentiation (by removing lactogenic hormones) does not alter TGF-β promoter activity. This type of regulation of TGF-β expression is specific for TGF-β1 since the level of TGF-β2 mRNA in the same epithelial cells is not affected by the absence of ECM.

An evaluation of the available data on TGF-β1 expression by different cell types reveals a major discrepancy between the TGF-β1 synthesis in vivo and in cell culture. In vivo, only a defined subset of cell populations expresses TGF-β1 (Wilcox and Derynck, 1988; Thompson et al., 1989; Fitzpatrick et al., 1990; Gatherer et al., 1990; Millan et al., 1990; Pelton et al., 1990a,b, 1991; Schmid et al., 1990), whereas in culture its expression is virtually ubiquitous. On the basis of our current data, we believe that this discrepancy can be explained by the presence or absence of basement membrane, since the absence of ECM results in a significant increase in TGF-β1 expression. There is considerable evidence that TGF-β is able to induce the synthesis of various ECM proteins (Massagué, 1990; Roberts and Sporn, 1990; Kahari et al., 1991). Also mammary cells cultured on plastic substratum produce high levels of ECM proteins (Streuli and Bissell, 1990), which may be a result of the high level of TGF-β1 expression. Thus, the absence of ECM induces TGF-β1 expression which in turn stimulates the synthesis of ECM proteins in an autocrine fashion. We have now documented that, once the ECM is deposited, TGF-β1 gene expression is strongly downregulated. This may explain the base-line expression of ECM proteins we observe when cells synthesize an endogenous basement membrane (Streuli and Bissell, 1990). It is thus possible that some type of ECM-induced negative feedback loop regulates TGF-β1 expression.

Our results also indicate that caution has to be exercised when evaluating the physiological relevance of TGF-β1 synthesis using cells cultured in the absence of basement membrane, i.e., the commonly used cell culture conditions. The expression of various ECM proteins and proteins involved in cell-matrix interactions, such as proteases, protease inhibitors and integrins, is strongly modulated by TGF-β. It is thus conceivable that their expression levels may be dependent upon the autocrine control of endogenously synthesized TGF-β, as we have recently demonstrated for a TGF-β1 overexpressing tumor cell line (Arrick et al., 1992). Thus, the enhanced TGF-β1 synthesis in the absence of ECM may also affect the levels of synthesis of these proteins under autocrine control of TGF-β, resulting in expression levels which are not representative of the physiological conditions in vivo.

We have shown that the presence of EHS matrix, a basement membrane preparation, and an endogenously synthesized basement membrane keep the TGF-β1 in an uninduced state, while a type I collagen matrix by itself does not downregulate TGF-β1 expression. Which component of the basement membrane affects TGF-β1 expression is unknown at present. It is, however, likely that the responsible factor must be deposited in an insoluble form in the basement membrane, since the conditioned medium from cells on plastic, which contains a variety of soluble ECM proteins, did not affect the TGF-β1 promoter activity. However, if a simple

negative feedback mechanism exists, it is likely that an ECM component whose expression is under control of TGF-β is responsible for the downregulation of TGF-β1 expression. The mechanism by which the cell recognizes the absence or the presence of ECM is presumably based on signaling through cell surface receptors for the appropriate matrix components. Occupation of the receptors would then result in a repressed transcriptional activity, whereas the TGF-β1 transcription would be induced in the absence of the ligand.

It has been established already that ECM components can influence gene expression. Our previous work using the same mammary epithelial cells has shown that cell-ECM interactions in the absence of cell-cell interaction and polarity is sufficient to induce expression of β-casein and that this induction of gene expression requires a signal transfer mediated through integrins (Streuli et al., 1991). In addition, interaction between the cells and the ECM, and more explicitly integrin-mediated interaction with fibronectin fragments, induces collagenase and stromelysin gene expression (Werb et al., 1989). Our current study indicates that basement membrane has the ability to modulate the transcription of TGF-β1, a factor that determines the cell-matrix interaction. On the basis of this finding, it would be no surprise if the expression of other regulators of cell-matrix interactions, such as the bone morphogenetic proteins or other members of the TGF-β superfamily, also would be modulated by cell-ECM interactions.

The transcription and mRNA levels of TGF-β1, -β2, and -β3 are differentially regulated and this is presumably related to their different promoter structures. Since the expression of TGF-β1, and not TGF-β2, is strongly affected by ECM, it is likely that this specific modulation is related to the presence of a TGF-β1 specific promoter element that is absent in the TGF-β2 promoter, e.g., an AP-1 site (Malipiero et al., 1990). We have recently demonstrated the presence of an ECM-responsive enhancer in the 5' region of the β casein promoter (Schmidhauser et al., 1992). The best inducer of TGF-β2 and -β3 currently known is retinoic acid (Glick et al., 1989). Whereas TGF-β2 mRNA levels can be enhanced up to 10-30-fold by retinoic acid, the level of TGF-β1 mRNA is increased only 1.5-3-fold. These studies, however, were performed using cells cultured on plastic substratum, resulting in an already high level of TGF-β1 transcription in untreated cultures; it is therefore possible that a greater difference could be observed if cells were cultured on ECM. The only dramatic induction of TGF-β1 mRNA expression reported so far has been the auto-induction of TGF-β1 by TGF-β itself (Van Obberghen-Schilling et al., 1988; Bascom et al., 1989). We now show that a dramatic difference in TGF-β expression can be achieved also by the absence or presence of ECM. These findings suggest that both the levels of active TGF-β1, as well as the cell-ECM interactions may be major determinants of the rates of TGF-β1 synthesis by epithelial and other cells in vivo.

The regulation of TGF-β1 and not TGF-β2 expression by ECM suggests that these two TGF-β forms have different normal physiological roles in vivo. The regulation of TGF-β2 by retinoids (and steroids) suggests that TGF-β2 may play a role in various differentiation and morphogenetic processes during development. On the other hand, TGF-β1 expression in vivo may be important in the deposition and establishment of basement membrane and extracellular matrix. In addi-

tion, auto-induction of TGF-β1 expression suggests a role in localized amplification of the TGF-β1 effects. Thus, expression of TGF-β1 would be upregulated whenever the interaction of the cells with the extracellular matrix or basement membrane is disturbed, e.g., following wounding (Sieweke et al., 1990).

In conclusion, our data show that the expression of the TGF-β1 gene, but not the TGF-β2 gene, is strongly affected by the absence or presence of ECM and suggests a possible feedback loop in vivo, whereby the loss of of the proper cellular interactions with the ECM induces TGF-β1 expression. TGF-β1 then in turn induces the synthesis of several ECM proteins. When conditions are right for the ECM components to come together to form a basement membrane, TGF-β1 expression is suppressed. Further studies will be needed to further define this regulation at the molecular level and to understand its relevance in vivo.

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