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Metallothionein Promotes Laminin-1-induced Acinar Differentiation *in Vitro* and Reduces Tumor Growth *in Vivo*[1]

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ABSTRACT

Laminin-1 was found previously to promote the morphological differentiation of a salivary gland cell line (HSG) into acinar-like structures with polarized nuclei (1). Here, microarray analysis showed that laminin-1 induced mainly one gene family of proteins, the metal binding metallothioneins (MTs), out of more than 10,500 cDNAs screened. Northern and protein analyses demonstrated that MT was increased some 5–10-fold by laminin-1 as early as 6 h after incubation. Cells transfected with this gene formed 3–5-fold larger acinar-like structures on exposure to laminin-1 *in vitro* and smaller, more differentiated tumors *in vivo*. We conclude that MTs are important in acinar cell morphological differentiation and may have novel functions other than metal binding.

INTRODUCTION

Laminins are a family of basement membrane glycoproteins important for epithelial and endothelial cell adhesion, migration, growth, and differentiation (1). At least 15 different isoforms have been described to date, but the activity of laminin-1 has been most characterized. Laminin-1 promotes the morphological differentiation of a human salivary gland cell line (HSG), which was derived from a human submandibular tumor (2). When cultured on laminin-1 gels, HSG cells form acinar-like structures with polarized nuclei within 24–48 h, whereas the cells grown in its absence form a monolayer. Such acini are also formed in microgravity (suspension) culture in the presence of laminin-1, whereas in its absence, undifferentiated cell aggregates are observed. A biologically active site in the carboxyl G4 domain of the α 1 chain of laminin-1 that contributes to this morphological differentiation has been identified, AG73, arg-lys-arg-leu-glu-val-glu-leu-ser-ile-arg-thr. When peptide AG73 was added to either standard or microgravity culture, it promoted acinar-like formation, but the cells were not fully polarized. It is likely that other sites on laminin-1 act together with this site to promote acinar differentiation. The cell surface receptor for this sequence was identified as syndecan-1, a cell surface heparan sulfate-containing proteoglycan (2). On the basis of these findings, we propose that multiple sites on laminin-1 contribute to the morphological differentiation of these cells.

Whereas we are beginning to understand that the interactions at the cell surface are important in salivary gland cell differentiation, we know very little about the genes involved. Here, we have used gene profiling to identify those that are important for laminin-1-induced salivary gland cell acinar differentiation. Surprisingly, mainly one gene family, MT,[3] was found to be elevated by HSG cells cultured in the presence of laminin-1. MTs are a group of low molecular weight intracellular proteins that contain high numbers of cysteinyl residues but no aromatic amino acids or histidine. MTs have high affinity for essential metals, such as zinc and copper, as well as nonessential metals, including cadmium and mercury, and might, therefore, serve in the intracellular storage, transport, and metabolism of essential metals (3).

Immunohistochemical studies have shown that expression of the MT gene appears early in mammalian development (4), with immunoreactivity found in fetal liver, small intestine, pancreas, and kidney (5–7). Most of the functional MT genes in higher eukaryotes can be induced by metal ions (8) via metal-responsive elements. MT gene expression is also controlled by glucocorticoids, various cytokines, and growth factors (9). Despite detailed knowledge of the factors that induce MT, it has been difficult to discern a specific biological role for the protein.

Recently, it has been postulated that metalloregulatory functions are important for cell proliferation and differentiation (10, 11). Here we have found that expression of this gene was elevated in HSG cells at both the mRNA and protein levels by laminin-1. Furthermore, cells transfected with MT formed larger acini when cultured in the presence of laminin-1 *in vitro* and smaller, more differentiated tumors when injected s.c. in mice. We conclude that MTs may have additional functions in salivary gland cells beyond metal binding and may be important in cell differentiation. Alternatively, metal binding may be involved in pathways necessary for morphological differentiation.

MATERIALS AND METHODS

Cells and Culture. HSG cells (12) were cultured in DMEM/Ham's F-12 (1:1) containing 5% fetal bovine serum (Biofluids, Rockville, MD), 100 units/ml penicillin, and 100 μg/ml streptomycin as described previously (2). Acinar formation was achieved in the presence of laminin-1 either on the culture dish (2–6 mg/ml, 200 μl/well in a 48 well dish; Ref. 13) or in the rotary culture system with high aspect ratio vessel bioreactors (Synthecon, Houston, TX; 0.2 mg/10 ml; Ref. 2) for the indicated times. After culture, acinar size was quantified using the Metamorph software program (Universal Imaging Corporation).

Gene Profiling. Cells, cultured with or without laminin-1, were harvested at the indicated times, washed once with PBS, and pelleted. Polyadenylated RNA was extracted using FastTrak polyadenylated RNA extraction kit (Invitrogen, San Diego, CA). mRNA was radiolabeled and hybridized to the cDNA filters containing a total of 10,000 known and unknown genes (GF200-204; Research Genetics,) according to the manufacturer's instructions. After hybridization, the membranes were exposed in a PhosphorImager (Molecular Dynamics, Piscataway, NJ). Each membrane was then stripped and reused in a second experiment with RNA prepared from a new experiment and applied to opposite filters. Each condition (minus and plus laminin) was analyzed several times with different filters. The PhosphorImager results were quantified and analyzed using the P-SCAN program (NIH; Ref. 14). Filters from Clontech containing 588 known genes were also analyzed but no changes in genes were found.

Northern Analysis. Cells cultured with or without laminin-1 were harvested at the indicated times and washed once with PBS. Total RNA was extracted using RNeasy extraction kit (Qiagen, Santa Clara, CA). Five μg of RNA were separated on 1% agarose formaldehyde gels, transferred to nytran membranes (Shleicher and Schuel, Keene, NH), and probed with MT-1f or other selected cDNAs labeled by random priming (Prime-it II; Stratagene, La Jolla, CA).

Cloning and Transfection of MT-1f. Full-length human MT-1f cDNA (clone no. 460955) was obtained from IMAGE Consortium. The cDNA was

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[3] The abbreviations used are: MT, metallothionein.

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amplified using the oligonucleotide primers MT5Eco (CGGAATTCCGGCT-TGCAATGGACCCC) and MT3Eco (CGGAATTCCGGCATCAGTCGCAG-CAGC), which contain *Eco*R1 or *Bam*H1 sites. The PCR product was digested with *Eco*R1/*Bam*H1 and cloned into pRK-mc plasmid (a generous gift from Dr. K. Yamada, National Institute of Dental and Craniofacial Research, NIH, Bethesda, MD) to express MT-1f protein with the myc-tag fused to its NH_2 terminus. HSG cells were transfected by electroporation (0.17 V, 960 μF) with pRK-MycMT plasmid together with pCMV Script plasmid. Clones were selected by neomycin resistance and tested for MT expression by direct ELISA on cells, using anti-myc antibodies. The cells were fixed with 3.7% formaldehyde in PBS (room temperature, 15 min), permeabilized with Triton for 5 min at room temperature, blocked with 3% BSA, reacted with the primary and secondary antibodies, and detected with the chemiluminescence ELISA reagent. Positive clones were also immunostained using both anti-myc and anti-MT antibodies (Dako, Carpinteria, CA) to verify the expression homogeneity of the clone. Cells and acini were also viewed by confocal microscopy after staining with the MT antibody.

Characterization of MT-expressing Cells. Mock- and MT-transfected cells were tested for proliferation in 96-well plates using the MTS assay (Promega, Madison, WI). Cell viability was also analyzed in the absence or presence of various concentrations of metal ions (0.1–0.6 mM $CuCl_2$ and 0.05–0.2 mM $ZnCl_2$). Cells were cultured on laminin-1 gels (2–6 mg/ml will form a lose gel at 37°C, 1 ml/25-mm dish) and the size of the acinar-like structures was measured as described above.

Tumor Growth. Mock and MT-transfected cells were injected s.c. in the upper dorsal area of athymic nude mice using 10^6 cells in 0.5 ml of either PBS or Matrigel (Collaborative Biomedical, Bedford, MA). Four mice were used for each cell line, and the experiment was carried out three times with similar results. Tumors were measured with calipers (l × w × h) at various times, and tumor weights were determined at the time of sacrifice, approximately 4–6 weeks after injection. Tumors were fixed in PBS-formalin, sectioned, and stained with H&E. A blinded observer trained in pathology analyzed the tumor histology slides from both experiments.

RESULTS

Identification of Laminin-1-induced Genes. Gene expression was studied using HSG cells that were shown to differentiate into acinar-like structures when cultured on a laminin-1 gel (2). Four conditions were analyzed including cells at 6 and 18 h cultured on either plastic or laminin-1 gel-coated plastic, and cells at 6 and 18 h in the absence and presence of laminin-1 in solution in microgravity (2). In the four different culture conditions analyzed, only a limited number of genes were differentially expressed out of the >10,000 cDNAs present on the commercially available microarrays (Table 1). We analyzed four different cell cultures at 6 h and three additional ones at 18 h. Because of the multiple analyses, many false positives were eliminated. Acinar formation on the laminin-1 gel is observed after 12–18 h, whereas no differentiation is observed on plastic alone (Fig. 1, *A* and *B*). HSG cells were also cultured under microgravity

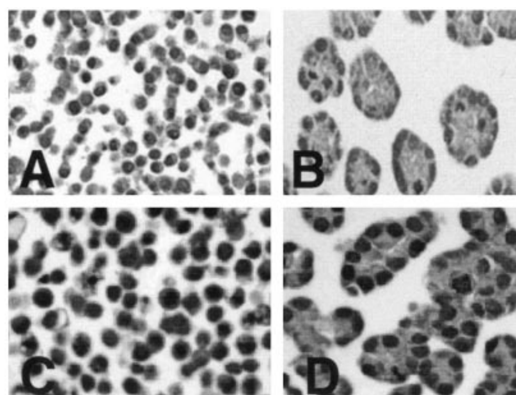

Fig. 1. Appearance of HSG cells after 18 h of culture. *A,* cells on plastic. *B,* cells on laminin-1 gel. *C,* cells in bioreactor. *D,* cells in bioreactor with laminin-1. *A* and *B* are ×20. *C* and *D* are ×32.

conditions in a rotary cell culture bioreactor to eliminate the effects on gene expression of cell attachment and spreading on plastic. Cells cultured for 18 h in the bioreactor without laminin-1 remained mainly as a single cell suspension (Fig. 1*C*). However, in the presence of laminin-1, morphological differentiation, as determined by acinar formation, was observed after 18 h (Fig. 1*D*; Ref. 2). Differential gene expression was analyzed after 6 h to identify genes that may regulate this differentiation process. Laminin-1-induced cDNAs encoded for two members of the MT gene family, *MT-If* and *MT-Ib*, in these 6-h cultures (Table 1). At 18 h in the presence of laminin-1, small acini were observed, and a third member of the MT gene family, *MT-II*, was induced. Because three different MTs were induced by laminin-1, we focused our subsequent studies on these genes.

MT Expression Levels in HSG Cells. Northern blot analysis was used to validate the array results. MT-If expression was increased at least 5-fold in HSG cells cultured with laminin-1 for 6 or 15 h in the bioreactor and for 6 h in standard tissue culture conditions (Fig. 2). This increase was observed consistently by Northern blot analysis although changes in *MT-If* expression had not been observed on the arrays. Elevated levels of MT immunostaining with an antibody that detects both MT-I and -II were observed when cells were exposed to laminin-1. This antibody specifically stains HSG acini formed in the presence of laminin-1 (Fig. 3, *B* and *C*), and little or no background levels were observed with those cells cultured without laminin-1 on either plastic or in the bioreactor (Fig. 3, *A* and *C,* respectively). MT immunostaining was mainly localized to the cytoplasm of the cells in the acini (Fig. 3, *B* and *D*). These data suggest that laminin-1 induces both the mRNA and protein levels of MT in HSG cells.

Overexpression of MT in HSG Cells. The induction of MT expression by laminin-1 suggested that MT may play a role in laminin-1-dependent cell differentiation. Therefore, we overexpressed *MT-If* in these cells. Overexpressed clones were selected based on protein levels as detected by direct ELISA on the cells, and the increased expression was confirmed by immunostaining. We chose to characterize two clones that showed 100% expression of MT. MT mRNA levels in these clones were 5–8-fold higher when compared with parental or mock-transfected cells. Cell proliferation in the parental, mock-transfected, and MT-overexpressing cells was not altered (data not shown). We additionally tested the ability of the cells to grow in the presence of heavy metals. Adding low concentrations of copper and zinc to the culture medium promoted cell growth. However, high amounts were toxic to the cells. Parental and mock-transfected cells were more sensitive to copper than the MT-transfected cells (IC_{50} equal to 0.14 and 0.36 mM, respectively). No difference was observed in the sensitivity to zinc.

Table 1 *Differentially expressed cDNAs*

Condition	cDNA id	Accession no.	Identity
6h, bioreactor +ln[a]	245990	N55459	MT 1-F
	232772	H72722	MT 10B/L
18 h, bioreactor	770887	AA34416	ESTs similar to BAT3
	29054	R40850	α-centractin
	811091	AA485673	ESTs
18h, bioreactor +ln	129585	R16596	EST similar to MT
6h, plastic	46415	H09167	ESTs
	813536	AA455605	P13k
	154465	R54850	Related to biphenyl hydrolase
6h, plastic +ln	741067	AA478436	SWI/SNF complex 60 Kda
18h, plastic +ln	295923	N73536	ESTs
18h	241658	H89843	ESTs
18h	305554	N90217	ESTs

[a] ln, laminin-1; ESTs, unknown genes; P13k, phosphotidylinositol 3′-kinase; SWI/SNF, multisubunit protein complex that regulates chromatin assembly; BAT3, HLA-B associated transcript 3, function unknown.

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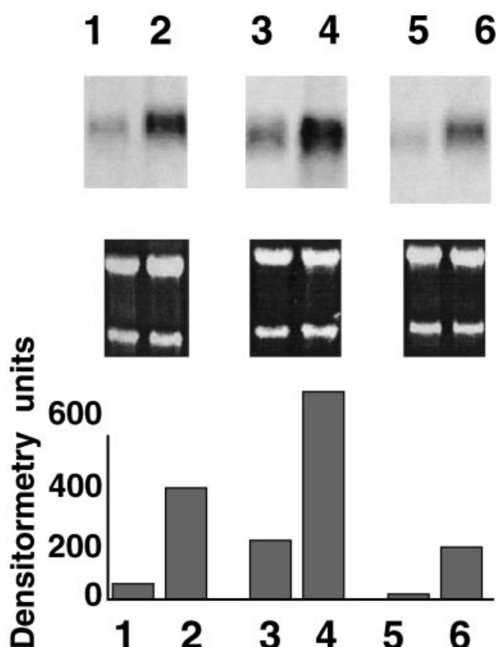

Fig. 2. Northern blot analysis for MT-1f mRNA levels in HSG cells in the absence or presence of laminin-1. Cells were cultured for 6 h (*Lanes 1, 2, 5,* and *6*) in either the bioreactor (*Lanes 1* and *2*) or in standard culture conditions (*Lanes 5* and *6*). Cells were also analyzed at 15 h in the bioreactor (*Lanes 3* and *4*). Laminin-1 was present in cultures for which the mRNA levels are shown in *Lanes 2*, *4,* and *6* as indicated and resulted in increased mRNA levels. The *bottom panel* shows the quantitation of the bands after normalization for loading of RNA, which is shown in the *middle panel*.

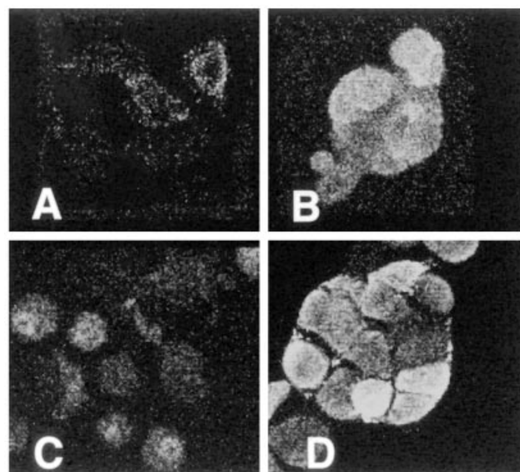

Fig. 3. MT expression in HSG cells transfected with MT and cultured in the presence of laminin-1 for 18 h. HSG cells were cultured on plastic (*A* and *B*) or in the bioreactor (*C* and *D*) in the presence (*B* and *D*) or absence (*A* and *C*) of laminin-1. Laminin-1 induced the expression of MT based on immunostaining of the cells.

MT Expression Induces Morphological Changes in HSG Cells. When cultured as a monolayer, MT-overexpressing HSG cells lose their cuboidal shape and spread more than the parental cells (data not shown). Cells also grew in aggregates and were hard to separate into a single cell suspension without trypsin treatment. When plated at low cell density on plastic, the MT-expressing cells were well spread and appeared larger than the parental cells. We next tested the ability of these cells to form acini in the presence of laminin-1. With parental or mock-transfected HSG cells, acinar-like structures were usually observed only after 12–18 h in the presence of laminin-1 (2), whereas acinar-like structures were already observed after 6–8 h with the MT-overexpressing cells. The overexpressing cells formed 3–5-fold larger acini at 24 h than mock-transfected cells (Fig. 4). These results suggest a role for MT in the morphological differentiation of HSG cells.

MT Expression Decreases Tumor Size in Nude Mice. The effect of MT expression on HSG cell growth and differentiation *in vivo* was determined. HSG cells are tumorigenic and were injected s.c. into nude mice either as a single cell suspension or mixed with basement membrane (Matrigel), which is known to facilitate tumor growth (15). In the presence of Matrigel, tumor growth was accelerated, and tumors were detected 1 week after injection. Without Matrigel, tumor formation was observed 3–4 weeks after injection. In both cases, MT-transfected cells formed smaller tumors as measured by tumor weight after harvesting (Table 2) or by tumor volume (not shown). The MT-transfected tumors were more morphologically differentiated than the control tumors (Fig. 5) and could be classified as an adenocarcinoma. When MT-expressing cells were coinjected with Matrigel, round, acinar-like structures formed, mainly at the tumor edge. No clear morphology was observed with the poorly differentiated control tumors. In the control tumors, the nuclei were enlarged (with less cytoplasm), and more mitotic cells were observed than in the MT-transfected tumors. In the control tumors, the cells aggregated into less organized nests, but no nuclear alignment was observed. Taken together, these results demonstrate that MT expression promotes a more differentiated morphology of HSG cells *in vitro* and *in vivo,* and reduces tumor growth *in vivo*.

DISCUSSION

We have demonstrated previously that laminin-1 plays a role in the morphological differentiation of HSG cells into acinar-like structures (2). Here, we identify MTs as the major laminin-1-induced genes, which may play a role in acinar formation. Several isoforms of MT were induced by laminin-1 as detected by microarrays. This induction

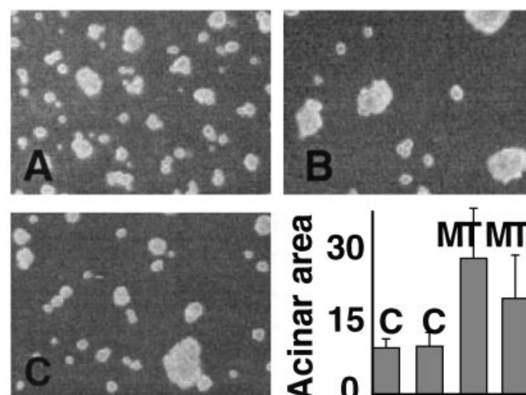

Fig. 4. MT transfection increases acinar size in the presence of laminin-1. *A,* control (PMCV). *B,* transfected clone MT5. *C,* transfected clone MT15. Shown after 24 h in the presence of laminin-1. Quantitation of acinar size of two control mock-transfected clones and two MT-transfected clones was performed at 24 h of culture in the presence of laminin-1; *bars,* ± SD.

Table 2 *Tumor weights of mock and MT transfected cells*

Cells injected alone formed smaller tumors than those coinjected with Matrigel. Cells injected alone were harvested at 7 weeks while those coinjected with Matrigel were harvested at 3 weeks. In both cases, the MT-transfected cells formed smaller more differentiated tumors.

	Tumor weight (gm)	
	Cells alone	Cells + Matrigel
Control (mock)	0.70 ± 0.15	0.70 ± 0.09
MT-transfected	0.23 ± 0.07	0.44 ± 0.06

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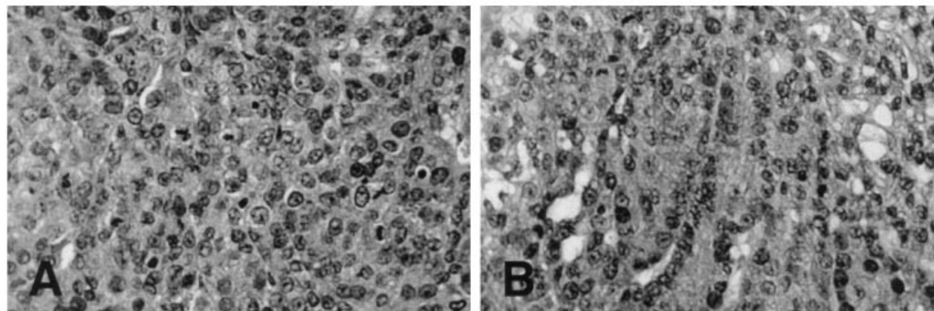

Fig. 5. Effect of MT transfection on the histological appearance of the tumors. Mock (PCMV) and transfected (MT5) cells were injected in the presence or absence of Matrigel. *A,* histology from the control tumor; *B,* histology from the tumor formed from transfected cells. The MT-transfected cells form smaller, more differentiated tumors.

was additionally verified by Northern blot analysis and protein immunostaining. At least a 5-fold increase in the mRNA expression of *MT-1f* and *MT-1b* was observed after a relatively short incubation with laminin-1 (6 h), and after a longer time period (15 h), the level of another member of this gene family, *MT-II*, was increased 5–10-fold. Immunostaining of the acini formed by HSG cells cultured with laminin-1 showed an increase in the MT proteins. These results suggest that MTs may play a role in laminin-dependent differentiation of HSG cells. Acinar formation can serve as a model for the differentiation of the salivary gland. Immunostaining of adult mouse salivary glands with anti-MT antibodies revealed specific staining of the ductal but not acinar cells (not shown). MT expression was demonstrated previously (16) in ductal basal cells and in myoepithelial cells of the normal salivary gland. It is possible that HSG cells in the presence of laminin-1 are actually forming ductal-alveolar structures.

HSG cells were cultured in the presence of laminin-1 both in conventional culture and in microgravity. We were concerned that the faster adherence of the cells to laminin-1 might induce genes unrelated to differentiation. The microgravity precluded genes possibly induced by cell adhesion. MT was induced by laminin-1 in both culture conditions, and the extent of the induction is similar. These data demonstrate that laminin-1 either as a gel or in solution can promote acinar formation and regulate gene induction.

The extracellular matrix in general, and laminin in particular, have a role in the embryonic and postnatal development of salivary glands (16–19). During mouse salivary gland embryogenesis, the laminin α 1 chain is located around the terminal clusters of cells, which continue to branch to form the ductal tree by day 13. At later stages, other laminin α chains are found.[4] Whether laminin regulates MT expression in developing salivary glands is not known.

After demonstrating that MTs are one of the major genes upregulated by laminin-1 during differentiation of HSG cells, we determined whether these genes play a role during this process. Overexpression of MT-1f in HSG cells led to a clear change in cell morphology when cultured on plastic and to a dramatic increase in the size of the acini formed in the presence laminin-1. There was no effect on cell proliferation, and it is likely that the larger acini are attributable to increased cell aggregation. There was no effect on amylase expression, a marker for the functional differentiation of the cells, as MT overexpressing cells demonstrated the same level of amylase as mock-transfected or parental cells when cultured on plastic or on Matrigel (not shown). *In vivo* we also did not detect a change in mucin production by the transfected cells as measured histochemically with mucicarmine.[5] Therefore, we suggest that MTs play a role in the morphological differentiation, but their role in the functional differentiation of HSG cells is unknown. Laminin-1 alone induces morphological differentiation but does not increase amylase expression. Growth factors in combination with laminin-1 are required for functional differentiation as determined by amylase expression (20). A correlation between MT expression and cell differentiation was described in other *in vitro* systems. For example, during differentiation of CaCo-2 cells, a human colon adenocarcinoma cell line, MT levels are increased (21). Also, during the differentiation of 3T3L1 mouse fibroblasts to adipocytes, a transient peak in MT levels is observed (22). On the other hand, during differentiation of myoblasts to myotubes there is a decrease in MT content with a change in localization from the nucleus to the cytoplasm (23). Moreover, in the latter case, H9C2 cells with higher levels of MT differentiated more slowly when compared with L6 cells. Thus, MTs play a role in cellular differentiation in a cell-specific manner.

The mechanism for the regulation of morphological differentiation by MT is not known. MT has been implicated in cell survival, metabolism, signal transduction, cell cycle, and extracellular matrix proteolysis. We did not find changes in the transfected cells with regard to proliferation or survival.[5] It is possible that other processes involving MT are important in cell differentiation either directly or indirectly. Laminin-1-dependent MT-induced expression appears to be specific to HSG cells and other cells (PC12 and prostate epithelial cells), which differentiate in the presence of laminin-1. We could not detect induction of MT RNA in other cell lines tested, including B16-F10 melanoma, HT1080 fibrosarcoma, MDA-231 breast carcinoma, and PC3 prostate carcinoma cells, when cultured on laminin-1 gels.[5]

MT expression plays a role in differentiation *in vivo* as well as *in vitro*, because overexpressing HSG cells form smaller and more differentiated tumors in nude mice. Many investigators have studied MT expression in tumors with variable findings depending on the tumor cell type. The most relevant to our system is a report by Sunardhi-Widyaputra *et al.* (16), which describes MT expression in salivary gland tumors. They found variable expression that could be a reflection of the morphological heterogeneity, which correlated with the degree of differentiation and maturation of the tumor cells. On the basis of their observations, these authors suggest that MT is necessary for the growth and differentiation of actively growing cells. Similarly, in hepatocarcinoma, there is a correlation between the differentiation stage and MT expression (24, 25). In this case, expression of MT and concentrations of zinc and copper decreased as the tumors became less differentiated. In contrast, in other human malignancies, such as carcinoma of the thyroid, testicular embryonic carcinoma, pancreatic carcinomas, and adenocarcinoma of the breast, overexpression of MTs correlated with a poor prognosis (26–29). In most of these studies, MT expression is based on immunohistochemistry, using antibodies that detect both MT-1 and -2. It may be that different isoforms of MT iso-proteins are related to the degree of differentiation of various tumor types.

In summary, we demonstrate here that only a surprisingly few

[4] M. P. Hoffman, unpublished observation.
[5] Unpublished observations.

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genes are induced >5-fold during laminin-1-dependent differentiation of HSG cells. We additionally demonstrate that the most frequently detected gene (*i.e., MT-1f*) has an effect on HSG cell morphology and acinar formation *in vitro,* and is correlated with smaller, more differentiated tumors formed by these cells *in vivo*. This is the first demonstration of a relationship between the extracellular matrix and MT expression, which is known to be induced by a variety of other factors. It is not clear yet if this observation extends to all cell types.

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