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Extracellular Matrix Components Induce Endocrine Differentiation *in Vitro* in NCI-H716 Cells

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Endocrine cells occur in ±30% of colorectal adenocarcinomas. The significance of this phenomenon in terms of tumor behavior is still controversial. Endocrine differentiation in colorectal cancer cell lines is almost confined to tumor xenografts* in vivo*, suggesting that endocrine differentiation might be regulated by epithelial-stromal interactions. This hypothesis was studied in the cecal adenocarcinoma-derived cell line NCI-H716 by comparing the expression of chromogranin A protein and messenger RNA in vivo and* in vitro *and by attempts to induce differentiation* in vitro*. We found that chromogranin A expression, which was strongest* in vivo*, could be significantly enhanced* in vitro *by culturing tumor cells in the presence of native extracellular matrix, on fibroblast feeder layers, and in a defined medium with basic fibroblast growth factor. The results suggest that the extracellular matrix induces endocrine differentiation through factors (e.g., basic fibroblast-growth factor) that may be produced by stromal cells and after secretion bind to the extracellular matrix. (Am J Pathol 1993, 142:773–782) (Am J Pathol 1993, 142:773–782)

Normal colonic epithelium contains columnar cells, goblet cells, and endocrine cells (ECs), which are all considered to be the progeny of a common endodermal stem cell, presumed to reside in the mucosal crypt base.[1–7] ECs in the intestines have been widely studied for their role in maintaining gut functions through production and secretion of a wide array of polypeptide hormones and biogenic amines.[8–10] Comparatively little is known on the role of ECs and their synthesis products in regulating intestinal growth[11] and on the position occupied by ECs in the spectrum of intestinal differentiation.[12] The potential relevance of endocrine cells for the regulation of growth and differentiation in the normal epithelium also has a bearing on neoplasia. In the large intestine, endocrine differentiation in tumors covers a spectrum, ranging from highly differentiated, relatively indolent carcinoid tumors to poorly differentiated, extremely aggressive small cell undifferentiated carcinomas,[13,14] via a heterogeneous group of combined exo- and endocrine tumors, including adenocarcinomas, which in ±30% of cases contain scattered endocrine tumor cells.[15] Although tumors with endocrine differentiation might have a relatively poor prognosis,[16–18] this matter has not been completely resolved. A more aggressive behavior could be related to a relatively low differentiation level of endocrine cells[19] or to secretion of growth regulating neurohormonal peptides by endocrine tumor cells.[20] To study the biological relevance of endocrine differentiation in adenocarcinomas, *in vitro* models are needed. However, endocrine characteristics in colorectal tumor cell lines are rare and have mainly been found in xenografts.[21] This suggests a role for the extracellular matrix (ECM) in generating endocrine features. From embryological studies it is known that the development of intestinal endocrine cells depends on contact between epithelium and appropriate mesenchyme.[22] Epithelial stromal interactions conceivably may also be necessary for the induction of the endocrine phenotype in tumor cells. This hypothesis was studied in the NCI-H716 cell line, derived from ascitic fluid of a patient with a poorly differentiated cecal adenocarcinoma.[23] In the original description of the cell line, endocrine differentiation was demonstrated by 3,4 dihydroxyphenylalanine-decarboxylase activity and dense core secretory granules. Commonly occurring colorectal tumor markers such as CA 19-9, TAG-72, and CEA were absent. We recently further characterized this

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cell line and, in addition to endocrine characteristics, also identified mucin production and the presence of high affinity receptors for several neurohormones, which makes the cell line NCI-H716 a suitable model in which to study endocrine differentiation in colorectal adenocarcinomas.[24] In the current study, we compared endocrine differentiation *in vivo* and *in vitro* and attempted to identify the factors governing the expression of the endocrine phenotype *in vitro*. As parameter for endocrine differentiation, we studied expression of chromogranin A (CGA), a 75 to 85 kd, soluble, monomeric acidic protein present in neurosecretory granules of practically all neuroendocrine cell types, and currently the most sensitive marker for endocrine differentiation in normal and neoplastic tissue.[25]

Materials and Methods

Tumor Cells

NCI-H716 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD).

In Vitro *Studies*

Cells were cultured in 25-cm^2 flasks or 6-well plates (Costar) at 37 C in an atmosphere of air with 5% CO2. The culture medium consisted of Dulbecco's modified Eagle's minimal essential medium (DMEM, Flow Laboratories, McLean, VA), either serum-free, with 10% fetal bovine serum (Boehringer Mannheim, Germany) or 10% dialyzed fetal bovine serum, supplemented with penicillin (100 IU/ml), streptomycin (100 µg/ml), and fungizone (2.5 µg/ml). Culturing conditions were modified by addition of the differentiation-inducing agents sodium butyrate (NaBT, 2 mmol/L) dimethylsulfoxide (2% v/v), dimethylformamide (1% v/v), trans-retinoic acid (35 µmol/L), or phorbol myristate acetate (10 ng/ml). Dimethylsulfoxide was obtained from Merck, Darmstadt, Germany; all other chemicals from Sigma, Chemical Co., St. Louis, MO.

Furthermore, cells were cultured in fibroblast-conditioned medium and in serum-free, defined medium (DMEM with hydrocortisone 50 nmol/L, insulin 5 µg/ml, transferrin 5 µg/ml, sodium selenite 5 nmol/L, ethanolamine 100 µmol/L, putrescine 1 µmol/L, spermidine 4 µmol/L, spermine 4 µmol/L, and 0.1% bovine serum albumin) in the presence or absence of basic fibroblast growth factor (bFGF, 10 ng/ml) and transforming growth factor-β (TGF-β, 1 ng/ml). The culturing substrate was modified by coating vials with soluble extracted extracellular matrix components (types I, III, and IV collagen, fibronectin, laminin, and heparan sulfate proteoglycan (HSPG), all 100 µg/ml). Cell adhesion to type IV collagen was further analyzed by culturing cells in calcium- and magnesium-free medium, and by pretreating coated surfaces with a polyclonal rabbit anti-type IV collagen antibody (produced in our laboratory[26]) and with 110 mmol/L dithiothreitol or 120 mmol/L iodoacetamide (Sigma).

In addition, the cells were cultured on amnion membranes and on various mesenchymal substrates. Amnion membranes were stripped by washing in 1 mol/L sodium chloride (NaCl), 2 mmol/L N-ethylmaleimide, 20 mmol/L ethylenediaminetetraacetic acid (EDTA), and 4% deoxycholate and by scraping with a rubber policeman. Lamina propria was prepared from fragments of colon mucosa obtained from fresh colectomy specimens, by removal of the crypt epithelium with 1 mmol/L EDTA/1 mmol/L EGTA/0.5 mmol/L dithiothreitol in phosphate-buffered saline as described by Whitehead et al.[27] Several types of human fibroblasts were used as feeder layers for the tumor cells: adult fibroblasts, explanted from dermis of human skin obtained at autopsy, fetal fibroblasts, explanted from umbilical cord and embryonal fibroblasts, purchased from ATCC. Fetal rat mesenchymal cells were isolated from the intestines of 16-day-old Lewis rat fetuses.[28] Also, tumor cells were grown on surfaces conditioned by confluent layers of fibroblasts, after removal of the fibroblasts with 25 mmol/L NH4OH. Three-dimensional culturing was performed in two types of extracellular matrix gels. Vitrogen 100, a gel made from purified, pepsin-solubilized bovine dermal collagen, consisting of 95 to 98% type I collagen and 2 to 5% type III collagen, was purchased from the Collagen Corporation (Palo Alto, CA). Basement membrane Matrigel, a solubilized basement membrane preparation containing laminin, type IV collagen, heparan sulfate proteoglycans, entactin, nidogen, and several growth factors (TGF-β, fibroblast growth factor (FGF), tissue plasminogen activator, etc.), was purchased from Collaborative Research Incorporated, (Bedford, MA).

In Vivo *Studies*

Immunodeficient female CD nu/nu mice (Charles River Wiga, Sulzfeld, Germany), aged 3 to 5 weeks, were inoculated in the dorsal subcutis (n = 6) or in the wall of the cecum (n = 6) with suspensions of 3 to 5 $\times$ 10^8 single tumor cells in phosphate-buffered saline.

Cell and Tissue Processing

Cells growing in suspension were collected by aspiration; attached cells were removed by gentle

Table 1. *Applied Antibodies (Clone, Source, Type, and Dilution)*

Specificity	Antibody
Endocrine differentiation	
Chromogranin A	LK2H10 (Hybritech-mouse mc-1:12,500)
Intercellular adhesion	
L_tCAM	6F9 (Eurodiagnostics-mouse mc-1:80)
Integrin receptors	
α2 (VLA2)	Thromb/4-CLB mouse mc-1:10
α3 (VLA3)	I143-CLB mouse mc-1:10
α6 (VLA6)	G0H3-CLB rat mc-1:10
β3	C17-CLB mouse mc-1:10
Secondary antibodies	
Rabbit anti-mouse HRP	DAKO p270–1:200
Rabbit anti-rat HRP	DAKO p162–1:100

CLB, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam.

cell-scraping. After washing twice in ice-cold phosphate-buffered saline, cells were immersed (3 hours, 4 C) in fixative containing ethanol (100%), formalin (36%), and glacial acetic acid (97%) (15:4:1 v/v). Cells were resuspended in 4% agarose gel before routine tissue processing and paraffin embedding. Xenografts removed from nude mice were fixed and processed similarly. For assay of cell surface receptor molecules, cytocentrifuge preparations of cultured tumor cells were prepared on poly-L-lysine-coated slides and fixed in 1:1 (v/v) methanol/acetone (10 minutes, −20 C).

Immunocytochemistry

Paraffin sections (4 μ) were immunocytochemically stained, by a standard indirect immunoperoxidase technique as previously described.[29] Endocrine differentiation was identified with a monoclonal antibody against human CGA (LK2H10,[30] Hybritech, San Diego, CA). The presence of cell-adhesion molecules was detected with a panel of antibodies recognizing integrin receptor molecules and LCAM (Table 1). Paraffin-embedded or frozen sections of normal colonic mucosa were chosen as appropriate positive controls. As negative controls primary monoclonal antibodies were omitted or replaced by normal mouse serum. Negative controls were consistently negative.

Quantification and Statistical Analysis

Immunohistochemical expression of CGA in NCI-H716 cells grown in various culturing conditions was compared with control cells grown in suspension or attached to type IV collagen. A readily apparent increase in the fraction of CGA immunoreactive cells was semiquantitatively scored, as indicated in Table 2 (see also Figure 4). Experiments were performed at least in triplicate. The effect of basic (b)FGF on CGA immunoreactivity was compared with control cells and evaluated by counting CGA immunoreactivity in 2×10^3 cells per experiment ($n = 3$). These data were statistically analyzed by *t*-test for independent groups with separate variances.

Northern Blot Analysis

The expression of the chromogranin A gene in NCI-H716 cells grown *in vitro* and *in vivo* was compared by Northern blot analysis of messenger (m)RNA. RNA was extracted (overnight, 0 C) from a subcutaneous xenograft tissue homogenate with 3 mol/L LiCl/6 mol/L ureum. After centrifugation (10,000 rpm), RNA was purified with a mixture of phenol, chloroform, and isoamylalcohol (25:24:1 v/v) and precipitated with 96% ethanol. Lysis of tumor cells cultured in standard conditions was performed with 0.65% NP40, 10 mmol/L vanadyl-ribonucleoside-complex (GIBCO, Laboratories, Grand Island, NY, and Bethesda Research Laboratories, Bethesda, MD) in 10 mmol/L Tris (pH 7.8), 0.15 mol/L NaCl. After centrifugation, 1% sodium dodecyl sulfate and 10 mmol/L EDTA were added to the supernatant, and RNA was extracted and precipitated as described. RNA was size-fractionated by overnight electrophoresis at 1.5 V/cm in 1% agarose/6% formaldehyde gel in MOPS buffer (20 mmol/L 3-morphiline

Table 2. *Effect of Substrate on Endocrine Differentiation*

Substrate	Adhesion*	CGA†
Coll.IV	+	−
Coll.I/III	−	−
Laminin	−	−
Coll.IV/laminin	+	−
Fibronectin	−	−
HSPG	−	−
Coll.IV/HSPG	+	++
Fibroblasts	+	+
Colon ECM	+	+
Amnion	+	++
Vitrogen 100	0	−/+
BM Matrigel	0	++

*Adhesion: − = no, + = yes, 0 = not applicable. †CGA: Immunohistochemical Chromogranin A expression: − = no increase compared to standard culture; + = increased, <50% of cells positive; ++ = increased, >50% of cells positive.

propanesulfonic acid, 5 mmol/L sodium acetate, 1 mmol/L EDTA) and transferred to Hybond-N+ filters. Chromogranin A messenger RNA was detected with a 1.8-kb complementary DNA probe (clone pHCG-A, ATCC). As a positive control, RNA from a human pheochromocytoma was hybridized; RNA from cultured HT-29 colon carcinoma cells was used as a negative control. Parallel hybridization for β actin messenger RNA was performed as an internal standard for the total amount of RNA, with a 1.3-kb complementary DNA probe (courtesy of Dr. T. Berkvens, University of Leiden).

The Northern blots were prehybridized overnight at 42 C in 50% formamide, 1 mol/L NaCl, 1% sodium dodecyl sulfate, 10% dextran sulphate, and 0.1 mg/ml denatured salmon sperm DNA. For hybridization, denatured CGA and β actin probes radiolabeled with ^{32}P by the random primer method[31,32] were added. After overnight incubation, the filters were washed in 2 × standard saline citrate (2 × 15′, RT), 2 × standard saline citrate and 1% sodium dodecyl sulfate (2 × 15′, 60 C). The filters were exposed to Kodak-XAR film, at −70 C with an Ilford intensifying screen. Autoradiograms were scanned by laserdensitometry (Ultrascan 2202, LKB, Sweden). The relative amount of CGA messenger RNA was calculated as the ratio of the densitometrically measured hybridization signals of CGA and β actin.

Electron Microscopy

Cells or tissues were fixed for 2 hours in 0.1 mol/L phosphate-buffered 2.5% glutaraldehyde, postfixed (1 hour) in 0.1 mol/L phosphate-buffered 1% osmium tetroxide, dehydrated in graded ethanol series, and embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate. To study the interaction between NCI-H716 cells and fibroblasts, co-cultures of confluent fibroblast feeder-layers and tumor cells were grown on Thermanox coverslips (Nunc Inc., Naperville, IL) and processed accordingly.

Results

Cell Culture

NCI-H716 cells *in vitro* grew in suspension as floating aggregates. Adherent growth could be achieved by coating the culture vessels with type IV collagen, which resulted in cell attachment and growth in sheets and three-dimensional cell clusters. This interaction with type IV collagen appeared to be Ca^{2+} and Mg^{2+} dependent. Treatment of type IV collagen–coated surfaces with a polyclonal rabbit anti-type IV collagen antibody did not block cell adhesion. This antibody is directed against epitopes in the triple helix domain and is immunoreactive with reduced and non-reduced type IV collagen.[26] Reduction of type IV collagen with 110 mmol/L dithiotreitol and 120 mmol/L iodoacetamide, abolished cell adhesion, although type IV collagen immunoreactivity could still be demonstrated on the coated surface. This indicates that cell adhesion is mediated by the globular carboxy-terminal domain of the type IV collagen molecule, where disulfide bonds are present to stabilize the associations between collagen molecules.[33,34]

Coating of culturing vessels with type I or III collagen, laminin, fibronectin, or HSPG did not result in cell adhesion. Culturing on the basement membrane side of stripped amnion membranes resulted in good attachment of the cells. Moderate attachment was observed on extracellular matrix prepared from colonic mucosa. When cells were grown on feeder-layers of fetal rat mesenchyme and various types of human fibroblasts (irrespective of type), this resulted in firm and selective adherence to the mesenchymal cells (Figure 1A). NCI-H716 cell attachment was also observed on surfaces conditioned by fibroblasts. Ultrastructural observations demonstrated close cell contacts of a non-junctional type between NCI-H716 cells and mesenchymal cells (Figure 1B). Similar close contacts were also seen between tumor cells, and these appeared to increase in number when they were cultured on feeder-layers of fibroblasts. No extracellular matrix components appeared to be deposited at the interface between epithelial and mesenchymal cells.

NCI-H716 cells grown in suspension showed a low level of LCAM (E-cadherin, uvomorulin[35]) expression on the plasma membrane. LCAM expression increased by culturing on amnion membranes (Figure 2A), indicating an increase in intercellular adhesion. Integrin immunoreactivity was found independent of culturing conditions. The cells strongly expressed α2β1 (Figure 2B), which has been shown to be a cell-surface receptor for collagen types I, II, III, and IV[36–39] or for both collagen and laminin, depending on the cell type.[40,41] α3β1, a receptor for collagen, laminin, and fibronectin,[42] was only weakly expressed. β3 integrin receptors, interactive with several ligands (e.g., vitronectin, fibrinogen, thrombospondin[43]), were also strongly expressed. NCI-H716 cells did not express α6β1, which has been identified as laminin receptor.[44]

Xenografts

All 6 mice inoculated subcutaneously developed tumors after an average period of 5 weeks. Of the 6

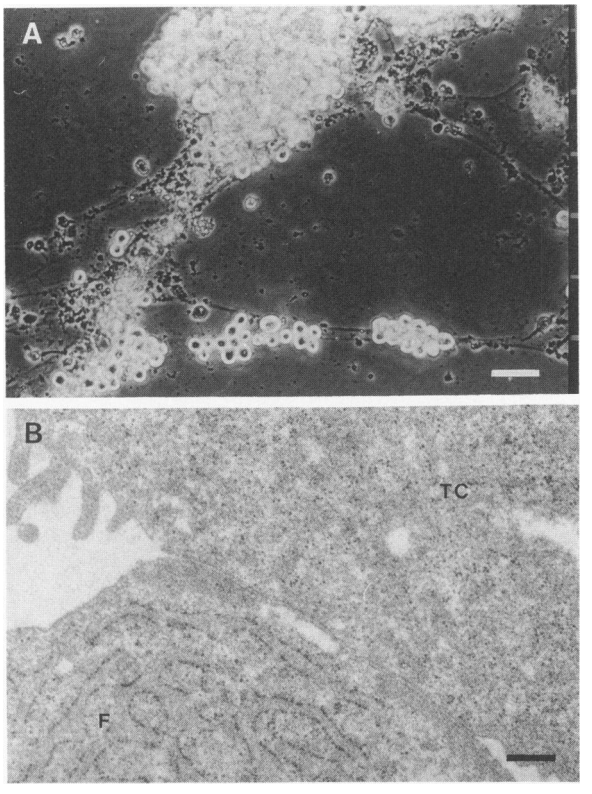

Figure 1. **A**: *Selective adherence of NCI-H716 cells to feeder-layers of fibroblasts. This specific interaction occurs independent of the type of fibroblasts. No attachment is observed to the fibroblast-free culturing surface (bar = 60 μ).* **B**: *Transmission electron micrograph demonstrating the close intercellular contact between a NCI-H716 tumor cell (TC) and a fibroblast (F). The contact is of a non-junctional type. No extracellular matrix is deposited at the intercellular interface (bar = 420 nm).*

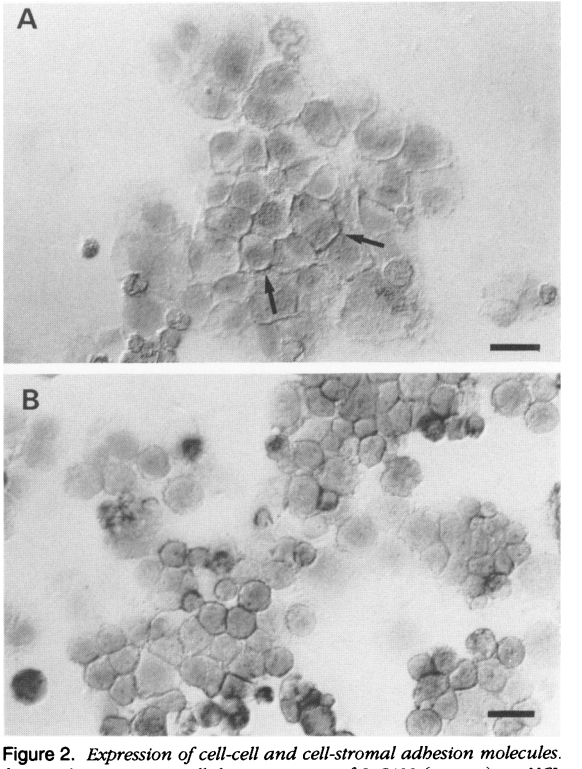

Figure 2. *Expression of cell-cell and cell-stromal adhesion molecules.* **A**: *Membranous intercellular expression of L-CAM (arrows) on NCI-H716 cells cultured on amnion membranes.* **B**: *Strong expression of the α2β1 (VLA2) integrin receptor in a similar pattern (indirect immunoperoxidase with DAB, bars = 25 μ).*

mice inoculated intracecally, 4 were sacrificed after 8 weeks but did not show tumor growth. The remaining 2 mice were sacrificed after 16 weeks, and one showed tumor growth. Histology of subcutaneous and intracecal xenografts was identical, showing a poorly differentiated adenocarcinoma (Figure 4B).

Phenotyping

Endocrine differentiation was demonstrated by immunostaining for CGA and by electron microscopy (dense core neurosecretory granules, ϕ approximately 200 nm) (Figure 3). CGA expression was sporadically observed in cells growing in suspension (<1% of the cells) but was strongly enhanced in xenografts (>50% of the cells) (Figure 4, A and B). Modifications of the culture medium did not lead to induction of the endocrine phenotype. In proliferation-restricting conditions (medium with dialyzed serum or serum-free medium), cell growth was clearly impaired but this was not associated with altered CGA expression.

Endocrine characteristics were considerably enhanced by culturing on fibroblasts, colonic mucosal lamina propria, and amnion (Figure 4, C and D, Table 2). Cell-adhesion to type IV collagen alone or in combination with laminin did not lead to enhanced endocrine differentiation, whereas adhesion to a combination of type IV collagen and HSPG resulted in significant increase of CGA immunoreactivity (Figure 4E). Neither adhesion to surfaces conditioned by fibroblasts nor culturing in fibroblast-conditioned media caused any induction of endocrine differentiation. Endocrine features were slightly increased by culturing in Vitrogen 100 and strongly

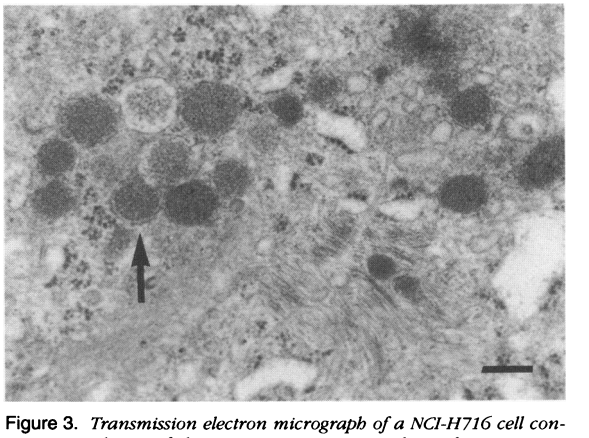

Figure 3. *Transmission electron micrograph of a NCI-H716 cell containing a cluster of dense-core secretory granules with an average diameter of 200 nm (arrow) (bar = 200 nm).*

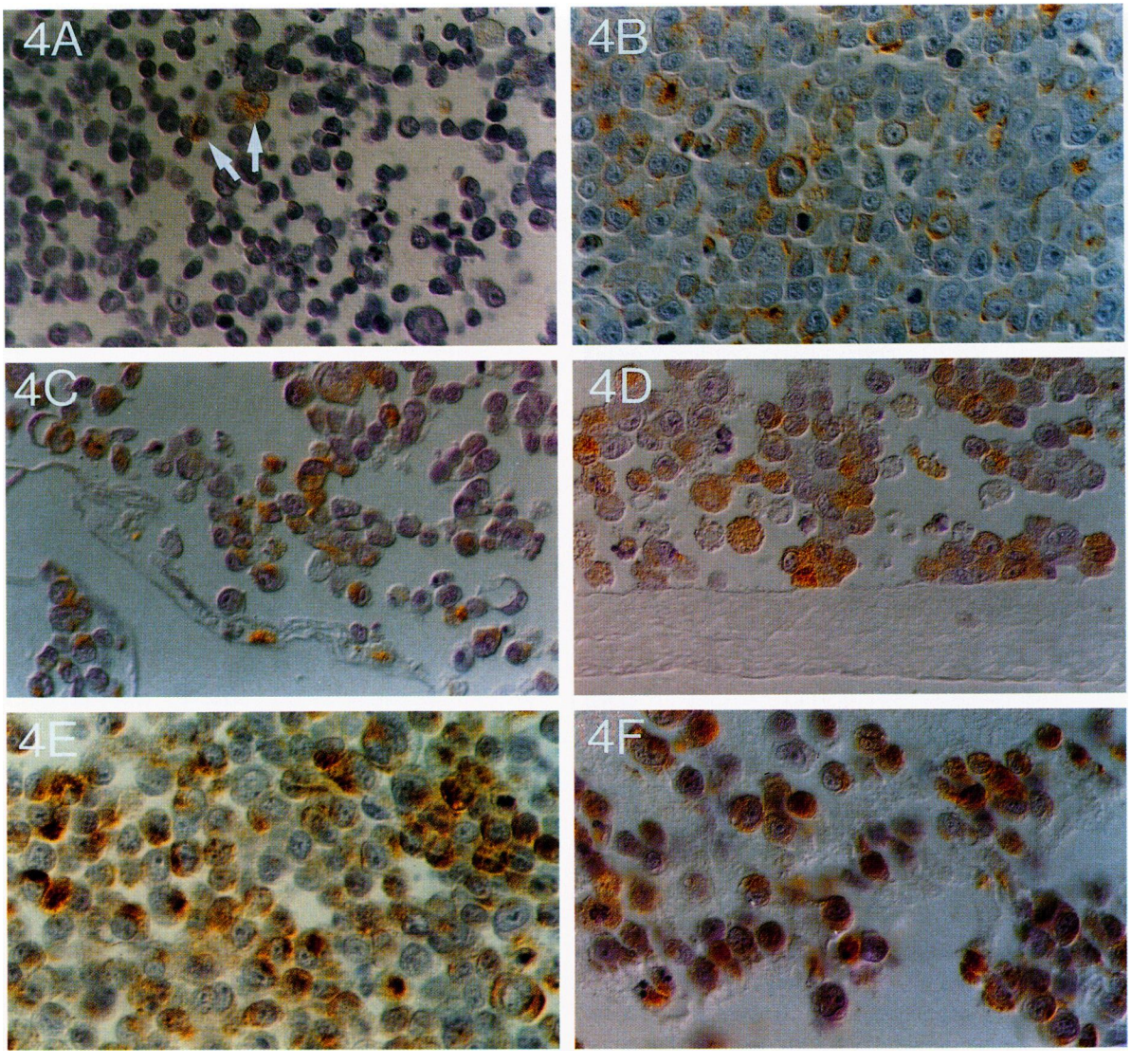

Figure 4. *CGA immunoreactivity in NCI-H716 cells in various conditions.* **A**: *Standard culturing conditions (suspension/attached to type IV collagen–coated surfaces). Rare cells show weak immunostaining (arrows).* **B**: *Subcutaneous xenograft. A poorly differentiated adenocarcinoma, in which a major proportion of cells shows CGA immunoreactivity.* **C** *to* **F**. *Increased CGA immunoreactivity in NCI-H716 cells cultured on various extracellular matrix components (compare with* **A**). **C**: *Co-culture with fibroblasts.* **D**: *Cells grown on amnion membrane.* **E**: *Cells grown on surfaces coated with type IV collagen combined with heparan sulfate proteoglycan.* **F**: *Cells cultured in basement membrane Matrigel (indirect immunoperoxidase with DAB, ×360).*

increased by culturing in basement membrane Matrigel (Figure 4F). When cells were cultured on type IV collagen–coated surfaces in a defined serum-free medium, addition of bFGF (10 ng/ml) resulted in enhanced endocrine differentiation (Figure 5), whereas TGFβ had no effect.

Northern Blot Analysis

As reported previously,[24] the complementary DNA probe for human CGA strongly hybridized with RNA extracted from a subcutaneous xenograft of NCI-H716 and from the human pheochromocytoma. A sequence of approximately 2.1 kb was detected, which is the expected size of the messenger.[45] Only a weak signal was found in RNA from cultured NCI-H716 cells, whereas the non-endocrine colon cancercell line HT-29 was negative. The CGA/β actin messenger RNA ratio *in vivo* showed an approximately tenfold increase compared with cultured cells (Figure 6).

Discussion

For the study of endocrine differentiation in normal and neoplastic intestinal epithelium, *in vitro* models are indispensable. Colorectal adenocarcinoma cell lines with endocrine differentiation are extremely

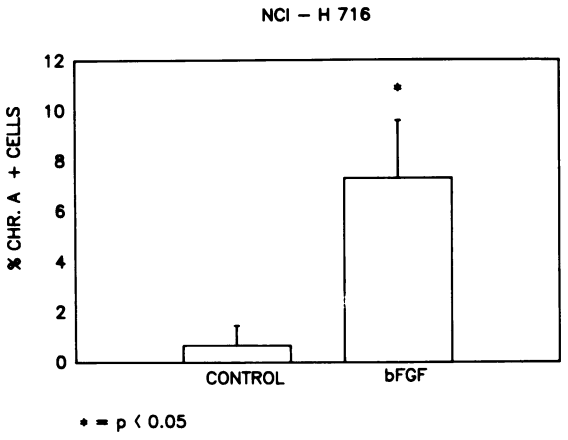

Figure 5. *Increased immunohistochemical expression of chromogranin A in NCI-H716 cells cultured for 5 days on type IV collagen–coated surfaces in a defined, serum-free medium with bFGF (10 ng/ml), as compared to control cells. Bars represent the mean percentage and standard deviation of chromogranin A–positive cells in three experiments (P < 0.05; t-test for independent groups with separate variances).*

rare, despite the relatively high incidence of endocrine differentiation in colorectal adenocarcinomas.[46] Apart from the NCI-H716 cell line, derived from a poorly differentiated cecal adenocarcinoma with endocrine characteristics,[23] the only other adenocarcinoma cell line of human origin convincingly demonstrating endocrine features is the HRA-19 cell line, which was derived from a moderately differentiated rectal adenocarcinoma.[21] This cell line contains cells of each of the three large intestinal lineages,[5] making it an attractive model to study differentiation in colorectal epithelium. However, endocrine cells are only encountered in HRA-19 xenografts in nude mice, suggesting that some factor present *in vivo* may be necessary to induce the endocrine phenotype. Recently, it was shown that endocrine differentiation of HRA-19 cells in xenografts depends on direct contact between tumor cells and host stromal cells.[47] In both murine and human cells systems, extracellular matrix components have been demonstrated to play an important role in intestinal epithelial differentiation.[22,48–50] Similar results have been obtained from embryological studies involving recombination of embryonal intestinal epithelium with mesenchyme from various parts of the developing intestine.[51,52] These studies have demonstrated a leading role for mesenchyme in the evolution of the endocrine phenotype. Endocrine differentiation can be induced when epithelium is recombined with the appropriate type of mesenchyme.

The present study on NCI-H716 cells primarily focused on the mechanisms involved in the induction of endocrine differentiation. Interestingly, as in the HRA-19 model, expression of a general neuroendocrine marker was significantly more intense in xenografts than in cultured cells, suggesting a role for the ECM or mesenchymal stroma in induction of the endocrine phenotype. We tested this hypothesis by performing differentiation induction experiments *in vitro*. The main purpose was to identify which factor(s) would be capable of enhancing endocrine differentiation *in vitro*. In the experiments either the culture medium was modified, by adding differentiation-inducing agents, or the culturing substrate was modified to expose several extracellular matrix components. None of the agents that are known to induce differentiation in colon cancer cell lines augmented endocrine differentiation. Growth restriction did not lead to altered differentiation either.

The experimental modifications of the culturing substrate indicate that cell adhesion is a prerequisite for induction of the endocrine phenotype. When cells were seeded on surfaces coated with type I or III collagen, laminin, fibronectin, or HSPG, the lack of cell adhesion was paralleled by the absence of phenotypical changes. However, cell adhesion to an individual ECM component alone also failed to induce endocrine differentiation. On type IV collagen, NCI-H716 cells did adhere but did not show a significant increase of endocrine characteristics. The cells also adhered to a combination of type IV collagen and laminin, but enhanced endocrine differentiation was not seen. In combination with the absence of the $\alpha 6\beta 1$ laminin receptor on NCI-H716 cells, this finding does not substantiate a major role for laminin in the induction of endocrine differentiation.

However, growth on surfaces coated with a combination of type IV collagen and HSPG resulted in both adhesion and induction of endocrine

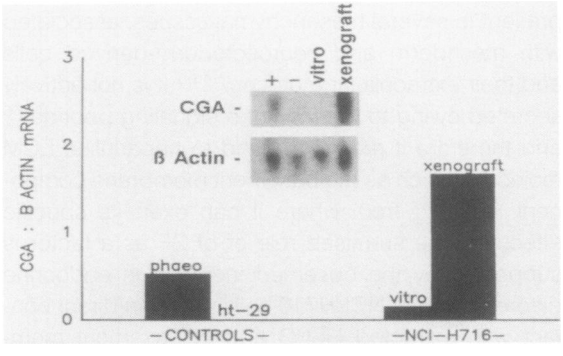

Figure 6. *Northern blot analysis. Comparison of CGA messenger RNA expression in NCI-H716 cells growing* in vitro *under standard conditions and in a subcutaneous xenograft. A human pheochromocytoma was used as a positive control (+); HT-29 colon carcinoma cells as a negative control (–). Hybridization for β actin was performed as an internal standard for the amount of RNA. Bars represent densitometric quantification of CGA messenger RNA, corrected for β actin messenger RNA (CGA:β actin ratio). The amount of messenger RNA for CGA in NCI-H716 cells shows an approximate tenfold increase* in vivo *versus* in vitro.

characteristics. Apparently, type IV collagen is necessary for cell adhesion and HSPG induces differentiation. Contrasting with individual ECM components, endocrine differentiation was found to be highly stimulated by cell growth on intact, naturally occurring stroma (amnionic membranes, stripped colonic mucosa, fibroblasts). This indicates a role for cell-surface adhesion molecules in mediating the induction of endocrine differentiation. ECM receptors differ from other cell-surface receptors (e.g., peptide hormone receptors) in their lower binding affinity and much higher concentration at the cell surface.[53] Therefore, these receptors probably cooperate in binding cells to combinations of matrix ligands rather than to individual components. In support of this, individual ECM components usually fail to elicit the cellular responses induced by the same components in a complex matrix. An ECM did not appear to be indispensable: co-culturing on feeder-layers of fibroblasts induced endocrine differentiation without involvement of ECM components at the intercellular interface. The inductive effect of fibroblasts seemed to be primarily dependent on the close contact with the tumor cells: culturing of tumor cells on type IV collagen–coated surfaces in fibroblast-conditioned media did not induce endocrine differentiation.

The enhancement of endocrine differentiation by direct contact with fibroblasts and the absence of this effect when employing fibroblast-conditioned culturing media suggests that one or more factors produced by cultured fibroblasts could have local paracrine activity as a differentiation inducer. Such a factor would be present in concentrations in a conditioned medium that were too low but would accumulate in sufficient amount in the ECM to account for the differentiation-inducing effect of complex stroma. One possible candidate is bFGF. This factor is present in several mesenchymal tissues, associated with mesoderm and neuroectoderm-derived cells and their extracellular matrices.[54,55] It is not actively secreted owing to the lack of a signaling peptide,[56] and therefore it remains bound to heparinlike ECM molecules such as the basement membrane component HSPG,[57] from where it can exert its specific effects.[58] The surmised role of bFGF as a factor is supported by the observed increase in endocrine differentiation of NCI-H716 cells grown in direct contact with extracted HSPG and in basement membrane Matrigel. The latter substance contains basement membrane components and several growth factors, among them, bFGF. Moreover, a specific effect of bFGF was confirmed by the observed increase of CGA expression, after addition of bFGF to cells growing in a serum-free, defined culture medium.

As the mesenchymal origin of bFGF was not investigated in our study, an alternative source for such a factor can not be excluded. NCI-H716 cells are known to produce substances that are involved in autocrine growth regulation.[59] Conceivably, these tumor cells could also produce factors inducing differentiation. In this respect, it is noteworthy that bFGF has been demonstrated in secretory granules of adrenal medullary chromaffin cells,[60] where it maintains differentiation by enhancing survival and transmitter storage of cultured cells.[61] If this would also apply to NCI-H716 cells, direct contact with ECM components might cause release of similar factors from the tumor cells and lead to enhanced endocrine features by auto- or paracrine mechanisms. The presence of heparinlike growth factor–binding substances in the ECM might modulate this response.

In summary, in the NCI-H716 cell line, endocrine differentiation is readily observed *in vivo* but is scarce *in vitro*. Endocrine differentiation can be induced *in vitro* when tumor cells interact directly with specific extracted basement membrane components, natural extracellular matrix, or mesenchymal cells. Therefore, the induction of endocrine differentiation involves a combination of cell adhesion, probably mediated by cell adhesion molecules of the integrin family, and the influence of local factors that function by auto- or paracrine mechanisms and/or are bound to ECM molecules.

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