

## INDUCTION OF CARCINOEMBRYONIC ANTIGEN EXPRESSION IN A THREE-DIMENSIONAL CULTURE SYSTEM

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### SUMMARY

MIP-101 is a poorly differentiated human colon carcinoma cell line established from ascites that produces minimal amounts of carcinoembryonic antigen (CEA), a 180 kDa glycoprotein tumor marker, and nonspecific cross-reacting antigen (NCA), a related protein that has 50 and 90 kDa isoforms, in monolayer culture. However, MIP-101 produces CEA when implanted into the peritoneum of nude mice but not when implanted into subcutaneous tissue. We tested whether three-dimensional (3D) growth was a sufficient stimulus to produce CEA and NCA 50/90 in MIP-101 cells, because cells grow in 3D *in vivo* rather than in two-dimensions (2D) as occurs in monolayer cultures. To do this, MIP-101 cells were cultured on microcarrier beads in 3D cultures, either in static cultures as nonadherent aggregates or under dynamic conditions in a NASA-designed low shear stress bioreactor. MIP-101 cells proliferated well under all three conditions and increased CEA and NCA production three- to four-fold when grown in 3D cultures compared to MIP-101 cells growing logarithmically in monolayers. These results suggest that 3D growth *in vitro* simulates tumor function *in vivo* and that 3D growth by itself may enhance production of molecules that are associated with the metastatic process.

**Key words:** cell adhesion; cancer; colon; proteases; metastasis; sheer stress.

### INTRODUCTION

Carcinoembryonic antigen (CEA) is a 180 kDa glycoprotein that is released by human colonic epithelial cells into either the lumen of the gastrointestinal tract or the circulation. The function of CEA is not well-defined but as a member of the immunoglobulin supergene family it may be involved with intercellular adhesion (19). Interestingly, an elevated concentration of CEA in the blood of patients who undergo resection of an apparently localized bowel carcinoma is often associated with the presence or subsequent appearance of metastases in the liver or lung (7,27). Experimental hepatic metastasis by human colorectal carcinomas in athymic nude mice is also associated with the serum level of CEA in the patient from whom the tumor is obtained (4,11) or CEA expression by the tumor cells (25). While CEA may be an adhesion molecule, it also has a humoral effect on metastasis because systemic pretreatment with CEA increases the ability of human colorectal carcinoma cells to colonize the liver, even when the neoplasm does not itself produce CEA (14). Thus, CEA is associated with the metastasis of colorectal carcinoma cells and conditions that increase the production of CEA by colorectal cancer cells may increase their potential for metastasis.

Poorly differentiated MIP-101 human colorectal carcinoma cells secrete little CEA *in vitro* in monolayer culture (26). Monolayer cultured MIP-101 cells form tumor nodules when implanted in subcutaneous tissue but do not produce either CEA or spontaneous metastases (26). Furthermore, monolayer cultured MIP-101 cells rarely

produce experimental metastases in liver or lung when injected intrasplenically or intravenously into nude mice (26). However, MIP-101 cells implanted in the peritoneum of nude mice develop nodules that produce CEA and form spontaneous hematogenous metastases in liver and lung (26). Because MIP-101 cells were originally isolated from the peritoneal cavity of a patient (17), they appear to produce CEA and spontaneous metastases when implanted in the same or orthotopic site from which they were harvested. The conventional interpretation of these findings invokes the seed-soil hypothesis of Paget (21), which states that the metastatic precursor cell (the right seed) will only produce a metastasis if planted in the appropriate organ site (the right soil). Thus, the microenvironment of the host permits or even supports the malignant behavior of a cancer. In the MIP-101 model, the microenvironment of the peritoneum appears to actively support the production of CEA and metastases by MIP-101 cells while the microenvironment of the subcutaneous tissue does not.

However, certain characteristics of the malignant phenotype may not depend on the interaction between host and neoplastic cells, but depend instead on the growth of the neoplastic cells in three-dimensional (3D) conformations that mimic the growth pattern of tumor cells *in vivo*. Rak and Kerbel have found that both clonal dominance (22) and resistance to chemotherapy (8) are facilitated when cells grow as 3D spheroids rather than as two-dimensional (2D) monolayers. Furthermore, the behavior of the tumor cells mimics their function *in vivo* without interaction with stromal cells (8).

Our purpose was to determine if 3D growth *in vitro* induced the expression of CEA in poorly differentiated MIP-101 human colorec-

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tal carcinoma cells that do not produce CEA in monolayer cultures. We tested this hypothesis by evaluating the effect of growth in both static and dynamic 3D culture systems on MIP-101 cells. MIP-101 cells were cultured on microcarrier beads and allowed to form clusters that were then analyzed for the expression of CEA. Previously, we have shown that human colon carcinoma cell lines cocultivated with stromal cells in a NASA-designed 3D culture system induces differentiation and mucin production that is morphologically similar to that in tumors implanted in nude mice (5). Such differentiation and mucin production does not occur in similar cocultures in 2D monolayers (5). Thus, we determined whether 3D growth was associated with the induction of CEA expression in MIP-101 cells that normally only express CEA when implanted orthotopically in nude mice. We compared growth in standard monolayer cultures to that in nonadherent bacterial petri dishes (static 3D culture system) or in the NASA-designed rotating wall vessel (RWV, a dynamic 3D culture system in which cells on microcarrier beads are rotated around the horizontal axis under low shear stress). We observed that production of both CEA and nonspecific cross-reacting antigen (NCA) 50/90 were stimulated by 3D growth.

#### MATERIALS AND METHODS

**Cell cultures.** For dynamic 3D cultures, MIP-101 colon carcinoma cells were seeded into four RWVs (Synthecon, Inc., Houston, TX.), with 5 mg/ml Cytodex 3 microcarrier beads (Sigma Chemical Co., St. Louis, MO) at 15 cells per bead and the vessels were adjusted to rotate at 30–35 rpm. As a static 3D culture control, 35-mm nonadherent petri dishes (Optilux, Falcon, Lincoln Park, NJ) were seeded with MIP-101 cells and microcarrier beads at concentrations identical to that in the vessels. In addition, 25-cm<sup>2</sup> flasks were seeded with MIP-101 cells as a 2D monolayer culture control. All cells were cultured in GTSF-2 medium (6). The experiment was conducted for 14 d and cultures were replenished as needed (every 24 h decreasing to every 8 h). Cell and medium samples were harvested every 4 d. Quick frozen cell samples ( $1 \times 10^7$  cells) were harvested for western blot analysis at Days 0, 8, 12, and 14. Cell samples were harvested on Days 4, 8, and 14 and these specimens fixed in Omnifix (Xenetics, Tuetin, CA) for immunohistochemical studies. At the end of the experiment, growth curves were based on nuclei counts. Frozen cell samples were analyzed on western blots for CEA.

**Immunohistochemistry and western blots.** Immunohistochemical analysis was performed on paraffin-embedded serial sections of MIP-101 microcarrier bead cultures. Five micron thick sections were prepared by RMC, Inc. (Tucson, AZ) and stained with an anti-CEA monoclonal antibody (Boehringer Mannheim Biochemicals, Indianapolis, IN). The murine primary antibody was identified with a rabbit anti-mouse antibody conjugated with horseradish peroxidase and the reaction developed with diaminobenzidine.

Cells ( $\sim 1 \times 10^7$ ) were extracted in 300  $\mu$ l of lysis buffer [60 mM n-Octyl- $\beta$ -D-glucopyranoside (Sigma), in 25 mM MES, pH 6.5 (Research Organics, Cleveland, OH), in ddH<sub>2</sub>O with protease inhibitors] and produced approximately 1 mg/ml of protein. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was then conducted in 7.5% gels under reducing conditions with 50  $\mu$ g of protein loaded per lane. Samples were electrophoresed at 60V for 18 h and transferred to 0.45  $\mu$ m nitrocellulose membranes. The western blots were performed with either a murine IgG monoclonal anti-CEA antibody (Zymed Laboratories, San Francisco, CA) or 228.2, a murine IgG raised to NCA 50/90 that was provided courtesy of Dr. J. L. Elting, (Molecular Therapeutics, Miles Laboratories, West Haven, CN). Blots were blocked with 10% dry milk in 0.1% Tween-20 and washed with 0.1% Tween-20 in 0.15 M phosphate-buffered saline (PBS), pH 7.4. The primary antibodies were detected with a sheep anti-mouse IgG conjugated with peroxidase (Amersham Life Science, Buckinghamshire, UK) using the enhanced chemiluminescence process (Amersham). Quantitation of the relative amounts of CEA or NCA proteins detected in the western blots was achieved by scanning the blots with a flat bed scanner (Microtek 600ZS, Microtek Lab, Torrance, CA) into Photoshop 2.5.1 (Adobe Systems, Mountain View, CA) on a Macintosh Quadra 950 (Apple Computer Co., Cupertino, CA). Images were then analyzed in IPLab (Signal Analytics Corp., Vienna, VA) and compared to blots of partially purified NCA and CEA proteins as internal standards.

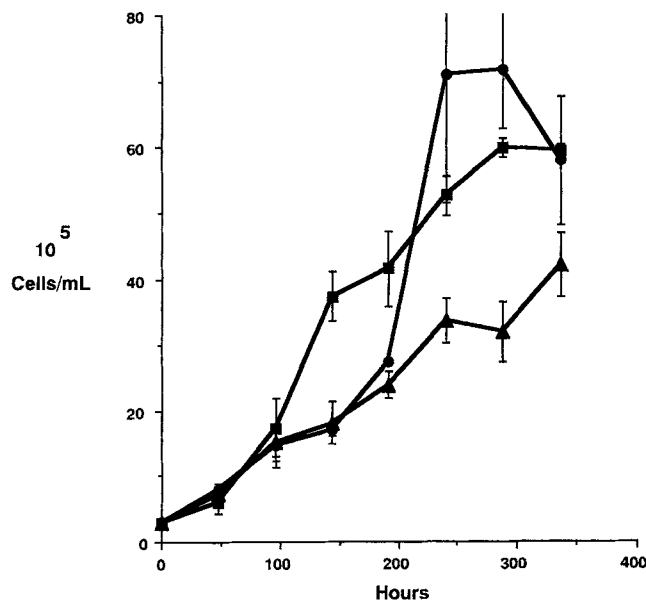


FIG. 1. Growth curves of MIP-101 cells under different conditions.  $3 \times 10^5$  cells/ml were incubated in monolayers in T-25 flasks (■, T-25) or on Cytodex 3 microcarrier beads in either nonstick petri dishes (●, Petri) or in the NASA-designed rotating wall vessel (RWV) (▲). Results are presented as mean  $\pm$  SEM from four separate RWV experiments and two separate experiments each for T-25 and petri dish cultures.

**Statistics.** Results are presented as the mean  $\pm$  standard error of the mean. Comparisons among means were performed by one-way analysis of variance using StatView II (Abacus Concepts, Berkeley, CA) on an Apple Macintosh computer (Cupertino, CA). Level of significance was 5% or less.

#### RESULTS

**Growth of MIP-101 cells in vitro.** MIP-101 cell cultures were initiated at  $3 \times 10^5$  cells/ml either as monolayer cultures in T-25 flasks or on microcarrier beads at 15 cells/bead in either static (in 35-mm petri dishes) or dynamic (low shear stress RWV cultures) 3D cultures. Cells grew well in all three conditions with the monolayer cultures plateauing at Day 8 (Fig. 1). The petri and RWV cultures continued to proliferate the full 14 d. Doubling times during the first 8 d averaged 45.8, 56.1, and 52.3 h for the Petri, RWV, and T-25 cultures, respectively. Viability was 95% or greater at each time point by trypan blue dye exclusion. Although monolayer cultures remained predominantly as monolayers at least through 8 d (Fig. 2A), both the static (Fig. 2B) and dynamic (Fig. 2C) cultures produced multilayer cell cultures indicating 3D growth. After 8 d, the monolayer cultures began to pile up and were no longer strictly monolayers. Thus, all three conditions led to growth of viable MIP-101 cells, although the monolayer culture entered a plateau phase by 8 d that was not apparent in either the static or dynamic 3D culture system.

**Detection of CEA in the cytoplasm of 3D MIP-101 cultures.** Monolayer and 3D cultures of MIP-101 cells were harvested and analyzed for expression of CEA-related proteins by immunohistochemistry. Monolayer cultures in the exponential phase did not react with the anti-CEA monoclonal antibody (Fig. 3). While trace expression of CEA-related proteins was evident by Day 4 in the RWV cultures, the expression increased in 8- and 14-d cultures (Fig. 4A–C). Cul-

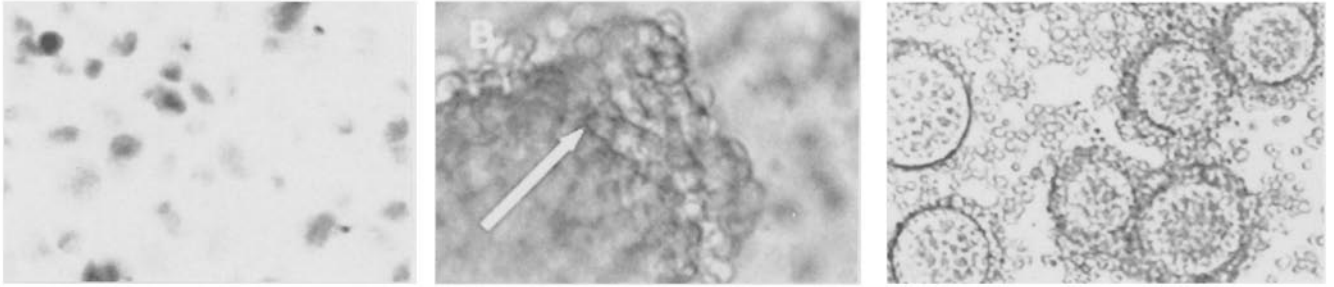


FIG. 2. Pattern of growth under different conditions. MIP-101 cells were cultured as monolayers in T-25 flasks (A), in static 3D cultures on microcarrier beads in nonstick petri dishes (B), or in dynamic 3D cultures in the rotating wall vessel (RWV) (C). MIP-101 cells form multilayer structures on microcarrier beads in both petri and RWV cultures. A and B are at  $\times 40$ , whereas C is at  $\times 10$ . Arrow in B denotes the edge of the microcarrier bead.

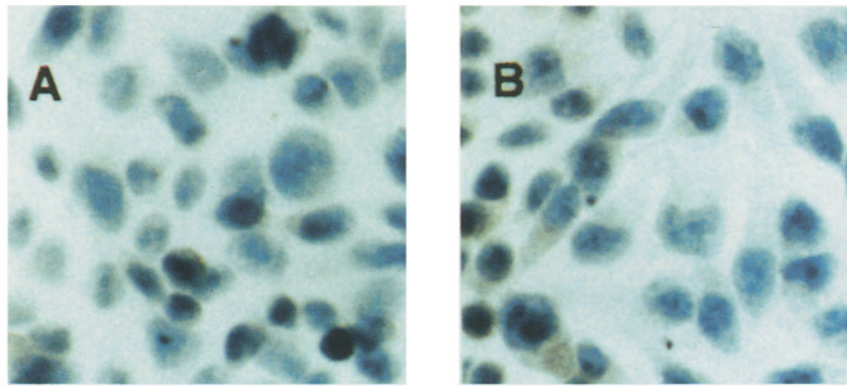


FIG. 3. Immunohistochemistry of MIP-101 cells growing as monolayers. MIP-101 cells were cultured for 6 d in T-25 flasks and then incubated without primary anti-carcinoembryonic antigen (CEA) antibody (A) or with an anti-CEA monoclonal antibody (B). After completion of staining as described in "Materials and Methods," CEA was not detected in MIP-101 cells. Magnification =  $\times 60$ .

tures in the petri dishes were similar, although slightly less intense at each time point (Fig. 4 D–F). Expression was localized in the cytoplasm and was not present in plasma membranes of MIP-101 cells (Fig. 4 C and F).

**Identification of CEA and NCA proteins in MIP-101 cultures.** Because the immunohistochemical analysis may identify either 180 kDa CEA or 50 or 90 kDa NCA, we sought to determine which CEA-related proteins are expressed in MIP-101 cells grown under the different conditions. Western blots of extracts of MIP-101 cells were probed with monoclonal antibodies directed to NCA or CEA. Extracts of MIP-101 cells growing exponentially in monolayer culture displayed small amounts of CEA and both 50 and 90 kDa NCA proteins (Fig. 5 A and B). However, extracts of either the petri or RWV 3D cultures grown for 12 d expressed increased amounts of both 180 kDa CEA and the 50 kDa NCA proteins (Fig. 5 A and B). When densitometry was performed and the amounts of proteins compared to standards of partially purified CEA or NCA (data not shown), the amount of 180 kDa CEA was increased 6.2-fold in the petri culture and 4.2–7.4 in the RWV cultures. It is not clear why one of the RWV cultures did not produce CEA, although it clearly contained NCA (Fig. 5 A and B). Although the amount of 90 kDa NCA was not increased significantly in the 3D cultures (ratios of RWV:monolayer culture extracts of 90 kDa NCA were only 0.9–2.4, while the petri dish ratio was 1.2), the amount of 50 kDa NCA was significantly

increased in the 3D cultures (9.4-fold in the petri dish culture and 3.8–10.0-fold in the RWV cultures compared to the amount in the monolayer cell cultures). This demonstrates that MIP-101 cells grown in a 3D culture system produce increased amounts of mature CEA and NCA proteins with a relatively greater increase in the amount of 50 kDa NCA.

## DISCUSSION

CEA functions *in vitro* as an intercellular adhesion molecule since it causes homotypic cell aggregation when transfected into cells that do not aggregate otherwise (1,20). Human colorectal carcinoma cells (13,15) and normal colon epithelial cells (10) also adhere to CEA attached to a solid phase through homophilic bonds. However, a role for CEA as an adhesion molecule *in vivo* has been difficult to define. CEA stimulates experimental and, possibly, clinical metastasis by a mechanism that does not involve cell adhesion because systemic pretreatment increases hepatic sequestration of weakly metastatic non-CEA expressing lines. Thus, CEA increases metastatic potential by some humoral effect that enhances sequestration of tumor cells in the liver.

We now report that 3D growth stimulates expression of CEA-related proteins in MIP-101 cells. Previous reports have shown that expression of CEA is increased by differentiation agents and cyto-

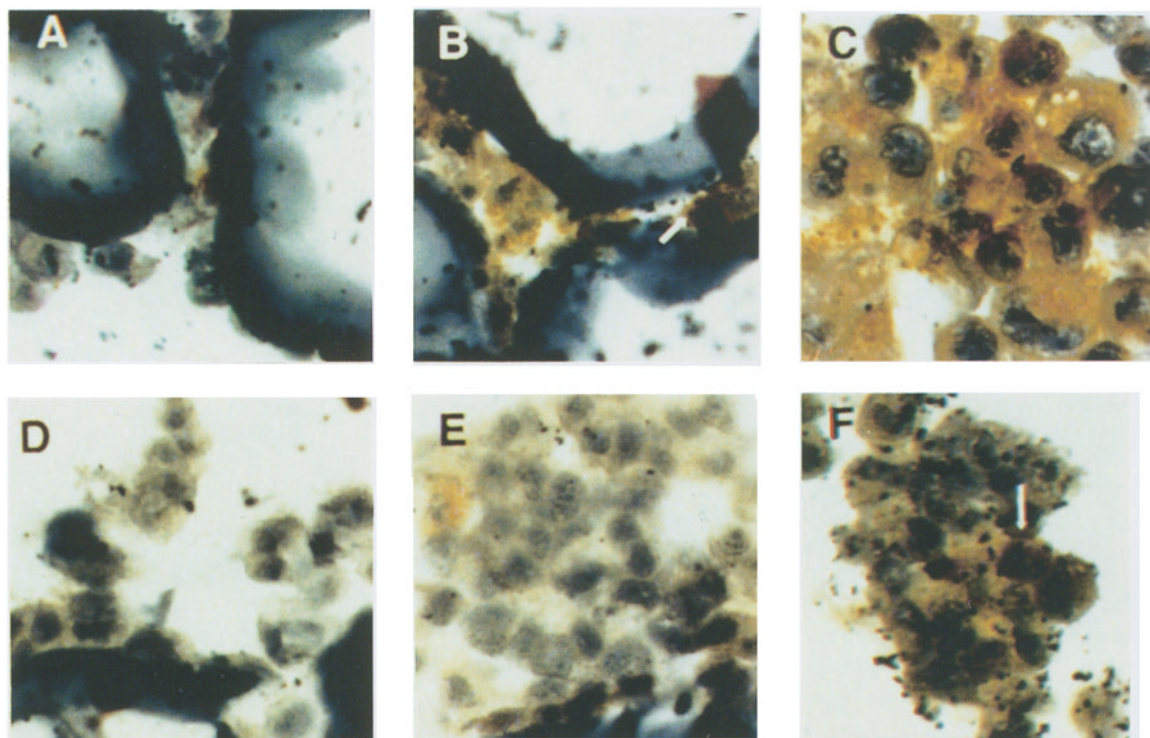


FIG. 4. Detection of carcinoembryonic antigen (CEA) in 3D CEA cultures. MIP-101 cells were grown on microcarrier beads in either the rotating wall vessel (RWV) (A-C) or in static microcarrier cultures in nonadherent petri dishes (D-F). On Days 4 (A,D), 8(B,E), or 14(C,F), cultures were harvested, fixed, and processed for CEA immunohistochemistry. CEA expression was not evident at 4 d in either RWV (A) or petri (D) cultures. However, CEA expression was evident at Day 8 (arrows, B and E) and increased at Day 14 in the Petri culture (F) but even more in the RWV culture (C). CEA is localized in the cytoplasm and does not mark intercellular boundaries as would be expected if it were present in the cell membrane. Magnification =  $\times 60$ .

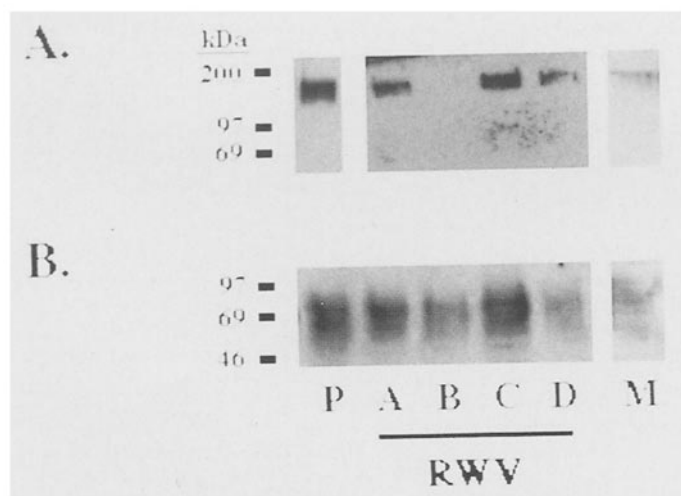


FIG. 5. Western blots of carcinoembryonic antigen (CEA) (A) and non-specific cross-reacting antigen (NCA) (B) expression in MIP-101 cells cultured under different conditions. MIP-101 cells were grown either in monolayer cultures (M), on microcarrier beads in nonadherent petri dishes (P), or in the rotating wall vessel (RWV) (A-D).  $10^7$  cells were extracted and analyzed for the content of 180 kDa CEA (A) or 50 and 90 kDa NCA (B). The results indicate that the expression of both CEA and NCA proteins is increased in 3D cultures of MIP-101.

kinases (18), as well as interferons (24). It is possible that 3D growth stimulates the production of transcription factors that may also be activated by cytokines. Clearly, further work is necessary to determine whether the regulation of CEA and NCA 50/90 occurs at the transcriptional, posttranscriptional, or translational level.

The expression of CEA-related proteins in MIP-101 in 3D culture is relatively weak and confined to the cytoplasm. CEA and NCA are bound to the plasma membrane through a glycosylphosphatidylinositol (GPI)-linkage (23). Release of CEA from cells may occur by cleavage of the GPI-linkage by an endogenous phosphatidylinositol-specific phospholipase (9). Because MIP-101 cells do not appear to localize CEA-related proteins to the plasma membrane, they may lack the ability to form the appropriate GPI-linkage. This may also explain why little CEA is released into the medium in cultures with high viability and low shear stress, since CEA may not be released from viable cells if it is not attached to the cell surface. An earlier report suggested that CEA was released by MIP-101 cells into the medium (12), but this may have reflected cell lysis in initial experiments in the RWV with higher shear stress from more turbulent conditions. The elevated blood levels of nude mice bearing intraperitoneal MIP-101 may reflect the lysis of tumor cells as tumor growth outstrips blood supply (14) rather than secretion of CEA from viable cells.

Production of CEA-related proteins appears to be greater in the RWV than in the static nonstick petri dish culture. The RWV may

provide better nutrient mixing than the static culture since the rotation provides some mixture of microcarrier bead aggregates through the medium, whereas the static system depends on simple diffusion for nutrient transfer. Further work is necessary to determine whether other metabolic effects are altering gene expression, since RWV cultures have similar glucose and oxygen consumptions as monolayer cultures do, but produce more hydrogen ion and carbon dioxide on a per cell basis (data not shown). This observation may suggest that anaerobic metabolism is increased in the RWV and that this is associated with the expression of CEA by MIP-101 cells.

Our present data suggest that the greatest increase in expression of CEA-related proteins occurs in the expression of 50 kDa NCA, followed by smaller increases in the expression of 180 kDa CEA without much change in the expression of 90 kDa NCA. The relatively greater increase in NCA expression is consistent with the relative increases that occur in NCA and CEA expression by human bowel epithelium during neoplastic progression. As normal colon epithelium becomes malignant, the relative expression of NCA also increases more than the increase in CEA expression (2). Since both 50 and 90 kDa NCA have the same peptide but different degrees of glycosylation, the present results suggest that production of the peptide may be increased but that posttranslational modification by increased glycosylation does not follow in parallel.

In summary, CEA is associated with the development of metastasis by human colorectal carcinoma. To the best of our knowledge, this is the first observation that 3D culture induces expression of CEA. Whether CEA or NCA expression is merely associated with or causes metastasis remains to be elucidated and was not evaluated in these experiments. Nonetheless, CEA expression is a marker of metastatic potential in MIP-101 cells. Future studies will seek to elucidate the mechanism by which 3D growth induces expression of CEA-related proteins. In addition, these experiments support the use of 3D culture techniques to investigate host-tumor interactions. Clearly, orthotopic implantation into the peritoneum of nude mice permits CEA expression, but may not actively stimulate it. Conversely, the subcutaneous tissue of nude mice must actively inhibit CEA expression that would otherwise occur in a 3D nodule. Similar differences in the metastatic behavior of human colon carcinomas have been noted by Fidler and colleagues (3,16), who suggest that the environment that is similar anatomically to the original site in which the cancer arose or metastasizes (the orthotopic site) induces production of proteases and other molecules that facilitate metastasis, whereas the subcutaneous tissue inhibits the expression of these molecules and inhibits metastasis. Thus, 3D growth may regulate gene expression that is not evident in monolayer cultures and that is further modulated in the host micro-environment.

#### ACKNOWLEDGMENTS

Supported by Grants NAG9-520 and NAG9-650 from the National Aeronautics and Space Administration and R01 CA 42857 from the Department of Health and Human Services.

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