Human Carcinoembryonic Antigen, an Intercellular Adhesion Molecule, Blocks Fusion and Differentiation of Rat Myoblasts

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Abstract. Human carcinoembryonic antigen (CEA), a widely used tumor marker, is a member of a family of cell surface glycoproteins that are overexpressed in many carcinomas. CEA has been shown to function in vitro as a homotypic intercellular adhesion molecule. This correlation of overproduction of an adhesion molecule with neoplastic transformation provoked a test of the effect of CEA on cell differentiation. Using stable CEA transfectants of the rat L6 myoblast cell line as a model system of differentiation, we show that fusion into myotubes and, in fact, the entire molecular program of differentiation, including creatine phosphokinase upregulation, myogenin upregulation, and β-actin downregulation are completely abrogated by the ectopic expression of CEA. The blocking of the upregulation of myogenin, a transcriptional regulator responsible for the execution of the entire myogenic differentiation program, indicates that CEA expression intercepts the process at a very early stage. The adhesion function of CEA is essential for this effect since an adhesion-defective N domain deletion mutant of CEA was ineffective in blocking fusion and CEA transfectants treated with adhesion-blocking peptides fused normally. Furthermore, CEA transfectants maintain their high division potential, whereas control transfectants lose division potential with differentiation similarly to the parental cell line. Thus the expression of functional CEA on the surface of cells can block terminal differentiation and maintain proliferative potential.

Human carcinoembryonic antigen (CEA) (Gold and Freedman, 1965), is a member of a family of cell surface glycoproteins which appear at high levels in the blood of patients bearing a wide variety of tumors including colon, breast, and lung carcinomas (Zimmermann et al., 1988; Cournoyer et al., 1988), a phenomenon that has led to its widespread use as a tumor marker in the prognosis and management of colon cancer (Shuster et al., 1980; Terry et al., 1974). The basis for this marked elevation in blood levels of CEA is likely due to an increased accessibility to the vascular system in tumors, with some workers finding no increase in cellular CEA production (Abbasi et al., 1992) and others a true increase due to malignant transformation (Higashide et al., 1990); our evidence favors the latter (Boucher et al., 1989).

CEA is a member of the immunoglobulin supergene family (Williams, 1987; Paxton et al., 1987) consisting of a processed leader sequence, a V-like amino-terminal domain, six C2-set-like repeat domains in homologous pairs denoted A1B1, A2B2, and A3B3, and a short COOH-terminal sequence which is processed to leave a glycosphatidylinositol (GPI) bond to the external cell membrane (Fig. 2) (Beauchemin et al., 1987).

CEA has recently been demonstrated to act in vitro, at least, as a calcium-independent, homotypic, intercellular adhesion molecule (Benchimol et al., 1989). The adhesion function could be of relevance to the observed correlation between CEA family overproduction and cancer. We have suggested an instrumental model (Benchimol et al., 1989) based on the following rationale: the adult colonic epithelium consists of a monolayer of polarized cells which divide at the base of the crypts and move up the sides, undergoing a precise terminal differentiation as they progress to be eventually sloughed off into the lumen of the bowel; CEA production in these cells tends to be low and apical; early embryonic epithelium, on the other hand, is multilayered and CEA is found over the entire cell surface (Benchimol et al., 1989); colonic carcinomas also frequently consist of multilayered epithelial cells with CEA expressed over the entire cell surface.

We have suggested that this apparent reversion to an embryonic multilayered epithelial architecture in adult carcinomas could be due to an imbalance in the presentation of adhesion molecules on the surface of colonocytes arising from inappropriate production of CEA; this, in turn, could lead to a disruption of differentiation leaving cells with
proliferative potential and providing a means for the selective outgrowth of cells with further oncogenic mutations (Benchimol et al., 1989). In this model, tissue architecture and cellular differentiation in heterogeneous cell populations is considered to be critically dependent on the accurate spatiotemporal display of a number of specific cell adhesion and factor receptor molecules on the cell surface (Edelman, 1984, 1986; Hatta et al., 1987; Fujimori et al., 1990). The effect of the ectopic introduction of CEA on the cell surface is envisioned as being potentially disruptive of normal cell–cell interactions which depend on these molecules.

As a test of this "instrumental" tissue architecture model, we have examined the effect of ectopic production of CEA in a well characterized differentiating system, the rat L6 myoblast. These cells, after reaching confluence in culture, undergo cellular alignment followed by fusion and the formation of multinucleated myotubes (Yaffe, 1968; Richler and Yaffe, 1970; for review see Wakeland, 1985). After proliferation to near confluence, the cells withdraw from the cell cycle before differentiation (Okazaki and Holtzer, 1966), a process which may be inhibited by growth factors (Linkhart et al., 1980; Lathrop et al., 1985; Florini et al., 1986), or oncogene activation (Olson et al., 1987; Payne et al., 1987; Gossett et al., 1988; Rahm et al., 1989). The process of cell cycle withdrawal is associated with an upregulation of helix-loop-helix transcriptional regulator proteins such as MyoD and myogenin (Wright et al., 1989), which have been shown recently to interact with the tumor suppressor retinoblastoma protein, pRB, a cell cycle regulator (Gu et al., 1993). Normal differentiation and fusion are associated with modifications of cell surface glycoproteins (Holland et al., 1984) and are inhibited by glycoprotein processing inhibitors (Gillix and Sanwal, 1980; Holland and Herscovics, 1986; Spearman et al., 1987) and lectins (Den et al., 1975; Parfett et al., 1983). Other necessary modifications include alterations in the expression of intercellular adhesion molecules, such as a shift in the isoform of the neural cell adhesion molecule (Dickson et al., 1990), and changes in the level of cadherins (Knudsen et al., 1990; Pouliot et al., 1990). Thus, the L6 myoblast represents a well studied model system which depends on multiple signals, including factors, membrane bound receptors and adhesion molecules, for its differentiation program.

We have isolated stable CEA cDNA transfectant clones of L6 myoblasts and have measured their ability to fuse and differentiate. In agreement with the above model, independently selected transfectants producing relatively low amounts of CEA are unable to differentiate and fuse whereas nonproducers, control CEA anti-sense transfectants, myoblasts transfected with an adhesion-defective NH2-terminal deletion mutant of CEA (CEAAN), and CEA transfectants treated with adhesion-blocking peptides, differentiate and fuse normally.

**Materials and Methods**

**Cell Culture and Differentiation**

The rat L6 myoblast cell line was the kind gift of Dr. P. C. Holland of the Montreal Neurological Institute. All experiments were performed using cells cultured for fewer than six passages after receipt. Cells were grown in monolayer culture in plastic tissue culture flasks or petri dishes containing D-MEM supplemented with 10% horse serum (HS) at 37°C in a humidified atmosphere of 5% CO2 and 95% air. The medium was changed every 2–3 d.

To promote fusion and differentiation, cultures were seeded at 104 cells/cm2 in 100- or 150-mm tissue culture plastic petri dishes and grown in D-MEM + 10% HS without changing the medium for 5 d, after which the medium was replaced with D-MEM + 2% HS and the cells cultured for an additional 3 d. For fusion index determination, cells were fixed with 2.5% glutaraldehyde and stained with haematoxylin and eosin. The fusion index was calculated as the number of nuclei within cells containing three or more nuclei divided by the total number of nuclei counted. A minimum of 500 nuclei in four randomly selected areas was counted for each determination.

**Isolation of Transfectants**

30 μg of full length CEA cDNA (Beauchemin et al., 1987) in the sense or antisense orientation in the expression vector, p91023B (courtesy of R. Kaufman, Genetics Institute, Boston, MA) or 30 μg of CEAA N in pDCDKdhfr (a gift from S. Oikawa, Suntory Limited, Osaka, Japan) and 3 μg of pSV2neo were cotransfected by electroporation with three pulses of 350 mV from 500 μF (Bio-Rad Gene Pulser) into 106 L6 myoblasts. CEAA N has a deletion between the Pvu II and Ace I restriction sites in its cDNA, resulting in the removal of amino acids 32–106 in the 108-amino acid N domain of CEA, and the introduction of an arginine residue between tyrosine residues at positions 31 and 107 (or positions 65 and 141, including the leader [Beauchemin et al., 1987])—(Oikawa et al., 1991). After incubation for 10 min on ice, cells were grown for 48 h in regular growth medium after which positive clones were selected by the addition of 400 μg/ml of the cytotoxic drug G418 (Sigma Chem. Co., St. Louis, MO). To prevent the depletion of myoblasts with high fusion potential, cells were not permitted to achieve confluence or differentiate during transfection and selection. Clones were picked by micromanipulation under direct microscopic vision and were checked for their levels of CEA production using whole cell sonicates and a CEA double monoclonal antibody clinical kit (Abott CEA-EIA Moneclonal; Abbott Laboratories, North Chicago, IL). This assay is highly specific and could not detect the truncated product of the CEAA N cDNA. The latter was therefore analyzed using immunoblotting and immunofluorescence with anti-CEA polyclonal antibody. Verification of cell surface expression of CEA or CEAA N was performed by immunofluorescence with mono- and polyclonal antibodies followed by both microscopic examination and analysis with a fluorocytometer (FACScan, Becton Dickinson, Immunocytometry Sys., Mountain View, CA) as described previously (Zhou et al., 1993).

**Immunoblot Analysis**

Cells from monolayer cultures in the late exponential phase of growth were removed from culture dishes with PBS containing 15 mM sodium citrate, washed twice in cold PBS, and lysed by sonication in PBS at 4°C. The protein concentration of the sonicates was determined by the Bio-Rad protein assay (Bio-Rad Labs., Richmond, CA). 50 μg of each of the samples was boiled in Laemmli's buffer (Laemmli, 1970) for 4 min. Proteins were resolved by electrophoresis (Laemmli, 1970) on a 7.5% Tris-glycine-SDS-polyacrylamide gel under standard conditions. The proteins were transferred to a nitrocellulose membrane (Towbin et al., 1979) and the blot was probed successively with anti-CEA polyclonal rabbit antibody and alkaline phosphatase-conjugated Affipure goat anti–rabbit IgG(H+L) (Jackson Immuno Research Laboratories, Inc., West Grove, PA). The labeled proteins were detected by reaction with 5-bromo-4-chloro-3-indolyl phosphate/nitro-blue tetrazolium substrate (Promega Fisher Scientific, Montreal, Quebec).

**Cell Proliferative Potential**

Replicate plates were seeded with 104 cells/cm2 in 100-mm plates and cultured as above for the induction of differentiation. At intervals cells were removed with trypsin, rendered single cell suspensions and various dilutions plated in 60-mm tissue culture plates, using alpha-MEM (Stanners et al., 1971) plus 10% FCS as growth medium. After 10 d of incubation at 37°C, triplicate plates were fixed in 10% formaldehyde in PBS and stained with 0.1% methylene blue. Colonies of 25 cells or more were enumerated microscopically. The plating efficiency was calculated by dividing the number of colonies obtained by the number of nuclei plated (nuclei rather than cells to allow for cell fusion). The number of nuclei plated was determined from nuclear counts of replicas of the differentiating cultures which were
fixed and stained as described above for the fusion index determination. Values were normalized to the plating efficiency on day 2 for each cell line. The absolute plating efficiency was between 78-93% for all experiments.

**Creatine Phosphokinase Determination**

Monolayer cultures of myoblasts at various stages of differentiation were harvested by scraping into ice cold PBS, sonicated, centrifuged at 15,000 rpm for 5 min and the supernatant kept at -70°C before assay. 25-50 μl of supernatant was analyzed for creatine phosphokinase (CPK) activity using the creatine kinase N-acetyl cysteine-activated ultraviolet system (Boehringer Mannheim Corp., Indianapolis, IN; GmbH Diagnostica) according to the manufacturer's protocol. The protein concentration of sonicates was determined by the protein assay (Bio-Rad Labs.). Results are expressed as μmol/min/mg protein.

**Northern Analysis**

Total cell RNA was isolated by the guanidium isothiocyanate procedure of Chirgwin et al. (1979). 20 μg of RNA was electrophoresed on 1.1 M formaldehyde, 1.5% agarose gels and transferred to nitrocellulose filters (Maniatis et al., 1982). Detection of myogenin and β-actin bands was accomplished with random primer 32P-labeled myogenin cDNA (courtesy of W. Wright, Houston, TX) or chicken β-actin cDNA hybridized at 42°C in 5x SSPE (1x SSPE is 0.18 M NaCl plus 10 mM NaPO₄ [pH 7.7] plus 1 mM EDTA), 5x Denhardt's solution, 50% formamide, 150 μg of heat denatured salmon testis DNA, 0.5% SDS, for 18 h. The membrane was then washed in 2x SSPE + 1% SDS at 20°C, twice in 1x SSPE + 0.1% SDS at 65°C, and twice in 0.1x SSPE + 0.1% SDS at 65°C. To control for the amount of RNA added, blots were stripped and reprobed with the 18S rRNA specific oligomer 5'-ACGGTATCTGATCGTCTTCGAACC-3', end-labeled with 32p using T4-kinase (Maniatis et al., 1982). Hybridization was performed in 5x SSPE, 10x Denhardt's, 250 μg/ml tRNA, 7% SDS, and 10% dextran sulfate, for 18 h at 37°C. The membranes were then washed twice in 1x SSC + 0.1% SDS at 20°C.

**Production of Bacterial Fusion Peptides**

Constructs consisting of the 108–amino acid N domain and the 178–amino acid A1B1, A2B2, and A3B3 domains of CEA fused to the E. coli CTP-CMP-3-deoxy-mannooctulosonate cytidylyltransferase (CKS) gene were expressed in E. coli and the products purified according to the protocol of Hass et al. (1991) as described previously (Zhou et al., 1993). After dialysis against D-MEM, the fusion peptides were added to differentiation medium to a final concentration of 1 mg/ml and incubation continued as indicated. In some cases medium was changed to medium containing fresh peptide after 2 days' incubation.

**Results**

**Effect of CEA Production on Fusion**

L6 myoblasts were cotransfected with pSV2neo and with CEA, antisense CEA, or CEAAN cDNAs, and stable transfectant clones were selected. CEAAN contains a deletion of the last 75 amino acids of the 108–amino acid NH₂-terminal domain, retaining the processed leader sequence, and is completely defective in adhesion function when expressed on the surface of CHO cell transfectants (Oikawa et al., 1991; Zhou et al., 1993). Transfectant clones were picked and cell surface expression of CEA was assessed using polyclonal and monoclonal antibodies. CEA production in positive clones was between 40 and 800 ng CEA/mg protein of total cell lysate using a calibrated double monoclonal antibody assay. This same assay, however, did not detect the product expressed by the mutant CEAAN transfectedants. A Western blot of electrophoretically separated total...
cell proteins from CEA and CEAΔN transfectants probed with polyclonal CEA antiserum is shown in Fig. 1. No CEA bands could be detected in lysates of the parental L6 myoblasts. Two CEA transfectant clones, which produced 104 and 837 ng CEA/mg protein, showed prominent bands of the expected molecular weight for fully glycosylated CEA. Most of the data presented below was obtained using the clone, L6-17, producing 104 ng CEA/mg protein, although similar results were obtained with all transfectant clones producing more than 100 ng CEA/mg protein (Table I). The three CEAΔN transfectant clones displayed intermediate levels of production of a protein with a predictably lower molecular weight (Fig. 1), which was shown by immunofluorescent staining with polyclonal anti-CEA antibody to appear on the surface of the cells (data not shown). Quantitative measures of the cell surface levels of CEA and CEAΔN were provided by cytofluorimetric profiles of various transfectant clones stained with anti-CEA antibody, including a CEAΔN high-producer population, L6(CEAΔN)-H, obtained by FACS sorting of the second peak of the CEAΔN transfectant (Fig. 1).

After reaching confluence and after the serum concentration of the growth medium is reduced from 10 to 2% 5 d after seeding the cultures, L6 myoblasts align and start to fuse into myotubes of multinucleated cells. A dramatic difference was observed in the ability of the L6 CEA transfectants to fuse; after 9 d in culture, these transfectants showed single cells which failed completely to align and fuse, whereas the parental cells and CEAAN transfectants showed extensive fusion (Fig. 2). The quantitation of this phenomenon is presented in Fig. 3. Seventy-nine percent of wild-type L6 myoblasts fused into myotubes by 9 d after plating; control CEAAN transfectants demonstrated a fusion index similar to the parental cells and CEAAN transfectants showed extensive fusion (Fig. 2). The quantitation of this phenomenon is presented in Fig. 3. Seventy-nine percent of wild-type L6 myoblasts fused into myotubes by 9 d after plating; control CEAAN transfectants demonstrated a fusion index similar to that of the parental cells (82%). In contrast, CEA transfectants showed no fusion whatsoever (Fig. 3). A complete absence of fusion was also observed for two other CEA transfectant clones producing more than 100 ng CEA/mg protein whereas a reduced fusion index of 21% was seen for a clone producing 40 ng CEA/mg protein (Table I); all these clones were obtained from independent transfections. Ten independent control clones, including CEAAN, the CEAAN high-producer, CEA antisense, and pSV2neo (only) transfectants, fused normally (Table I and data not shown). The results are thus unlikely to be due to the selection of spontaneous L6 variants that lack fusion ability (Kaufman and Parks, 1977). To rule out this possibility entirely, the nonfusing CEA transfectant L6-17 was cultured in the absence of G418 selection for 43 passages, in order to lose production of CEA; at passage 37, when CEA production was only 0.5 ng/mg, fusion ability returned fully to previous nontransfected levels (79%) (Fig. 2 and Table I); L6-17 maintained under selection for the same number of passages, on the other hand, was still completely unable to fuse (data not shown). The fact that this reversion to full differentiation capacity required loss of virtually all CEA production also shows that very little CEA is required to block fusion. Lastly, growth rate differences were not involved since there was no significant difference in the rate of proliferation of the transfectants compared with the parental myoblasts; in addition, the cell volume of

### Table I. Differentiation Parameters for L6 Transfectants

| Clone            | Transfected construct* | CEA production  | Fusion index | CPK1 (fold increase) |
|------------------|------------------------|-----------------|--------------|----------------------|
| L6               | —                      | 0               | 0.81         | 11                   |
| L6-AN           | pSV2Neo                | 0               | 0.84         | 15                   |
| L6-27           | CEA-antisense          | 0               | 0.78         | 18                   |
| L6-23           | CEA-antisense          | 0               | 0.63         | nd**                 |
| L6-16           | CEAΔN                  | >100†           | 0.82         | 19                   |
| L6-16H          | CEAΔN                  | >300            | 0.50         | nd                   |
| L6-18           | CEA                    | 0               | 0.83         | 13                   |
| L6-73           | CEA                    | 40              | 0.21         | nd                   |
| L6-17           | CEA                    | 104             | 0            | nd                   |
| L6-71           | CEA                    | 212             | 0            | nd                   |
| L6-82           | CEA                    | 837             | 0            | 1                    |
| L6-17-37(−)†     | CEA                    | 0.5             | 0.79         | 10                   |
| L6-17-43(−)†     | CEA                    | 0.2             | 0.76         | 10                   |
| L6-17-N†        | CEA                    | 104             | 0.47         | nd                   |
| L6-17+A3B3†     | CEA                    | 104             | 0.58         | nd                   |
| L6-17+N+A3B3†   | CEA                    | 104             | 0.51         | nd                   |
| L6-17+a1B1†     | CEA                    | 104             | 0.04         | nd                   |
| L6-17+a2B2†     | CEA                    | 104             | 0.01         | nd                   |

* All cell lines were cotransfected with pSV2Neo and expression constructs containing the indicated cDNA.
† The fold increase in CPK activity was calculated relative to the activity of nonfusing L6 cells.
† The L6-17 CEA transfectant maintained in culture without selection with G-418 for 37 and 43 passages, resulting in a reduction in the original level (104 ng/mg) of CEA production.
‡ Fusion peptides CKS-N, CKS-A1B1, CKS-A2B2, and CKS-A3B3 made in bacteria and containing the N, A1B1, A2B2, or A3B3 domains of CEA were added to 1 mg/ml to transfectant cultures in differentiation medium and incubated for 4 d.
§ Estimated from data shown in Fig. 1.
** Not done.

The Jornal of Cell Biology, Volume 123, 1993 470

Proliferating CEA transfectant and parental myoblasts was identical (data not shown).

CEA-mediated adhesion requires a double-reciprocal interaction between the V-like N domains and the membrane-proximal C2-like A3B3 internal domains of antiparallel molecules on apposing cell surfaces; thus treatment with N domain or A3B3 domain peptides made in bacteria can inhibit intercellular adhesion completely, while AIB1 and A2B2 peptides are much less effective (Zhou et al., 1993). Treatment of nonfusing L6 CEA transfectants in differentiation medium with either or both of the N and A3B3 peptides was found to restore differentiation and fusion to 50–60% with approximately the same kinetics as the parental L6 cells when exposed to differentiation medium, whereas the AIB1 and A2B2 peptides were without effect (Table I and Fig. 2). The concentration of the N and A3B3 peptides required to restore fusion capacity to the L6 CEA transfectants (1 mg/ml) was similar to that required to block aggregation of CHO CEA transfectants in suspension (2 mg/ml) (Zhou et al., 1993). These results strongly implicate the necessity for adhesive interactions between CEA molecules on apposed cells to block L6 differentiation and fusion.

**Effect of CEA on Molecular Measures of Differentiation**

Nonfusing myoblasts which still demonstrate all the molecu-
lar changes required for differentiation have been described (Pinset and Whalen, 1984; Olson et al., 1987). To explore the possibility that the nonfusing phenotype in CEA transfectants was due to an uncoupling of fusion from differentiation, we examined the production of CPK, a muscle specific enzyme which is upregulated in differentiated myoblasts (Olson et al., 1983), myogenin, a helix-loop-helix key transcriptional regulator protein whose production is induced early in L6 myoblast differentiation (Wright et al., 1989), and β-actin which is downregulated in developing myotubes (Endo and Nadal-Ginard, 1987).

**Creatine Phosphokinase**

CPK levels (Fig. 4, Table I) rose 10–20-fold after 7 d in culture in both L6 parental cells and in control CEAΔN transfectants. In contrast, CEA transfectants showed no increase in CPK activity and remained at basal levels throughout. CEA antisense, pSV2neo, and L6-A7 transfectants which had lost CEA expression due to prolonged passage without selection showed increases in CPK levels comparable to L6 parental cells (Table I). Thus, ectopic production of CEA completely blocks the normal induction of CPK with differentiation.

**Myogenin**

Northern blot analysis of total cell RNA with myogenin cDNA as a probe (Fig. 5) demonstrated a dramatic rise in myogenin mRNA in the parental L6 myoblasts and control

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**Figure 2.** (A) Depiction of cDNAs and corresponding protein domains for CEA and CEAΔN. (B) Morphology of parental L6 myoblasts, an L6 CEA transfectant clone, the same L6 CEA transfectant clone after 37 passages to lose CEA production, the L6 CEA transfectant clone, treated with 1 mg/ml CEA A3B3 peptide for 4 d and the L6 CEAΔN transfectant clone, under culture conditions promoting differentiation (see Materials and Methods). Cells were fixed in glutaraldehyde and stained with hematoxylin. (Magnification ×200).
transfectants, starting 5 d after plating, i.e., before the large increase in the fusion index (Fig. 3). In contrast, the CEA-producing transfectant clones showed absolutely no increase in myogenin mRNA (Fig. 5). Since the induction of this central transcriptional regulator represents a key early event in the myoblast differentiation program, this result indicates that CEA intercepts the process at a relatively early stage.

**Actin**

The same Northern blot used for the myogenin analysis was probed with β-actin cDNA. The predicted downregulation of actin mRNA in parental L6 myoblasts and control transfectants during fusion and differentiation was observed (Fig. 5). In contrast, the CEA L6 transfectant clone showed no reduction in the β-actin mRNA. The same blot rehybridized with a 24-mer probe specific for 18S RNA is shown to demonstrate that equal amounts of RNA were analyzed in each lane.

These results demonstrate that all three molecular aspects of myoblast differentiation, even relatively early events, were blocked by the production of CEA.

**Effect of CEA on Cellular Proliferative Potential**

The relative plating efficiency for colony formation by L6 myoblasts and various transfectants at different times during the differentiation cycle was measured (Fig. 6). The plating efficiency of the parental myoblasts and CEAΔN transfectants declined markedly with time in culture and with kinetics which indicate that irreversible withdrawal from the cell cycle, as measured by this assay, precedes fusion and differentiation. The residual proliferative capacity of ~20% at day 9 reflects the proportion of nonfused cells. Comparable declines in plating efficiency were seen in control pSV2neo transfectants (data not shown). In contrast, CEA transfectant clones retained their proliferative capacity, maintaining a relative plating efficiency of ~75% until day 9. Thus the expression of CEA abrogated terminal differentiation and allowed cells to maintain their division potential.

**Discussion**

The overexpression of CEA family members in many types of human tumors and their function in vitro as intercellular adhesion molecules raise the possibility that this correlation
Figure 5. Myogenin and actin mRNA levels in L6 parental myoblasts and transfectants with time in culture. L6 myoblasts (L6), L6 transfected with PSV2neo alone (L6neo), or L6 transfected with CEA (L6 CEA+), were grown in culture under conditions to promote fusion. At the indicated intervals, cells were harvested, and total cell RNA was prepared. Aliquots of the RNA were resolved by electrophoresis, transferred to a nitrocellulose membrane, and hybridized successively with probes for myogenin and β-actin, under high stringency conditions. The same blot as rehybridized with a labeled 18S ribosomal RNA-specific oligomer to give an independent measure of the amount of RNA electrophoresed in each lane. The major band just below the 18s marker represents the myogenin transcript (Wright et al., 1989); the significance of the other bands is not known.

Figure 6. Plating efficiency of L6 parental myoblasts and transfectants with time in culture. Parental L6 myoblasts (m) and CEA (●), or CEAΔN (▲), transfectants were plated and grown under conditions to promote fusion. At the indicated intervals the mean plating efficiency (colonies/nucleus plated) was determined. Values shown are the mean of at least three determinations and are normalized to the plating efficiency at day 2. Error bars represent the standard error of the mean. The fusion index curve for L6 parental myoblasts (---) is shown for comparison.

Eidelman et al. L6 Differentiation Blocked by Adhesion Molecule, CEA
other adhesion molecules required for fusion are not re-
the process (Dickson et al., 1990). As mentioned above, the
molecules or intracellular Cendo') by interception of normal
intercellular adhesive function of CEA is necessary for the
presence on the surface of a new adhesion molecule, CEA.

The interference envisioned could be extracellular ("ecto")
by direct interaction of CEA with other cell surface mole-
cules or intracellular ("endo") by interception of normal
transduced molecular event sequences by a CEA-induced se-
quence of cell fusion so that blockage of fusion would natu-
rally lead to retention of division capacity. This effect of
CEA again requires an intact NH2-terminal domain, i.e.,
its 28 N-linked sugar side chains. It could thus introduce a
negative charge in the intermembrane space which could in-
terfere with the adhesive function of other adhesion mole-
cules. This hypothesis has been suggested for the antiadhe-
resive effects of N-CAM seen in some situations, in this case
because of its highly negatively charged specialized poly-
sialic acid structure attached to a domain close to the cell
membrane (Rutishauser et al., 1988). Again, if this is the
mechanism of the CEA effect, it must also require CEA in-
termolecular adhesion since very high levels of the nonadhe-
sive CEAN molecule, with almost as many charged residues as CEA itself, on the cell surface allow L6 differen-
tiation. Adhesion by CEA molecules could be required,
however, since it would produce high local concentrations of
charge at apposed regions of the membranes, so that this
mechanism still seems plausible. Experiments are in prog-
ress to study directly a potential role of the terminal sialic
acid residues in CEA.

Considering endo effects, the failure of the CEA transfec-
tants to withdraw irreversibly from the cell cycle suggests
that CEA production inhibits a terminal differentiation path-
way which normally is induced after the cells reach conflu-
ence and experience a deficiency of mitogenic stimuli. This
could be due to the stimulation by CEA of a competing path-
way, thus representing an endo effect of CEA; BGP, a closely
related family member with transmembrane and cytoplas-
mic domains, for example, has been suggested to be as-
associated with protein tyrosine kinases in the cell membrane
(Afar et al., 1992) and recent results have shown an associa-
tion of CEA itself with src—family kinases (Draber, P., and
C. P. Stanners, unpublished results). Alternatively, loss of
proliferative capacity could simply be a terminal conse-
quence of cell fusion so that blockage of fusion would natu-
rally lead to retention of division capacity. This effect of
CEA again requires an intact NH2-terminal domain, i.e.,
presumably its intercellular adhesion function, given that the
CEAN transfectants lost cell division potential normally.

Additional work will be required to elucidate the nature of
the signals required for differentiation and loss of division po-
tential which the CEA—CEA interactions on the cell sur-
face distort or perhaps block. The generality of the phenom-
enon for the ectopic expression of other adhesion molecules
in other differentiating systems also deserves investigation.
Regardless of the mechanism involved, the failure to with-
draw from the cell cycle and differentiate suggests that aber-
rant expression of CEA, a human tumor marker, could rep-
resent one step in the progression of a cell towards an overt
malignant phenotype.

We thank Drs. P. C. Holland, O. Blaschuk, and Y. Pouliot for helpful dis-
cussions and Dr. Nicole Beauchemin for critical reading of the manuscript.

This work was supported by grants from the Medical Research Council of
Canada and the National Cancer Institute of Canada. F. Eidelman was
supported by a Medical Research Council of Canada Clinician Scientist
award.

Received for publication 22 February 1993 and in revised form 6 July
1993.

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