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Suppression of C8161 Melanoma Metastatic Ability by Chromosome 6 Induces Differentiation-Associated Tyrosinase and Decreases Proliferation on Adhesion-Restrictive Substrates Mediated by Overexpression of p21WAF1 and Down-Regulation of bcl-2 and Cyclin D3

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Metastatic tumors grow under conditions that restrict proliferation of non-metastatic, more differentiated cells. To investigate this prediction, we developed a simple adhesion-restrictive assay which allows proliferation of human metastatic C8161 melanoma, but prevents growth of neo 6.3/C8161 cells in which metastasis is suppressed by introduction of *neo*-tagged chromosome 6. We show that tyrosinase, a key enzyme in melanocytic cell differentiation, and expression of chromosome 6-encoded cell cycle modulators like p21WAF1 and cyclin D3 is selectively increased in C8161 tumors in which metastasisis is suppressed by chromosome 6. In the latter cells, growth arrest evidenced only under adhesion-restrictive conditions correlated with down-regulation of cyclin D3 and anti-apoptotic bcl-2. No comparable growth arrest or down-regulation was detected under comparable conditions in metastatic cells, which showed activation of invasion-associated MMP-9 92 kDa gelatinase B. Our data suggests that the metastasis-suppressing effects of chromosome 6 involving increased differentiation-associated tyrosinase and growth arrest on adhesion-restrictive substrates; are partly mediated by modulation of growth regulators, like p21WAF1 and cyclin D3.

Key Words: **metastasis suppression; invasion-associated MMP-9 gelatinase; differentiation; growth control.**

Metastasis is the culmination of the multistep process of tumor progression toward increasing malignancy. The ability to colonize a secondary site requires that cells successfully accomplish several highly restrictive tasks. Among them, metastatic tumor cells must survive harsh transit circumstances and then proliferate under conditions that do not allow growth for other tumor cells. Since differentiation and uncontrolled proliferation may be mutually exclusive, it follows that differences in differentiation-associated gene expression and behaviour under suboptimal or growth-restrictive conditions might be used to discriminate metastatic from nonmetastatic tumor subpopulations. Based upon the prediction that metastatic cells would adhere better and proliferate more under suboptimal adhesive conditions than their benign or normal counterparts, we adapted a cell culture assay (1) to test this hypothesis. Many normal cells do not adhere well to untreated polystyrene. Under conditions where they lack contact with an adhesive matrix, normal melanocytes undergo apoptosis (anoikis) (2, 3). In contrast, highly metastatic human melanoma C8161 cells not only adhered, but continued to proliferate. No comparable proliferation was seen after chromosome 6-mediated suppression of C8161 melanoma metastatic ability (4). Poorly metastatic or nonmetastatic melanoma cells obtained by introduction of chromosome 6 exhibited an intermediate phenotype: The cells were growth inhibited and aggregated, but did not undergo apoptosis by anoikis. Collectively, these data confirmed the finding that transformed cells are less susceptible to anoikis when grown on poorly adhesive substrates, and also suggested that this assay may be useful for discriminating cells at different stages of tumor progression (i.e., nonmetastatic from metastatic). To validate these assays and provide insights into the mechanisms by which introduced chromosome 6-mediated metastasis-suppression functions, we investigated whether expression of chromosome 6-encoded p21WAF1 (5) and cyclin D3 (6) is affected. We also investigated whether

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adhesion-restrictive conditions and this kind of metastasis suppression influenced expression of specific components important in the control of tumor cell growth and differentiation, extracellular matrix degradation, and apoptosis.

MATERIALS AND METHODS

Cells. C8161 is an amelanotic human melanoma cell that metastasizes widely following either subcutaneous (s.c.), intradermal (i.d.), or intravenous (i.v.) injection into athymic or SCID mice (Harlan Sprague Dawley, Indianapolis, IN). C8161.09 and C8161 transfected with empty neo vector are subclones isolated from C8161 by limiting dilution (4). Tum^+/Met^- neo6/C8161.2 and neo6/C8161.3 cells were derived after introduction of a neomycin-tagged normal human chromosome 6 by microcell-mediated chromosome transfer (4). Metastatic potential of the transfectants was significantly suppressed, but controls transfected with only vector were as metastatic as parental C8161. Specifically, neo 6/ C8161 cell metastatic potential was inhibited by at least 90% compared to the parental C8161 cell line (4, 5).

All cells were cultured in Dulbecco's modified Eagle's medium mixed 1:1 (*v/v*) with Ham's F12 medium (Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum (Life Technologies or Atlanta Biologicals, Atlanta, GA). neo6/C8161.3 cells and those transfected only with neo vector, were cultured in the presence of 500 μg/ml Geneticin (G-418, Life Technologies). Cells were routinely tested and found to be free of *Mycoplasma* contamination by the GenProbe assay (Fisher Scientific, Pittsburgh, PA).

Differential adhesion to substrate. Polystyrene bacterial-grade petri dishes (VWR Scientific, Cat #25384), when untreated or when coated with bovine serum albumin (10 mg/ml in phosphate-buffered saline for 24 h) (1), allowed spreading and proliferation of metastatic cells and favored formation of unanchored or poorly attached aggregates in non-metastatic variants of C8161 (1). Under similar conditions, anoikis was seen in normal melanocytes, Melan-A, as recently demonstrated for other normal human melanocytes (3).

The effect of differential adhesion on cell growth was assessed by measuring incorporation of 25 μCi ^{3}H-thymidine into DNA for 4 h. The results of this assay correlated well with cell number as determined by counting with a hemacytometer after detachment with hyaluronidase (0.1%) and trypsin (0.1%) in 0.15 M NaCl (pH 7.2). Relative DNA levels were confirmed fluorometrically.

Immune blotting. Cell extracts (150 μg protein) were prepared in lysis buffer (50 mM Tris–HCl pH 8, 120 mM NaCl, 50 mM NaF, 0.1 mM sodium vanadate, 5 mM EDTA, 10 μg/ml each of leupeptin, soybean trypsin inhibitor and aprotinin, 1 mM phenylmethylsulfonylfluoride, 0.4% NP-40), separated on 12% SDS–PAGE gels and bidirectionally blotted onto replica nitrocellulose membranes as described previously (7). After transfer, membranes were blocked with PBS containing 0.5% Tween-20 and 5% non-fat skim milk. Antibodies against p21WAF1, *bax* and *bcl*-2 were all purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody PEP 7 vs differentiation-associated tyrosinase, previously described (7) was kindly provided by Dr. V.Hearing, National Cancer Institute, Bethesda, MD. Whenever necessary, blot pairs were erased by heating (70°C; 30 min) with 62 mM Tris–HCl, 2% SDS, 100 mM β-mercaptoethanol followed by extensive washing with 0.1% Tween-80 in TBS, reblocking with skim milk and reaction with a different set of primary antibodies. In each case, detection was accomplished by reaction with protein A-peroxidase and ECL chemiluminescence (Amersham, Piscataway, NJ).

Gelatinase activity. Prior to assay, cells were seeded overnight in medium, without serum plus ITS supplement (insulin-transferrin-selenium) (Gibco # 71200-042) on 96 well microtiter tissue culture plates or on 96 well Falcon 3912 microtest III coated with 10 μg/ml of either laminin, chondroitin sulphate A, chondroitin sulphate B, or elastin (all tissue culture grade, from Sigma Chemical Co, St.Louis, MO). Activity assays were carried out at comparable concentrations (100 μg protein) in extracts lysed in RIPA buffer without protease or phosphatase inhibitors (9). After lysis, samples were run without prior boiling or exposure to reducing agents on 10% SDS–polyacrylamide gels containing copolymerized gelatin (2 mg/ml). After electrophoresis at 4°C, gels were washed 4 times in 2.5% Triton X-100, and left overnight in 0.15 M NaCl, 50 mM Tris–HCl p H 7.5, 10 mM $CaCl_2$, 0.05% Na N3. Then, gels were stained with 0.05% Coomassie blue to reveal areas of degraded gelatin (8, 9).

RESULTS

Metastatic, but Not Non-Metastatic Cells, Spread and Proliferate on Poorly Adhesive Substrate

We asked whether melanoma cell variants, isolated from the same tumor but with differing metastatic ability, (4, 5) exhibit differences in their proficiency to proliferate on substrates that usually do not support adhesion or proliferation of normal melanocytes. On bacterial grade, polystyrene dishes (± precoating with bovine serum albumin (10 mg/ml), normal, anchorage-dependent Melan A melanocytes not only failed to adhere but died by anoikis (i.e., condensed eccentric chromatin identified by Hoechst 33258 staining in 85% of cells; faster electrophoretic DNA migration; data not shown). Similar findings have been presented by others (3). In contrast, tumorigenic variants remained viable but exhibited differences in their abilities to spread and proliferate according to their metastatic propensity. Metastatic C8161 (parental, vector-only transfected and clone C8161.9) cells showed similar spreading and adherence under adhesion-permissve (+) or restrictive conditions (−); as exemplified with C8161 cells (lower Fig. 1). In contrast, non-metastatic neo6/C8161.2 and neo6/C8161.3 cells obtained by introduction of neo-tagged chromosome 6, failed to spread and adhere on the restrictive (−) substrate, showing increased cell–cell interaction (i.e., clumping, homotypic aggregation, as exemplified for the neo6/C8161.3 cells (lower Fig. 1). Whereas permissive conditions allowed a similar proliferation in either metastatic variant, the adhesion-restrictive substrate (−) inhibited growth preferentially in the non-metastatic neo6.3 than in metastatic C8161 (upper Fig. 1).

Cell counts confirmed that the metastatic (C8161 parent, vector-only transfected C8161 and C8161.9) cells proliferated on the non-permissive substrate at a rate about 10–20% below that of the same cells growing on tissue culture plates during the first 40 h; under comparable conditions non-metastatic cell growth was significantly more suppressed. Cell number in aggregates was determined by hemacytometer counts following dispersion with a solution containing hyaluronidase (0.1%) and trypsin (0.1%). Also, differential proliferation was measured by ^{3}H-thymidine incorporation. In several experiments, the vast majority (75–

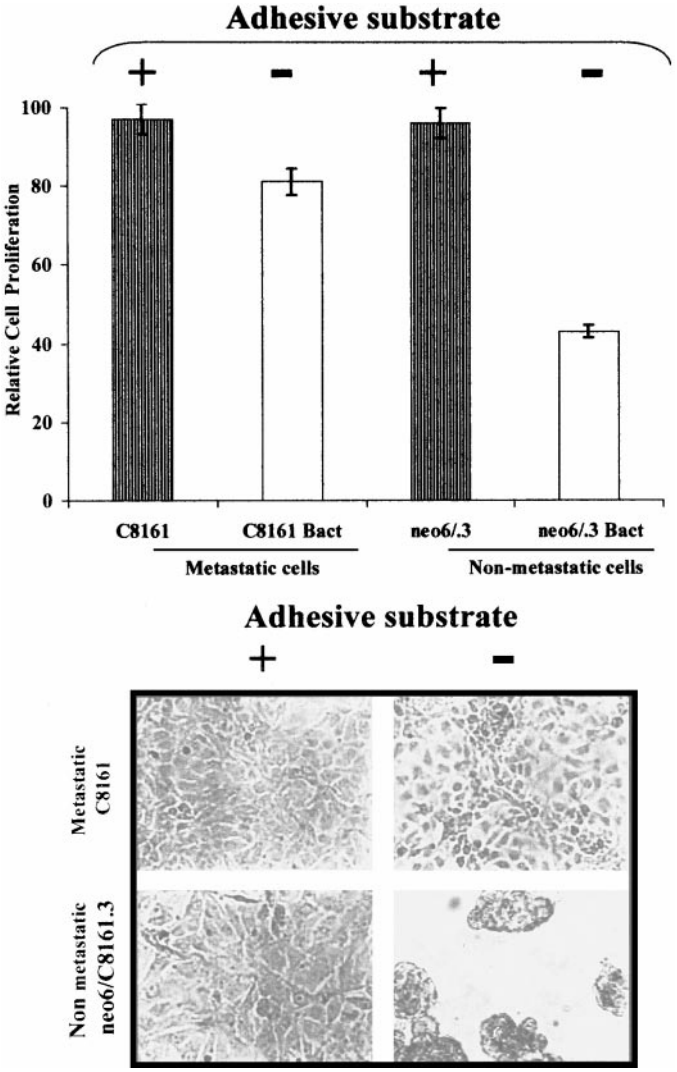

FIG. 1. Differential growth of human melanoma cells on adhesion-restrictive substrates. The upper panel shows relative average cell numbers, 40 h after seeding replicate cell aliquots in triplicate onto BSA-coated, bacterial grade petri dishes (−) or tissue culture plates (+). Note: The metastatic cells adhered and proliferated more on both substrates whereas non-metastatic cells only adhered and proliferated on the TC plates. The lower panels are representative photomicrographs of metastatic (C8161, vector-only transfected or C8161.9) cells and nonmetastatic neo6/C8161.3 (neo/6.3) or neo6/C8161.2 on adhesive tissue culture (+ or TC) or adhesion-restrictive bacterial (− or Bact) plates. Note also that the non-metastatic cells tended to aggregate and coalesce on the non-adhesive substrate.

90%) of metastatic cells were actively dividing on the non-adhesive substrate. In contrast, proliferation was significantly reduced (50%) for non-metastatic (neo6/C8161.2, and neo6/C8161.3 cells grown under the same conditions, as exemplified for neo6/C8161 in Fig. 1. Essentially identical conclusions were drawn in different experiments, if relative DNA content, measured fluorometrically, was used as an index for cell number (not shown).

Expression of Differentiation-Associated Tyrosinase Is Increased with Metastasis-Suppression

Since uncontrolled proliferation is mutually exclusive with differentiantion, and tyrosinase is a key enzyme in melanocyte-specific pigmentation (7), we investigated whether the greater proliferative ability of metastatic C8161 melanoma apparent on the adhesion-restrictive substrate correlated with lower levels of tyrosinase. In unpigmented C8161 melanoma, we detected two main isoforms of about 70 and 55 kDA (7). Both of these forms were barely detected in metastatic C8161 cells, but appeared greatly increased in neo 6.3/C8161 non-metastatic cells, obtained by introduction of neo tagged chromosome 6 fragments (4). This increase in tyrosinase in metastasis-suppressed C8161 melanoma, appeared constitutive, because it was apparent both under adhesion-permissive or restrictive conditions (Fig. 2).

Gelatinase B Is Inducible on Extracellular Matrix Components Only in Metastatic C8161 Cells

Gelatinase B (also known as 92 kDa collagenase or MMP-9) activity is reportedly expressed or is inducible in advanced metastatic melanomas, but not in early stage tumors (8). Inducibility of gelatinase B by UV

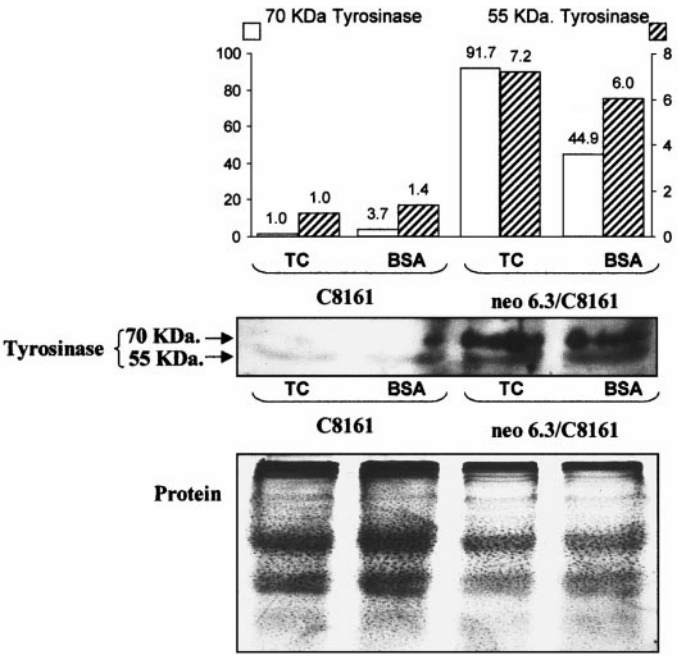

FIG. 2. Substrate-independent induction of Tyrosinase with chromosome 6 suppression of metastatic ability in metastatic cells under adhesion-restrictive conditions. Metastatic variants were cultured under adhesion-permissive (TC) or restrictive (Bact) conditions, for immune blotting of tyrosinase as indicated under Materials and Methods. Note significant increase in tyrosinase expression in neo6/C8161.2 cells, both under adhesion-permissive or -restrictive conditions with reference to lower protein profiles.

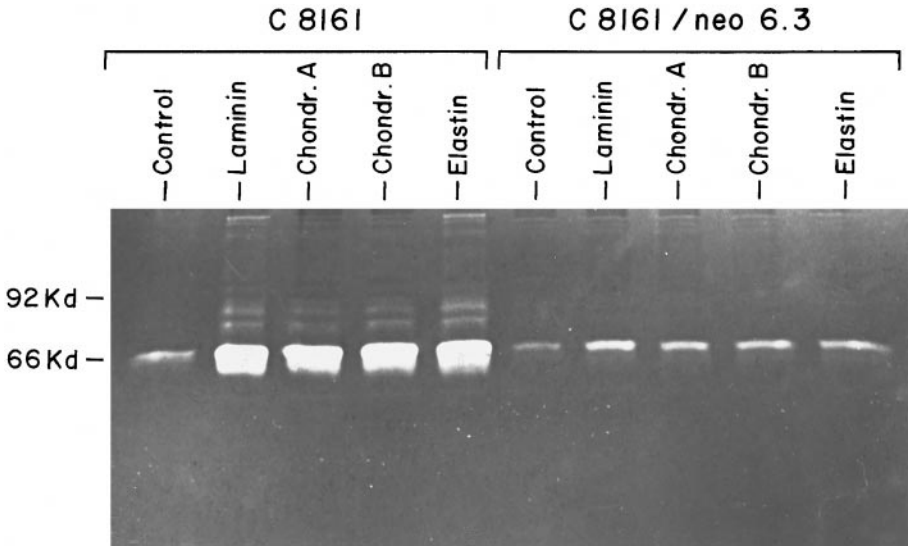

FIG. 3. Gelatinase B (MMP-9, 92 kD collagenase) is selectively induced in metastatic cells under adhesion-restrictive conditions. Gelatin zymography of C8161 and neo6/C8161.3 cells grown on adhesion-permissive (tissue culture plates) or bacterial-grade petri dishes) coated as indicated in each experiment. Note increase in MMP-9 activity and larger gelatinase activities only in metastatic C8161 cells cultured with specific extracellular matrix components.

treatment was compared in C8161 cells and chromosome 6-transfected C8161 cells. Induction of membrane-bound gelatinase B was measured by solubilization in detergent-containing extracts as described (8). MMP-9 was detected in metastatic C8161 cells, but only when they were cultured on various extracellular matrix components described in Fig. 3. No comparable activities were seen in the non-metastatic cells cultured under comparable conditions (Fig. 3). This result is consistent with prior reports showing inducibility of 92 kDa gelatinase only in C8161 metastatic cells (8, 9) and further validates that the *in vivo* metastasis suppression mediated by chromosome 6 indeed correlates with inability to induce MMP-9 gelatinase B (8, 9) and other large, slow-migrasting gelatinases (Fig. 3).

Selective Down-Regulation of bcl2 and Cyclin D3 Is Associated with Decreased Cell Proliferation of Non-Metastatic Cells under Adhesion-Restrictive Conditions

Since metastatic cells are migratory, modulation of extrinsic signals will impact expression of genes that control the balance between growth, cell cycle checkpoints, and programmed cell death. To determine whether the failure of nonmetastatic cells to spread or proliferate on a poorly adhesive substrate correlates with changes in the expression of genes involved in growth promotion (cdk4, and D-cyclins (10) or cell death (*bcl*2, *bax* (2, 11)), bidirectional immune blot analysis was conducted (10). On non-adhesive substrates, expression of the apoptosis inhibitor *bcl*2 by nonmetastatic cells decreased. Expression of the pro-apoptotic gene *bax* was unaffected (Fig. 4). In the metastatic parental C8161 cells, expression of *bax* and *bcl2* did not differ on the substrates. On the other hand, cdk4 and cyclin D1 were comparable between metastatic and non-metastatic cells grown irrespective of adhesion-restrictive or permissive conditions (Fig. 5).

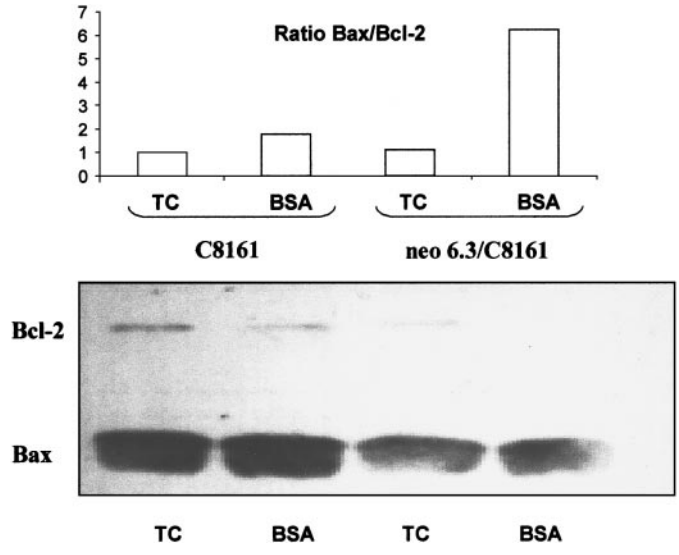

FIG. 4. Bcl-2 is preferentially decreased in non-metastatic cells under adhesion-restrictive conditions. Relative expression of bax and bcl-2 was measured after bidirectional immune blotting and densitometric analysis in C8161 and neo6/C8161.3 cells cultured under adhesion-permissive (tissue culture plates) or adhesion-restrictive (BSA-coated bacterial-grade petri dishes). Note that the bax/bcl-2 ratio is selectively increased under adhesion-restrictive conditions in non-metastatic cells.

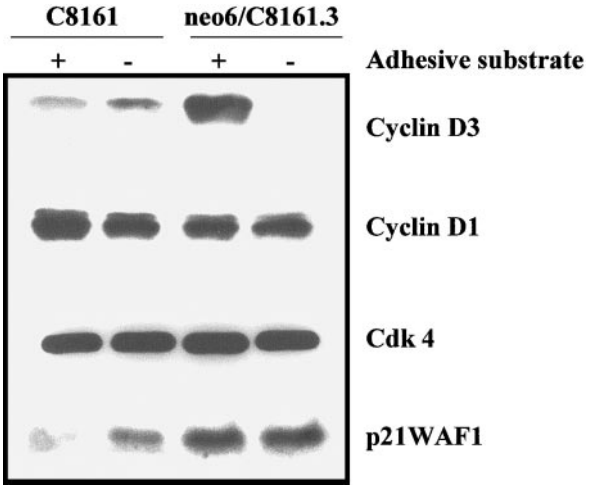

FIG. 5. Overexpression of p21WAF1 and down-regulation of cyclin D3 in non-metastatic cells under adhesion-restrictive conditions. Relative expression of cyclin D3, cyclin D1, cdk 4, and p21WAF1 was measured after bidirectional immune blotting in C8161 and neo6/C8161.3 cells cultured on adhesion-permissive (+) tissue culture plates or adhesion-restrictive (−) BSA-coated bacterial-grade petri dishes. Note that p21WAF1 is selectively increased in non-metastatic cells, and cyclin D3 is decreased in the latter cells under adhesion-restrictive conditions.

Similarly, expression of chromosome 6-encoded p21WAF1 (5) and cyclin D3 (6) genes was clearly increased on the adhesion-permissive substrate in neo 6.3/C8161 cells in which metastasis was suppressed by chromosome 6. However, cyclin D3 levels decreased when these cells were placed on adhesion-restrictive bacterial petri plates (Fig. 5).

DISCUSSION

The most lethal attribute of cancer cells is their ability to disseminate to distant sites. To metastasize, cancer cells must detach from the primary tumor, disseminate, arrest and colonize a secondary site. This is an extremely inefficient process (i.e., $<0.01\%$ of cells entering the blood stream successfully form clinically detectable macroscopic metastases (12, 13). Given the highly selective nature of the process, we predicted that the rare cells which successfully metastasize are more likely to survive and proliferate under suboptimal conditions than their non-metastatic counterparts.

To test this hypothesis, metastatic melanoma (C8161, C8161 transfected with neo empty vector and C8161.09) and related, but metastasis-suppressed cell lines transfected with neo-tagged chromosome 6 neo6.3/C8161) were evaluated for their ability to spread and proliferate on a poorly-adhesive substrate (BSA-coated polystyrene dishes) compared to their proliferation on permissive, tissue culture dishes. Results were compared to normal melanocytes (Melan A) cultured under identical conditions.In agreement with others (3) normal Melan A cells died when placed on BSA-coated plates (not shown). Metastasis-suppressed cells survived, but were growth inhibited. The morphology of the non-metastatic cells was also clearly different than that of the metastatic cells. Non-metastatic melanoma tended to cluster 24–36 h after plating while the melanocytes remained as individual cells and died by anoikis (3). In some experiments, as in the case of neo6/C8161.2 non-metastatic cells, they retracted readily if the plates were removed for 15–30 min from the incubator, an effect not seen with metastatic cells (Fig. 2). This suggests that: (a) the abilities of metastasis-suppressed cells to survive under suboptimal conditions may be dependent, at least in part, upon their ability to compensate by intercellular aggregation (14); (b) both neo6/C8161.2, neo6/C8161.3 showed inhibition of proliferation under adhesion-restrictive conditions. These observations clearly contrast with results obtained using metastatic melanoma (C8161 and C8161 transfected with neo empty vector). All the latter still adhered, spread well and proliferated on bacterial or BSA-coated bacterial culture dishes. Collectively, these results demonstrate (in the human C8161 melanoma, at least) that there is a correlation between the ability of tumor cells to proliferate on poorly adhesive substrates (1) and higher grade malignancy *in vivo* (4, 5).

For at least six days during culture on the adhesion-restrictive substrate, the morphology and growth rate of non-metastatic cells can be restored when the cells are plated back onto tissue culture plastic. It is not known how long the cell aggregates would remain in this semi-quiescent state, whether the cells would eventually undergo apoptosis, or whether metastatic variants might emerge from the population. The answers to these questions will require further experimentation but may provide insights into the phenomenon of dormancy of metastases, involving reciprocal regulation of cyclin D3 and p21WAF1.

Nonetheless, our results suggest two things. First, it appears that oncogenesis confers upon cells the ability to withstand poorly adhesive conditions, at least temporarily. This is consistent with previous studies showing increased propensity for anchorage-independent growth in soft agar. Second, the results suggest that differential proliferation on a poorly adhesive substrate may be an inherent property of metastatic cells, but resiliency requires verification using other models. The assay presented here is based upon differential spreading and proliferation on an adhesion-restrictive one-dimensional substrate unlike other assays that used three-dimensional agar, agarose or poly HEMA layers (reviewed in ref. 15). It should be noted that increased growth in soft agar (i.e., anchorage-independent growth) neither measures differential spreading, nor does it necessarily correlate with metastatic propensity (reviewed in ref. 16). Collectively, these findings show the importance of cell adhesion and

spreading in cancer metastasis, and they also highlight the complexity of such role(s).

Patterns of gene expression on the restrictive substrates were determined with particular focus on invasion-, differentiation-, growth control-, and apoptosis-related molecules. Prior studies demonstrated that 92 kDa gelatinases are important in the spreading of human metastatic melanoma tumors (8, 9). Here we show that adhesion-restrictive conditions and UV-mediated stress induce 92 kDa gelatinase B activity in metastatic, but not in the metastasis-suppressed melanoma cells. We also investigated whether the decreased proliferation of non-metastatic neo 6.3/C8161 on the adhesion-restrictive substrate was associated with an increased differentiation in these cells. For this, we compared expression of tyrosinase, a key enzyme in melanocyte-specific pigmentation (7). This revealed no influence of the different substrate on tyrosinase expression, but its clearly greater expression in non-metastatic cells.

The slower proliferation of non-metastatic cells under adhesion-restrictive conditions, also correlated with preferential down-regulation of the anti-apoptotic protein *bcl*2 rather than with an increase in pro-apoptotic bax. Although others have correlated apoptosis with increases in bax/bcl-2 ratio (11), in these studies, a greater level of bax to bcl-2 induced by the adhesion-restrictive substrate in non-metastatic cells is interpreted to correlate with propensity rather than actual execution of apoptosis by anoikis on a poorly permissive substrate (2, 3). Under the latter conditions, perhaps overexpression of the cyclin-dependent kinase inhibitor p21WAF1 (5) concomitant with down-regulation of cyclin D3 (6) promote growth arrest (Fig. 1) which should attenuate apoptosis. Others recently reported that overexpression of either p21WAF1 or that of bcl-2, can promote resistance to apoptosis in lung cancer cells (17). Also, increased intercellular interactions (14) seen in non-metastatic cells under adhesion-restrictive conditions contribute to diminish immediate execution of apoptosis in these cells (Fig. 1).

The results presented here clearly demonstrate that C8161 metastatic cells are more resilient to suboptimal adhesion conditions than their nonmetastatic counterparts. This is to be expected since they must survive harsh conditions during transit to secondary sites. By using a relatively simple modification of culture conditions, we have been able to mimic some aspects of the stresses (i.e., poorly adhesive substrates) encountered while tumor cells metastasize. The results with adhesion-restrictive substrates help to confirm that metastatic cells are distinct from their non-metastatic but tumorigenic counterparts. In summary, these studies define that chromosome 6-mediated suppression of melanoma metastasis involves not only a modulation of chromosome 6-encoded p21WAF1 (5) and cyclin D3 (6), known as regulators of differentiation (18, 19) but also induce tyrosinase, known to be associated with melanocytic differentiation (7). These results also support the notion that the suppression of metastasis by restoration of some chromosome 6 functions affects the ability of metastatic tumors to proliferate on adhesion-restrictive substrates. Additional application of these approaches should be helpful in establishing novel molecular markers to distinguish characteristics unique to cancer cells at different stages of tumor progression, and to characterize new genes and pathways involved in controlling tumor cell proliferation, differentiation and apoptosis, subject to regulation by adhesion-restrictive substrates.

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