5-Bromo-2-deoxyuridine regulates invasiveness and expression of integrins and matrix-degrading proteinases in a differentiated hamster melanoma cell

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SUMMARY

Cell interactions with the extracellular matrix play a critical role in regulating complex processes such as terminal differentiation and tumor progression. In these studies we describe a melanoma cell system that should be useful in addressing the regulation of cell-matrix interactions and the roles they play in regulating differentiation and cell invasiveness. CS (suspension)-1 melanoma cells are relatively well differentiated: they are melanotic, responsive to melanocyte-stimulating hormone, and express TA99, a melanosome membrane differentiation marker. Their repertoire of integrin receptors for extracellular matrix ligands is limited; in particular, they lack receptors for vitronectin, accounting for the observation that they are nonadherent when cultured in the presence of serum. CS-1 cells are noninvasive as well, and express low levels of both metalloproteinases and activated plasminogen activators. Treatment of these cells with melanocyte-stimulating hormone causes them to increase melanin production and assume an arborized phenotype, suggesting that it promotes their further differentiation. In contrast, treatment of CS-1 with the thymidine analog 5-bromo-deoxyuridine, converts them to a highly invasive cell population (termed BCS-1) that loses its differentiated properties and responsiveness to melanocyte-stimulating hormone, acquires a broad integrin repertoire (including vitronectin receptors), and expresses elevated levels of metalloproteinases and activated urokinase. From these observations and findings of others on BrdU treatment of other developmental lineages, we hypothesize that BrdU both suppresses differentiation and promotes invasiveness of CS-1 cells. The demonstrated manipulability of CS-1 cells should make them extremely useful for studying the regulation of both terminal differentiation and tumor progression in the melanocyte lineage.

Key words: melanocyte differentiation, invasion, adhesion receptors, proteinases

INTRODUCTION

Interactions of cells with extracellular matrix (ECM) play a critical role in cell behavior, growth control, and differentiated cell function. Cells interact not only with ECM ligands but also with matrix-bound components such as growth factors, proteinases, and their inhibitors. Although the underlying mechanisms are poorly understood, it is clear that growth factors can regulate the expression of ECM constituents and their receptors as well as proteinases (Heino et al., 1989) and that extracellular matrix, through its cell-surface receptors, can regulate the expression of growth factors, proteinases and tissue-specific gene products (Webb et al., 1990; Werb et al., 1989; Streuli et al., 1991; reviewed in Damsky and Werb, 1992). Thus, the regulatory hierarchies that govern processes such as terminal differentiation or tumor progression are complex.

Analyses of related tumor cell lines, such as the leukemias, which display distinct repertoires of differentiation markers characteristic of their tissue origin have been useful both for the study of tumor progression and for increasing our understanding of the normal differentiation of such cells. A similar effort is in progress to understand both melanoma tumor progression and normal differentiation of melanocytes. Thus, Houghton et al. (1982, 1987) proposed a putative melanocyte differentiation pathway based on the staining patterns of several monoclonal antibodies which differentially recognize melanoma cell lines that appear to be relatively well or relatively poorly differentiated. Albelda et al. (1990) examined the repertoire of integrins expressed by melanotic lesions at different stages.
of progression to a metastatic phenotype. The most consistent change accompanying the transition from the relatively benign radial growth phase to the much more aggressive vertical growth phase was a shift to expression of the αvβ3 integrin, which recognizes several ligands including vitronectin, fibrinogen, and thrombospondin. Using the M21 melanoma cell line and cloning out a cell line from it (M21L) that did not express αvβ3 integrin, and which does not express αvβ5 integrin, allowed us to test the hypothesis that ω subunit was a key component in the regulation of these cells. The results suggest both that the integrin repertoire expressed by melanoma cells is relevant to their invasive phenotype, and that understanding how interactions of cells of the melanocyte lineage with the ECM are regulated should be instructive for understanding melanocyte differentiation and tumor progression.

The studies presented here characterize a melanoma cell system, first described by Farishian and Whittaker (1979) and Knudsen et al. (1982) that should be particularly useful in addressing the regulation of cell-matrix interactions and their roles in regulating differentiation and cell invasiveness. A pivotal cell in this analysis is the CS (suspension)-1 cell. Our experiments show that the thymidine analog 5-bromodeoxyuridine (BrDU), known to regulate differentiation of many embryonic cell types, can reversibly convert this relatively well-differentiated, poorly adhesive, noninvasive melanoma cell line into a poorly differentiated, migratory, and invasive melanoma line (BCS-1). In the course of this conversion, the expression of differentiation-specific markers and of several integrins and proteinases is altered, such that the BrDU appears to regulate a specific pathway relevant to differentiation and invasiveness. In contrast, treatment of the CS-1 cells with melanocyte-stimulating hormone (αMSH) results in increased melanin expression and restricted growth rate, characteristics suggesting terminal differentiation.

**MATERIALS AND METHODS**

**Antibodies and reagents**

Fibronectin (Fn) and vitronectin (Vn) were purchased from Telios Pharmaceuticals. Laminin (Ln), purified from the Engelbreth-Holm Swarm (EHS) tumor, was kindly provided by Drs D. Hall and M. Reynolds, UCSF, or purchased from Collaborative Research (Lexington, MA). Collagen type IV (Col IV) was purchased from Collaborative Research. Fibrinogen (Fbg) was provided by Dr I. Charo, Cor Therapeutics (So. San Francisco, CA). Antibodies and their sources were: anti-integrin β1 cytoplasmic domain peptide, Drs K. Tomasselli and L. Reichardt, UCSF; anti-αv cytoplasmic domain peptide, Dr R. O. Hynes, MIT (Plantefab and Hynes et al., 1989); B1E5 rat anti-human α5 monoclonal antibody, which immunoprecipitates α5β1 from hamster cells but does not perturb it adhesion (Hall et al., 1990); function-perturbing anti-αvβ3 rat monoclonal antibody GoH3, Dr A. Sonnenberg, Netherlands Cancer Institute (Sonnenberg et al., 1986); mouse anti-αvβ3-specific monoclonal antibody, Dr Diane Horne, Oncogen (Seattle, WA) (Wayner et al., 1991); polyclonal rabbit anti-human vitronectin receptor (αvβ3), Telios Pharmaceuticals. Sheep anti-porcine 72 kDa gelatinase, sheep anti-human 92 kDa gelatinase, sheep anti-human stromelysin were kind gifts of Dr John Reynolds and colleagues, Strangeways Laboratories, Cambridge University, UK (Murphy et al., 1989). TA99 mouse anti-human monoclonal antibody against a melanosome membrane antigen was the kind gift of Dr A. Houghton and Dr A. Albino, Sloan Kettering Memorial Hospital, NY (Houghton et al., 1982, 1987).

**Cells and culture conditions**

The CM-1 and CS-1 hamster melanoma cell lines were established by Farishian and Whittaker (1979). CM (monolayer)-1 cells are amelanotic and adherent when grown on plastic in the presence of serum. CS (suspension)-1 cells were derived from CM-1 by continuous passage of cells that did not adhere under the culture conditions used. CS-1 cells are melanotic and are nonadherent when grown in the presence of serum. They can be induced to adhere and to stop making melanin by the addition of 2×10^{-6} M BrDU to the culture medium; the cells are then designated BCS-1. This effect is reversible: when the BrDU is removed, BCS-1 cells revert to the original CS-1 phenotype (Knudsen et al., 1982). BrDU-treated CM-1 cells are designated BCM-1. These four cell lines were routinely cultured in Dulbecco’s Minimum Essential Medium (DMEM) as described previously (Farishian and Whittaker, 1979; Knudsen et al., 1982). To study the adhesive and invasive properties of these cells, serum in the medium was replaced by either 0.2% bovine serum albumin (SFM-BSA; for short term assays) or 1% Nutridoma (Boehringer Mannheim, Indianapolis, IN), a medium supplement that lacks attachment factors (SFM-ND).

**Adhesion assays**

Cell attachment assays on defined ECM ligands were carried out as described previously (Kramer et al., 1989; Hall et al., 1990). Briefly, 96-well tissue culture plates with the wells containing 5 μg/ml of Fn or 10 μg/ml Ln, Vn, Fbg, or Col IV, in PBS, were incubated overnight at 4°C. Nonspecific adhesion to cells was blocked by adding 0.2% BSA in PBS for 2 h. Cells were harvested with 1 mM EDTA, washed once in SFM-BSA, and plated at a density of 2×10^5 cells/100 μl SFM-BSA. Cells were incubated at 37°C for 45-60 min. The plates were agitated on a rotary platform, nonadherent cells were removed, and attached cells quantitated as described (Kramer et al., 1989).

**Immunoprecipitation**

Cells were metabolically labeled in low glucose (1 g/liter) DMEM with N-acetyl-D-[3H]glucosamine (50 μCi/ml; New England Nuclear, Boston, MA) for 24 h, or in methionine-free DMEM with [35S]methionine (50 μCi/ml; Amersham, Arlington Hts, IL) for 6 h. Monolayers were washed, harvested, and washed with P-40 (NP-40)-containing buffer (10 mM Tris-acetate, pH 7.4, containing 0.5% NP-40, 75 mM NaCl, 0.5 mM EDTA and 0.5 mM phenylmethylsulfonylfluoride (PMSF)). The extract was trituated at 4°C for 20 min and centrifuged at 2,000 g for 12,000 g for 10 min each. The supernatant was then incubated with primary antibodies against integrins for 2 h or overnight at 4°C, and precipitated with secondary rabbit, rat or mouse antibodies conjugated to agarose (Sigma, St. Louis, MO). The bead-bound precipitates were washed as described (Hall et al., 1990). Immunoprecipitates were analyzed by SDS-PAGE under nonreducing conditions and detected by autoradiography.

**Responsiveness to αMSH and detection of melanin**

αMSH was prepared as a 1 mg/ml stock solution in 0.05 M acetic acid and stored at -20°C. It was added to cell cultures at 2×10^{-7} M. After a 48 h exposure, cells were assayed for melanin content, integrin profile and attachment behavior. Melanin content of cells in the presence and absence of αMSH was quantified by using the colorimetric assay of Foster et al. (1954) as modified by
Whittaker (1963). Melanin levels were compared in all cells during late log phase.

**Immunofluorescence**

Cells were plated on Fn-coated coverslips for 48 or 72 h in SFM-ND in the presence or absence of αMSH, then stained for the differentiation antigen TA99, using the procedure described previously (Damsky et al., 1992; Werb et al., 1989). Briefly, cells were either fixed for 10 min in 3% paraformaldehyde and permeabilized with acetone, or fixed in ice cold methanol for 3-5 min. After washing with PBS and incubation in PBS containing 0.2% BSA to block nonspecific binding, samples were stained with primary antibodies for 1-2 h and with fluorescein-conjugated secondary antibodies for 30 min. They were then mounted in Elvanol containing phenylenediamine to reduce bleaching of the fluorescent signal. Samples were examined in a Zeiss Axiophot phase-epifluorescence microscope and photographed with Kodak T-Max 400 film, which was developed in Accufine (Kodak).

**Detection of cell-associated and secreted proteinases**

Cells (5×10⁴) in 3 ml SFM-ND were plated for 48 h in 6-well (30 mm) culture dishes coated with Fn or Ln, so that all cell types would be adherent. The conditioned media were concentrated five-to ten-fold in Centraprep concentrators with a 10 kDa exclusion filter (Amicon Corp., Beverly, MA). Cell monolayers were washed with PBS and extracted in 1X Laemmli sample buffer containing 2.5% SDS but no reducing agent. The protein content of all samples was determined (Lowry et al., 1951), and equal amounts of cell extract or conditioned medium protein were electrophoresed under nonreducing conditions on 10% gels containing 1 mg/ml gelatin, 1 mg/ml casein, or 1 mg/ml casein plus 12 μg/ml plasminogen. Next, the gels were incubated with 2.5% Triton X-100 for 30 min at 37°C to remove SDS and then incubated for 18-24 h at 37°C in 50 mM Tris-HCl, pH 8, with 5 mM CaCl₂. Gels were stained with Coomassie blue to demonstrate gelatin-, casein- or casein/plasminogen-degrading enzymes as clear bands in a blue background (Unemori and Werb, 1986; Fisher and Werb, 1992).

**Immunoblotting**

Immunoblotting was used to verify the identity of metalloproteinases detected by zymography. Cells were plated on Fn or Ln in SFM-ND. Conditioned medium was collected after 48 h and concentrated up to four-fold, when necessary, as described above. Cell layers were washed with PBS and extracted with 2X Laemmli sample buffer. The protein content of all samples was determined (Lowry et al., 1951). Samples were analyzed on 10% gels under reducing conditions and transferred to nitrocellulose (Towbin et al., 1979). Nonspecific binding was blocked with 5% nonfat dry milk in Tris-buffered saline as described previously (Librach et al., 1991), and the blots were incubated with primary antibodies against the 92 kDa and 72 kDa gelatinases and stromelysin. The bound antibodies were detected with the appropriate secondary antibodies made in donkey or goat (Jackson Immunoresearch, West Grove, PA). After washing, the bands were demonstrated by the Enhanced Chemiluminescence Detection System (Amer sham Corp.) in a 1:1 dilution.

**Invasion assays**

To compare the invasiveness of the cell lines, Transwell culture chambers partitioned with a Nucleopore filter (8 μm pore size, 0.28 cm² surface area; Costar, Cambridge, MA) were used in 24-well plates. The filters were coated with 20 μl of a 2:1 mixture of Matrigel and SFM-ND (final Matrigel concentration 6-8 mg/ml protein). Then 1×10⁵ cells in 200 μl SFM-ND were added to the top of the chamber and 0.8 ml of the same medium to the bottom. Cultures were incubated for 24, 48 or 72 h at 37°C. When desired, a transition-state analog inhibitor of matrix metalloproteinases (compound 6A, 100-1000 nM; Reich et al., 1988; Librach et al., 1991) was included in the medium and co-polymerized with the Matrigel. Cultures were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, and prepared for scanning electron microscopy by standard procedures. Samples were mounted so that either the top or the underside of the filter could be examined, and were observed in a JEOL JSM-840 electron microscope. The presence of cells on the underside of the filter indicates that the cells have penetrated the Matrigel barrier and migrated through the pores of the filter (Librach et al., 1991). To quantify this assay, five representative photographs of the underside of each filter in the region of invasion were taken in a systematic fashion. Photographs were overlaid with a grid consisting of points forming equilateral triangles, and the number of cells that overlapped with grid points was counted. The percentage of grid points that overlapped with invading cells is equivalent to the percentage of surface area covered by cells (Weibel and Bolender, 1974). The data are the average of two filters per experiment and at least two different experiments.

**RESULTS**

**BrdU induces striking effects on adhesiveness and integrin expression in hamster melanoma cells**

Initial results reported by Knudsen et al. (1982) indicated...
that adding $2 \times 10^{-6}$ M BrdU to the serum-containing culture medium of the melanotic nonadherent CS-1 hamster melanoma cell line caused these cells to adhere, increase production of cell-surface adhesion receptors, and cease production of melanin over a period of several days. This phenotype resembled that of the adherent amelanotic parental CM-1 melanoma cell line, from which the CS-1 cells were originally derived (see Materials and Methods). The effects of BrdU were reversible: upon removal of the BrdU, the CS-1 cells detached and resumed production of melanin over a period of several days. Treatment of the parental CM-1 cells with BrdU did not result in any apparent morphologic changes, although the levels of adhesion receptors were increased over levels present in the untreated CM-1 cells.

To determine more specifically the basis for the differences in the adhesive behavior of CS-1, BCS-1, CM-1 and BrdU-treated CM-1 cells (BCM-1), we plated the four cell lines on substrates coated with defined matrix ligands in the absence of serum (Fig. 1). All four cell lines adhered to substrates coated with Fn or Ln and exhibited a fibroblastic morphology, although the CS-1 cells spread less extensively than the other lines. Thus, the inability of CS-1 cells to attach when grown in the presence of serum was not due to the absence of a Fn receptor or to any intrinsic inability to attach to an appropriate substrate. Both the CM-1 and CS-1 cell lines attached poorly to Col IV, but did adhere after treatment with BrdU. Finally, CS-1 cells did not attach to either Fbg or Vn, whereas the three other cell lines attached and spread readily. These results suggested that the CS-1 cell line lacked functional receptors for these molecules, both of which recognize a promiscuous vitronectin receptor integrin, $\alpha_V\beta_3$.

To determine the molecular basis of these striking BrdU-induced changes in adhesive behavior, we immunoprecipitated samples of the four cell lines representing equal protein content with subunit-specific antibodies against members of the $\beta_1$ and $\beta_3$ integrin families (Fig. 2). Precipitations were carried out under conditions of antibody excess so that the levels of various integrins expressed by the different cell lines could be compared. Both $^{35}$S]methionine and $[^3H]$glucosamine were used to label integrins metabolically. Results were similar with both labels. The integrin $\alpha_5\beta_1$ Fn receptor complex was expressed by all four cell lines, with the $\alpha_5$ subunit present as a ladder of closely spaced bands, presumably representing maturation states of this subunit, as has been shown in other studies (Fig. 2A). CS-1 cells appeared to express less of the $\alpha_5\beta_1$ complex than the other lines and relatively higher levels of the less mature (higher molecular weight) forms of the $\alpha_5$ subunit. Treatment of CS-1 cells with BrdU increased the level of precipitable $\alpha_5\beta_1$, and this material

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Fig. 2. Detection of integrins on control and BrdU-treated cells by immunoprecipitation. NP-40 extracts of $[^3H]$glucosamine-labeled cells were incubated with anti-integrin antibodies and precipitated with the appropriate agarose-conjugated secondary antibody as described in Materials and Methods. Lane 1, CS-1; lane 2, BCS-1; lane 3, CM-1; lane 4, BCM-1. (A) Rat anti-$\alpha_5$ (B1E5); (B) rat anti-$\alpha_6$ (GoH3); (C) rabbit anti-$\alpha_3$ cytoplasmic domain; (D) rabbit anti-$\alpha_V\beta_3$; (E) mouse anti-$\beta_5$.
BrdU regulates melanoma cell invasion and differentiation was enriched in the more mature forms. Thus, BrdU appeared to induce higher levels of the \( \alpha_5\beta_1 \) Fn receptor and accelerated its maturation.

Treatment of either CS-1 or CM-1 cells with BrdU also increased the levels of the \( \alpha_6\beta_1 \) integrin (Fig. 2B). Anti-\( \alpha_6 \) antibody did not co-precipitate any \( \beta_4 \) subunit in any of the cell lines. In an adhesion assay, the function-disrupting monoclonal anti-\( \alpha_6 \) antibody, GoH3 (Sonnenberg et al., 1986), inhibited CS-1 cell attachment to Ln by 75% but had no effect on the attachment of any of the other cell lines, indicating that \( \alpha_6\beta_1 \) was the major Ln receptor for CS-1 cells but that the other cell lines, including BrdU-treated CS-1 cells, had additional Ln receptors. One receptor that might fulfill this role is the promiscuous integrin receptor \( \alpha_3\beta_1 \), which recognizes Ln and several other ligands (reviewed by Hynes, 1992). Using an antibody against the cytoplasmic domain of \( \alpha_3 \), we detected a strong signal was evident. (A) CM-1 cells; no staining was evident. (B) CS-1 cells; about 30% of the cells, which attach well to Fn but remain rounded, expressed the TA99 antigen in a perinuclear zone. (C) BCS-1 cells; BrdU treatment suppressed TA99 expression. (D) and (E) \( \alpha \)MSH-treated CS-1 cells. After 3-day treatment with \( \alpha \)MSH, the CS-1 cells had spread and a subpopulation exhibited a highly arborized morphology. More than 50% of the \( \alpha \)MSH-treated CS-1 cells expressed TA99, and the granular nature of the staining is consistent with localization on an internal membrane compartment.
in CM-1 and both BrdU-treated cell lines, but none in the CS-1 cells (Fig. 2C). This pattern of expression may help to explain why attachment of CS-1 cells to Ln was susceptible to treatment with GoH3 whereas attachment of the other cell lines was not (data not shown). We could not test this idea directly because function-blocking anti-α3 antibodies, active on hamster cells, were not available. BrdU also induced attachment of both CS-1 and CM-1 cells to collagen. This was likely due to upregulation of expression of the α2 integrin subunit (not shown). It was not related to α3 or α1 expression since the CM-1 cells expressed high levels of α3β1 and attached poorly to collagen, and the α1 subunit was not detected in the β1 immunoprecipitates of any of the cell lines. Because of the lack of function-blocking antibodies to these subunits, the functional significance of the increased expression of α2β1 could not be tested directly.

Finally, BrdU induced striking changes in the expression of vitronectin receptors by CS-1 cells (Fig. 2D,E). Polyclonal anti-αvβ3 and a monoclonal antibody specific for the αvβ3 integrin precipitated material of 135 kDa and 97 kDa from CM-1 cells, characteristic of αv complexed with either β3 or β5. These components were not detected in CS-1 cells. After BrdU treatment, high levels of the αv complexes were detected in both cell lines by immunoprecipitation of metabolically labeled cells. Analysis of surface expression of αvβ3 by flow-cytometry confirmed that CS-1 did not express this complex at the cell surface, whereas the BCS-1 cells did (not shown). Thus, BrdU upregulated the expression of αv-containing integrins in CM-1 cells (Fig. 2D and data not shown) and appeared to induce them, de novo, in the CS-1 cells (Fig. 2D,E). That CS-1 cells apparently do not express receptors for vitronectin explains why they do not adhere to plastic when grown in the presence of serum. Taken together, the data presented thus far indicate that treatment of CS-1 cells with BrdU causes profound changes in their adhesive behavior, accompanied by enhanced expression of several integrins. In some cases (α3β1, α3β3), the integrins were expressed at relatively low levels in CS-1 cells and elevated by BrdU, whereas in other cases (α3β1, αv-containing complexes) the complexes were not detected in CS-1 cells and appeared to be induced, de novo, by BrdU.

**BrdU affects melanin production and responsiveness to melanocyte-stimulating hormone**

The other obvious change observed in CS-1 cells after BrdU treatment was repression of melanin production (Fig. 3). Only CS-1 cells are capable of expressing melanin. Quantification of melanin demonstrated that BrdU repressed CS-1 melanin synthesis to the level found in CM-1 cells. BrdU treatment of CM-1 cells had no effect on melanin production. Thus, BrdU is a negative regulator of melanin, the major differentiation-specific product of melanocytes. To determine whether responsiveness to αMSH was also regulated by BrdU, the CS-1, BCS-1 and CM-1 cell lines were treated with αMSH for 48 h. CS-1 cells responded by increasing their level of melanin production two-fold (Fig. 3) and slowing their growth rate (not shown). Melanin synthesis in the BCS-1 and CM-1 cells was not affected by αMSH. BrdU and αMSH also have opposing effects on expression of the melanocyte differentiation marker TA99, a melanosome membrane glycoprotein called tyrosinase-related protein-1, which is the product of the brown locus (Halaban and Moellman, 1990). This antigen is only detected on well-differentiated melanomas, nevi and mature melanocytes (Houghton et al., 1982, 1987). CS-1 cells, but not CM-1 cells, expressed TA99 (Fig. 4A,B). TA99 expression was depressed by treatment of CS-1 cells with BrdU (Fig. 4C) and further elevated by treatment of CS-1
cells with αMSH (Fig. 4D,E). Thus, CS-1 cells are susceptible to treatment with both αMSH and BrdU. The former promotes further differentiation, while the latter appears to suppress differentiation.

**BrdU regulates expression of two classes of matrix-degrading proteinases: the metalloproteinases and the plasminogen activators**

Using the substratum gel technique, we analyzed cell extracts and conditioned media from the hamster melanoma cell lines plated on either Ln or Fn, using gelatin-, casein- or plasminogen-casein-containing polyacrylamide gels to determine their repertoires of proteinases (Figs 5, 6). Extracts of CS-1 cells plated on either substratum, and their 48 h conditioned media, contained very low levels of 72 and 92 kDa gelatin-degrading proteinases. In contrast, extracts of BrdU-treated CS-1 cells plated on either Fn or Ln, and their conditioned media, produced lysis zones with the mobilities of both activated and proenzyme forms of the 92 kDa type IV gelatinase. CM-1 cells produced intermediate levels of activated and proenzyme forms of the 92 kDa gelatinase (Fig. 5A). Gelatinolytic activity was largely inhibited when similar gels were incubated in the presence of 1,10-phenanthroline, a zinc chelator, indicating that the activity was due primarily to matrix metalloproteinases (Fig. 5B). A stromelysin-like activity was also detected in conditioned media from CM-1 and BCS-1, but not CS-1, cells by casein gel zymography (Fig. 5C). Immunoblotting with antibodies against the 92 and 72 kDa gelatinases and stromelysin confirmed the identity of these components (data not shown). Only the 72 kDa gelatinase was detected in CS-1 cells, whereas all three enzymes were present in BCS-1 cells. Comparison of the enzyme activity profiles after electrophoresis in plasminogen-casein gels showed that both cell lines produced plasminogen activators (PA) (Fig. 6). However, the CS-1 zymograms contained material migrating like tPA (tissue-type plasminogen activator) and urokinase-type plasminogen activator (uPA), whereas the BCS-1 zymograms displayed material migrating like uPA and very low molecular mass PAs.

To determine whether the BrdU-induced differences in adhesive properties and expression of proteinases were reflected in altered invasive behavior, the four cell lines were plated on Matrigel-coated porous filters and assayed for their ability to traverse the Matrigel barrier and migrate to the underside of the filter. The results were analyzed by scanning electron microscopy (Fig. 7) and quantified by stereological methods (Fig. 8) (Weibel and Bolender, 1974; Librach et al., 1991). CS-1 cells formed small clusters scattered over the surface of the Matrigel, but did not invade even after 72 h in culture (Fig. 7A-C). BCS-1 cells, in contrast, aggregated in a single large nest-like structure in the center of the well within 24 h (Fig. 7D,E). Examination of the underside of the filter revealed extensive invasion of cells that was restricted to the area corresponding to the bottom of the nest (Fig. 7F), indicating that the cells had aggregated prior to any invasion. CM-1 cells also formed nest-like structures and invaded, although invasion was not detected for about 48 h (not shown). Thus, the relative invasiveness of the three cell lines correlated with the levels of metalloproteinases they expressed. Some BCS-1 cultures were incubated in medium containing different concentrations of the compound 6A, a transition state analog of the metalloproteinase family (Librach et al., 1991), to determine whether this proteinase family was required for invasion in this assay. The invasiveness of the BCS-1 cells was evaluated after 24 h. The 6A, in the range of 200-500 nM, blocked invasion (Fig. 8B). These concentrations were not toxic, since cells exposed to 500 nM grew as rapidly as control cells (not shown). Furthermore, the BCS-1 cells were able to invade after an additional 24 h in the absence of the 6A (not shown).

**DISCUSSION**

Table 1 summarizes the data obtained in our experiments. The cell lines are arranged from right to left in order of their apparent level of differentiation and from left to right in order of their observed level of invasiveness. The data are consistent with the idea that the CS-1 cells are developmentally arrested at a point in which they exhibit a relatively differentiated phenotype. In particular, they are...
Fig. 7. Comparison of the invasive activity of control (A-C) and BrdU-treated (D-F) CS-1 cells. Cells were plated in serum-free medium on Matrigel-coated Nucleopore filters with 8-mm pores in a Costar transwell filter. CS-1 cultures were fixed after 72 h (A-C) and BCS-1 cultures after 24 h (D-F). Filters were prepared for scanning electron microscopy. (A) and (D) Low magnification of the tops of the filters showing the morphology of the (A) CS-1 and (D) BCS-1 cultures on Matrigel. (B) and (E) Higher magnification of the same samples. (C) and (F) Low magnification of companion wells in the same experiment showing the undersides of the filters of the CS-1 (C) and BCS-1 (F) cultures after 72 or 24 h, respectively. Extensive invasion is evident in the BCS-1 culture after 24 h (F), but not in the CS-1 culture, even after 72 h (C). Inset in (F) shows the morphology of the BCS-1 cells that have invaded. CM-1 cells also invaded, but 48 h were required for them to reach a similar level of invasion as that found in BCS-1 cells after 24 h (not shown).
Fig. 8. (A) Quantitation of invasion activity of CS-1, BCS-1 and CM-1 cells at 24 and 48 h under control conditions. (B) Quantitation of BCS-1 invasiveness after 24 h in the presence of a transition state analog inhibitor of the metalloproteinase family, 6A. The undersides of Matrigel-coated filters were photographed and percent of surface area covered by invading cells was calculated as described in Materials and Methods. Two filters of each sample were scored in each experiment and the experiments were repeated twice. Errors bars represent s.e.m.

responsive to αMSH, express a melanosome membrane antigen (TA99), and produce melanin. Accompanying this phenotype is a very restricted profile of both adhesion receptors and proteolytic enzymes capable of degrading ECM, including basement membranes. CS-1 cells are able to differentiate further in response to αMSH: they produced more melanin and more TA99 antigen, and some cells assumed an arborized phenotype. As judged by the expression of melanocyte markers, the differentiated phenotype of CS-1 cells is suppressed by treatment with BrdU, a thymidine analog known to modulate the differentiation of several cell lineages (reviewed in Rutter et al., 1973; Tapscott et al., 1989). After BrdU treatment, CS-1 cells adhered to a broader range of substrates, resembling the adherent CM-1 cell line from which they were derived. Melanin production and response to αMSH were absent, and the adhesion receptor and proteinase profiles displayed increased complexity.

Relating the phenotype of the cells in this study directly to those of melanocytes at different stages of their differentiation has been difficult, as the integrin and proteinase profiles of the mammalian neural crest melanocyte precursor lineage have not been well characterized. However, since cells of the melanocyte lineage must be highly migratory and able to invade the epidermal basement membrane, such precursor cells are likely to have an opportunistic adhesion phenotype and express several proteinases. Results of several studies using chick neural tube explants as the source of a heterogeneous population of neural crest cells (Lallier et al., 1992; Erickson and Isseroff, 1989; Erickson, 1987) support the hypothesis that CM-1 cells and the BrdU-treated cells display adhesive and invasive phenotypes consistent with those expected of migratory and invasive neural crest-derived precursors of the melanocyte lineage. In particular, two integrins with promiscuous ligand recognition properties, α3β1 and αvβ3, are expressed strongly by the CM-1 cells and both BrdU-treated lines, whereas neither is expressed by the CS-1 cells. In addition, BCS-1 and CM-1 cells express higher levels of several metalloproteinases and low molecular mass forms of PA than CS-1 cells.

When melanocyte precursors reach their position in the epidermis, they undergo terminal differentiation, expressing markers for the melanosome membrane and enzymes in the melanin biosynthesis pathway (Houghton et al., 1982, 1987; Bennett, 1989). We attempted to determine which components are expressed by the CM-1, CS-1 and BCS-1 cells. The TA99 (GP75) melanosome membrane antigen, which is expressed on differentiated melanomas and melanocytes, was expressed on CS-1 and αMSH-treated CS-1 cells but not on the other lines, indicating that CS-1 cells are relatively well differentiated. Several other monoclonal antibodies that have been used as markers to stage

### Table 1. Adhesive and invasive properties of control and 5-bromodeoxyuridine-treated hamster melanoma cell lines

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<td></td>
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</tr>
<tr>
<td>Fibrinectin</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Laminin</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Collagen</td>
<td>−</td>
<td>−/+/−</td>
</tr>
<tr>
<td>Vitronectin</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>Integrin expression</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>α3</td>
<td>−</td>
<td>++</td>
</tr>
<tr>
<td>α5</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>α6</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>αv</td>
<td>−</td>
<td>++</td>
</tr>
<tr>
<td>β1</td>
<td>−</td>
<td>++</td>
</tr>
<tr>
<td>β3</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Melanin expression</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Proteases</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>72 kDa MMP</td>
<td>+/−</td>
<td>+</td>
</tr>
<tr>
<td>92 kDa MMP</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>uPA (kDa)</td>
<td>55</td>
<td>22</td>
</tr>
<tr>
<td>tPA</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Invasion</td>
<td>ND</td>
<td>(72 h)</td>
</tr>
</tbody>
</table>

MCS-1, CS-1 cells treated with α melanocyte-stimulating hormone; BCS-1 and BCM-1, 5-bromodeoxyuridine-treated CS-1 and CM-1 cells; MMP, matrix metalloproteinase; uPA, urokinase-type plasminogen activator; tPA, tissue-type plasminogen activator; ND, not determined.
melanomas and fetal and mature melanocytes in humans did not recognize these hamster cells.

The mechanism by which BrdU suppresses differentiation is poorly understood, but the phenomenon has been observed in many lineages: erythroid (Weintraub et al., 1972), muscle (Stockdale et al., 1964), cartilage (Abbott and Holtzer, 1968), as well as melanoma (Silaghi and Bruce, 1970; Kidson and DeHaan, 1990). The best studied case is muscle, in which BrdU treatment of embryonic chick myoblast cultures (Stockdale et al., 1964) or rodent myoblast cell lines (Wright and Aronoff, 1983) blocks myogenic differentiation and suppresses the myogenic determination gene Myo-D (Tapscott et al., 1989). The BrdU block is overcome if Myo-D is transfected into these cells (Tapscott et al., 1989). These studies suggest that BrdU interferes with some aspect of the commitment process in muscle and are consistent with the idea that BrdU exerts its effects on a small number of regulatory loci (discussed by Tapscott et al., 1989). It is possible that BrdU treatment of CS-1 cells suppresses the differentiated phenotype in cells of the melanocyte lineage in an analogous manner, perhaps making this system a useful one in detecting commitment genes in the melanocyte lineage.

This system is also potentially useful in studying the role of specific adhesion receptors in regulating the invasive phenotype of melanoma cells. The studies of Albelda et al. (1990), using melanoma tumor biopsy tissue, and others using melanoma cell lines (Leavesley et al., 1992; Fielding-Habermann et al., 1992; Seftor et al., 1992), using human melanoma cell lines differing genetically only in their expression of αv complexes, suggest that an aggressively invasive phenotype for melanoma cells in vivo requires expression of αv complexes. The balance in effects of the appropriate proteinases and their inhibitors is also likely to be critical. Since our cell lines differ in several aspects of their adhesive and invasive phenotype, it should be possible to determine which of these receptors and enzymes are critical for the invasiveness of CM-1 and BCS-1 cells by individually blocking their function by molecular, antisense or antibody methods. Alternatively, transfection of such critical components into CS-1 cells should promote their invasiveness.

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REFERENCES


BrdU regulates melanoma cell invasion and differentiation


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