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5-Bromodeoxyuridine (BrdU) regulates invasiveness of hamster melanoma cells

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5-BROMODEOXYURIDINE (BrdU) REGULATES INVASIVENESS OF HAMSTER MELANOMA CELLS

by

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THESIS

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This thesis is dedicated to my family

whose love and support have made this all possible.

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I would also like to thank the other my committee, Drs. Susan Fisher and Randall Kramer for their advice.

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Invasion assay and substrate gel zymography were carried out in collaboration with Patti Walter Chan.

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INTRODUCTION

Integrins are a family of cell surface glycoproteins consisting of two subunits, α and β that mediate cell adhesion. These transmembrane heterodimeric glycoproteins are thought to have the role in integrating the intracellular cytoskeleton with the extracellular matrix (ECM). There are 14 known α subunits and 8 β subunits known at this time and it is likely that more will be discovered. The α and β subunits in various combinations form at least 20 integrins (Ruoslahti, 1991; Hynes, 1992). The α and β subunits are noncovalently bound to one another, and both are required to bind ligand and also this association is promoted by divalent cations (Ginsberg, et al., 1988). Transport of the newly synthesized receptor to the cell surface takes place only after the subunits have combined (Cheresh and Spiro, 1987).

Initially, the integrins were categorized according to the β subunit they contained. However, in some cases, the same α subunit can become associated with more than one β subunit, for example, αV can become associated with β1, β3, β5, and β6. Moreover, most integrins recognize more than one ligand, and more than one integrin can bind to the same ligand, making it difficult to use the binding activity for identification (Hynes, 1992). The pattern of integrin expression varies between cell types and some integrins are clearly cell-type specific such as gp IIb/IIIa in platelets and LFA-1, Mac-1, and p150/95 only in leukocytes (Hynes, 1987). Perhaps more significantly, the specificity and affinity of a given integrin receptor on a given cell are not always constant (Hynes, 1992).

The expression of individual integrins appears to be regulated during development and can be modulated by agents that affect growth and

differentiation (Heino, et al., 1989). For example, transforming growth factor-β (TGF-β) causes a striking upregulation of integrins in some cultured cells. In WI-38 lung fibroblasts, TGF-β1 elevated concomitantly the expression of all α/β1 integrin subunits at the protein and/or mRNA level, their assembly into the corresponding α/β1 complexes, and their exposure on the cell surface (Heino, et al., 1989). Also, TGF-β1 elevated the expression of LFA-1 in human leukemia cells and vitronectin receptors in all cells examined including WI-38 human lung fibroblasts, 3T3-L1 mouse fibroblasts, and MG-63 human osteogenic sarcoma cells (Ignotz, et al., 1989). By modifying the balance of individual integrins, TGF-β1 might modulate those aspects of cell migration, positioning, and development that are guided by adhesion to extracellular matrices.

Integrin-mediated cell-ECM interactions are apparently highly complex. Extracellular matrices are made up of an insoluble meshwork of protein and carbohydrate that is laid down by cells and that fills most of intercellular spaces. Many integrins bind to extracellular matrix proteins such as fibronectin, laminin, collagens, vitronectin, fibrinogen, entactin, and tenascin. These extracellular matrices also bind many growth factors, proteinases and proteinase inhibitors. Interactions with the ECM localize these molecules to the pericellular environment and modulate their biological activities, therefore influencing many aspects of cell behavior, including movement, morphology, differentiation, and proliferation. The ability of tumor cells to invade through the dermal-epidermal basement membrane and then penetrate the interstitial extracellular matrix is dependent on their capacity to adhere to, interact with, and migrate through these matrices (Kramer, et al., 1989; 1991).

Normal cells proliferate rapidly under appropriate condition, yet regulate their growth when the cellular environment indicates. In contrast, the malignantly transformed cell does not fully respond to environmental stimuli signaling growth cessation (Herlyn, et al., 1990). In many cases, including the leukemias and melanomas, cancer cells resemble embryonic precursors of normal differentiated cells in terms of their phenotypes such as proliferation, migration, invasion and poorly differentiated nature. It can sometimes be useful to consider the idea of tumor progression from primary site to secondary metastatic site and differentiation process of embryonic cells as being inversely related. In other words, tumor cells with different phenotypes and levels of metastasis can sometimes be classified in a manner that resembles a developmental pathway; suggesting that tumor cells with increasing invasiveness and decreasing differentiation markers represent cells arrested at different stages of differentiation. Thus, it should be possible to learn not only about the metastatic process, but also about differentiation pathway from studying tumor cells. This has been the case with leukemias and normal leukocyte differentiation, and increasingly the case with melanomas and normal melanocyte differentiation as well.

During the early stage of embryogenesis, the melanoblast lineage of the neural crest migrate from the neural tube to the skin, where they differentiate into melanocytes (Kramer, et al., 1991). Melanoblasts are unpigmented precursor cells which may have premelanosomes, but contain no tyrosinase activity (Bennett, 1989). Once they reach their final epidermal sites, they remain associated with the epidermal basement membrane as they interact with keratinocytes and deposit melanin.

Malignant melanoma which is comprised 3% of all cancers is the leading cause of death from all diseases arising in the skin (Liotta, et al., 1987). Several histopathologic features are judged useful for assessing prognosis of malignant melanoma. It has long been recognized that the deeper the invasion of malignant melanoma into the skin, the worse the prognosis. In general, tumors measuring less than O.76mm in thickness have a uniformly favorable outcome, while patients with tumors greater than 3.6mm are at high risk for recurrent diseases and death (Breslow, 1970).

The major cause of morbidity and death for cancer patients is metastasis. Therapeutic modalities including surgery, chemotherapy and radiotherapy can now cure approximately 50% of the patients who develop a malignant tumor. The dispersed anatomic location of metastases, their size and age variation, and their heterogeneous cell composition prevent surgical removal and limit the response to systemic anticancer agents (Liotta, 1984). Local tumor invasion is one of the early important step in the complex multistep process which leads to metastasis initiation and growth. A critical challenge to clinical and basic cancer scientists is the development of improved prognostic methods to predict the metastatic aggressiveness of a patient's individual tumor (Hendrix, et al., 1987; Liotta, et al., 1987), and especially to control local invasion and identify and treat clinically silent micrometastases. During invasion which is the important step in metastasis, tumor cells must adhere to and penetrate the basement membrane and interstitial stroma. This process clearly involves the specific interactions between tumor cell surface adhesion receptors and components of the extracellular matrix.

The biochemical interactions between growth factors, proteolytic enzymes, their inhibitors, extracellular matrix proteins, and adhesion receptors are complex and may be altered during neoplasia, thereby influencing tumor

proliferation and invasion (Liotta, 1986). Invasion of the matrix is not merely due to passive growth pressure, but requires active biochemical mechanisms. Liotta and co-workers have proposed a three-step hypothesis of invasion describing the sequence of biochemical events during tumor cell interaction with the extracellular matrix. It consists of

(1) tumor cell attachment to a matrix substratum,

(2) local degradation of the matrix by tumor cell-associated proteinases, and

(3) tumor cell locomotion into the matrix modified by proteolysis.

The ability of a tumor cell to metastasize is determined, in part, by its adhesive interactions with other cells, and with components of the extracellular matrix (Ruoslahti and Giancotti, 1989). This is partly based on the observation in the early 1970s that fibronectin, a major component of the extracellular matrix was absent from the matrix of many tumorigenic cell lines (Ruoslahti, 1988; Hynes and Yamada, 1982).

One particular type of matrix, the basement membrane, appears to play a crucial role during the progression of invasive tumors and during hematogenous dissemination (Liotta, 1984). In particular, melanocytes adhere poorly to laminin while metastatic melanoma cells bind well to this ligand which is the main component of basement membrane (Liotta, et al., 1986). Melanoma cells selected for increased attachment to laminin exhibit much higher metastatic potential in lung colonization assays (Liotta, et al., 1986). Once the tumor cells enter the stroma, they gain access to lymphatic and blood vessels for further dissemination. During intravasation or extravasation, the tumor cells must penetrate the subendothelial basement membrane.

Studies by McGregor, et al. (1989) and Albelda, et al. (1990) reported that the β3-containing complexes were readily detected in most samples of tissue

sections of melanoma tumor, but that this β chain was not detected in normal skin melanocytes. This suggests that this integrin, which recognizes several ligands including most likely vitronectin, may be up-regulated in the transformed state. As described below, our hamster melanoma cell system also showed that the best differentiated, least invasive cell line had no αV complexes, while the more invasive lines expressed both αV/β3 and αV/β5. Also, ligand affinity chromatography on laminin-Sepharose columns showed that the melanocytes did not express detectable amounts of the α7/β1 complex. Many melanoma cell lines, in contrast, express high levels of this heterodimer (Kramer, 1989; 1991). Interestingly, the most metastatic cells do not. Indeed, different melanoma cell lines appear to differ from melanocytes and from one another in their expression of integrin receptors.

In addition, correlations have been found between the production of growth factors by malignant cells and their potential for distant metastasis (Nicolson, 1988). The production of certain growth factors such as TGF-β by the tumor cells themselves, or by host tissue, may alter integrin expression (Ignotz, et al., 1989; Heino, et al., 1989) and thus modulate adhesive properties of the cells. These alterations might modulate those aspects of cell migration, positioning, and development that are guided by adhesion to extracellular matrices.

One of the other important properties is an ability to degrade and penetrate extracellular matrix. Metalloproteinases and plasminogen activators are fundamental to the invasive phenotype of both normal and transformed cell types (Alexander and Werb, 1989). Metalloproteinases include interstitial collagenase, gelatinases, stromelysin that are dependent on Zn^{++} ions for activity (Werb, 1989a). Interstitial collagenase cleaves collagens of type I, II,

III, VIII, X, but does not degrade basement membrane collagen type IV, or type V collagen. The stromelysin is the another major metalloproteinase which has also been called proteoglycanase. Substrates for this proteinase are collagen type IV, V, gelatin, fibronectin, laminin, proteoglycan, elastin. There are two major gelatinases, 92-95kD and 68-72kD which degrade collagen type IV, V, VII, VIII and gelatin. Two distinct genes for plasminogen activators, the tissue-type plasminogen activator (tPA) and the urokinase-type plasminogen activator (uPA) contribute to the activation of plasminogen, the zymogen of plasmin. Plasmin can degrade some matrix components directly and can activate some members of the metalloproteinase family.

The overall activities of these proteinases in vivo are modulated in a complex fashion that includes proenzyme activation (Vater, et al., 1983) and the action of the naturally occurring inhibitors of the enzymes (Murphy, et al., 1985). Tissue levels of metalloproteinase activity are controlled by TIMP which forms tight-binding complexes with collagenase and stromelysin. At least two tissue inhibitors of metalloproteinase, TIMP-1 and TIMP-2 have been identified and found to be regulated independently (Howard, et al., 1991). Several studies (Hearing, et al., 1988; Cajot, et al., 1990) have suggested that plasminogen activator and plasminogen activator inhibitor (PAI) system may play a critical role in the regulation of extracellular matrix degradation during tumor cell invasion.

Growth factors appear to regulate the synthesis of specific matrix molecules as well as proteinases and their inhibitors which in turn, modify matrix components. Quiescent human fibroblasts expressed mRNA transcripts encoding the metalloproteinases and the tissue inhibitor of metalloproteinases (TIMP) when exposed to growth factors other than TGF-β. (Edwards, et al., 1987). Exposure of quiescent cells to growth factors in the presence of TGF-β

inhibited collagenase induction and a synergistic increase in TIMP expression. TGF-β alone did not significantly induce metalloproteinases or TIMP expression. This study suggests that TGF-β exerts a selective effect on extracellular matrix (ECM) deposition by modulating the action of other growth factors on metalloproteinases and TIMP expression. ECM components can also regulate expression of collagenase. When synovial fibroblasts are attached to intact Fn, they express low levels of stromelysin and collagenase. However, when they are attached to even large cell binding domains of Fn, they express elevated levels of both enzymes (Werb, et al., 1989b; Damsky, et al., 1992).

Study by Pepper, et al. showed that either basic fibroblast growth factor (bFGF) or TGF-β1 induced both urokinase-type plasminogen activator (uPA) and plasminogen activator inhibitor-1 (PAI-1) in bovine microvascular endothelial (BME) cells. However, net proteolytic balance was towards enhanced proteolysis in response to bFGF, while towards antiproteolysis in response to TGF-β1. Thus, the interplay between bFGF and TGF-β1 could be critical to achieve a proteolytic balance which is appropriate for normal capillary morphogenesis.

5-bromo-2'-deoxyuridine (BrdU) is a nucleotide analog which incorporates into nascent DNA instead of Thymidine during cell replication and alters gene expression, therefore inhibits the normal differentiation of cells (Rutter, et al., 1973). Previous data by Thomas and Damsky (JCB 109 abstr:319a #1751) suggested that in hamster melanoma cells, BrdU seemed to reverse the differentiated properties of a melanotic cell line. The next question to be asked was what other properties were altered that suggested BrdU might be causing

dedifferentiation. During embryonic development, the neural crest cells emigrate from the dorsal aspect of the neural tube and migrate throughout the body to form various cell types including pigment-producing melanocytes. We wanted to determine if the apparently less differentiated phenotype exhibited by BrdU treated cells corresponds to the phenotype of melanocyte progenitor cells. Therefore, we investigated whether invasive potential is regulated by BrdU by examining the expression of proteinases and their inhibitors, and the invasive behavior of BrdU treated and control melanoma cells.

MATERIALS AND METHODS

1. Cell cultures

Two variants of the hamster melanoma cell line RPMI 3460 (Moore, 1964), CS (suspension)-1 and CM (monolayer)-1 were used (Avdalovic and Aden, 1978; Farishian and Whittaker, 1979). The cells were maintained in Dulbecco's Modified Eagle's Medium (DME H-16 growth medium, 3g/L glucose) supplemented with 10% fetal calf serum (FCS), 2mM L-Glutamine, and gentamicine (final concentration of 50μg/ml). BrdU was kept frozen (-20°C) at $2x10^{-4}M$ and was added to cells in complete growth medium at a final concentration of $2x10^{-6}M$ (Knudsen, et al., 1982). Nonadherent CS-1 cells which grow as a suspension of aggregated and single cells could be induced to adhere to the substratum by BrdU (Avdalovic and Aden, 1978). Nonadherent cells induced to adhere to the substratum by BrdU are referred to as adherent

BrdU-treated CS-1 cells (BCS-1). CS-1 cells that failed to attach in culture with BrdU were discarded and the remaining adherent cells were cultured for use in experiments. Experiments on invasion and proteinase production were performed with cells in an identical, serum free media supplemented with 2% Nutridoma (Boehringer Mannheim, Indianapolis IN).

2. Immunoprecipitations

Cells for immunoprecipitations were metabolically labeled for 24 hours in low-glucose growth medium supplemented with 50μCi/ml ^{3}H-glucosamine. Labeled cells were harvested with EDTA and washed with PBS. They were then lysed in 1.0 ml NP-40 lysis buffer 30 minutes on ice with frequent pipetting. Lysates were centrifuged at 2000 rpm for 5 minutes and microfuged for 10 minutes to remove insoluble fractions and precleared on 100 μl packed Sepharose 4B beads (Pharmacia, Piscataway, NJ 08854) twice for 30 minutes each. Primary antibodies were then added and incubated for 2 hours on ice or overnight at 4°C with gentle agitaton. Rabbit anti-mouse or rabbit anti-rat (depends on primary antibody, 1:100 dilution) was added and incubated one hour on ice. Then, protein A conjugated sepharose beads were added (50μl packed) because it has high affinity to rabbit IgG. Beads were washed 3X with Tris, NP-40 and calcium (TNC), 1X TNC plus NaCl, 1X TNC plus SDS, and again 2X TNC. Beads were then boiled 5 minutes and loaded onto 7% SDS acrylamide gels for electrophoresis. After fixing and drying, gels were exposed to x-ray film at -70°C.

Antibodies we used :

Antibodies specific for integrin subunits were used to immunoprecipitate matrix receptor glycoproteins from CS-1 cells and its BrdU-treated counterparts.

1) Anti-α2 : A polyclonal antiserum raised in a rabbit against a peptide which corresponds to a sequence in the human cytoplasmic peptide of α2.
Dr. L. Reichardt. University of California, San Francisco

2) Anti-α3 : A polyclonal antiserum raised in a rabbit that recognizes a peptide corresponding to the cytoplasmic domain of the α3 integrin subunit.
Dr. R. Hynes. Massachusetts Institute of Technology, Cambridge MA

3) Anti-α4 : A polyclonal antiserum raised in a rabbit against the α4 cytoplasmic domain peptide.
Dr. Mats Johannson. La Jolla Cancer Research Foundation

4) Anti-α4 : A rat monoclonal antibody raised against mouse antigen.
Dr. Weissman. Howard Hughes Medical Institute

5) Anti-α6 : J1B5, a rat anti-human monoclonal antibody.
Dr. Caroline H. Damsky. University of California, San Francisco

We used anti-α3 and anti-α6 as positive controls.

3. Invasion assay

This assay measures invasion as the ability of cells to cross a barrier of Matrigel^{TM} (Collaborative Research Incorp. Bedford, MA), a commercially available basement membrane-like matrix prepared from the basement membrane of the Engelbreth-Holm-Swarm (EHS) yolk sac tumor. The major components of the reconstituted structures include laminin, type IV collagen, heparan sulfate proteoglycan, entactin (Kleinman, et al., 1982; 1986). Our in vitro invasion assay system used this reconstituted basement membrane-like matrix coating a polycarbonate filter thus simulating in vivo events of intravasation and extravasation of lymphatic and blood vessels. To determine invasiveness, cells were plated on 6.5mm Transwell inserts containing polycarbonate filter with 8μm pores (Nucleopore, Pleasanton, CA) in tissue culture well plates (Costar, Cambridge, MA). The upper surface of the filters was coated with 1:1 ratio of Matrigel^{TM} to DME containing 2% Nutridoma, but no serum (N-DMEM) and incubated at 37°C for 30 minutes to allow the matrix to gel. CS-1 cells ($1x10^5$) and BCS-1 cells ($1x10^5$) were plated on the filter in 200μl of serum free media. Medium (N-DMEM) was added to both the top and bottom of the culture. After 72 hrs, the Transwell inserts were washed and the cells were fixed with 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer. The samples were then processed for scanning electron microscopy. The filters were passed through increasing ethanol concentrations up to 100% for dehydration. While still attached to the inserts, the samples were critical-point dried, then the filters were cut off the inserts with a scalpel blade and mounted with either the upper or lower sides exposed for coating. The filters were sputter-coated with a thin layer of gold palladium, and the specimens were examined in a JEOL (JSM-840A) scanning electron microscope operating

at 10 kV. For quantitation of invaded cells, we took 3 photograghs (150X) of each filter underside which covered the whole central area in which invasion was observed. We overlaid the photographs with a grid consisting of 120 bars and counted the number of ends of the bars that overlapped with cells. Then calculated the data as the percentage of grid ends that overlapped with invading cells. For each condition tested, we examined two filters and repeated twice.

4. Substrate gel zymography

Cell extracts and conditioned media were subjected to SDS substrate gel electrophoresis in 10% polyacrylamide gels. Proteinase substrates were gelatin (3mg/ml, denatured type I collagen; Sigma, St. Louis MO) for the detection of metalloproteinases, and casein (3mg/ml; Sigma) and plasminogen (2μg/ml) for the characterization of plasminogen activators. These substrates are readily cleaved by connective tissue-degrading proteinases and are easily incorporated into the polyacrylamide gels. Serum free conditioned media derived from cell cultures were spun at low speed to remove floating cells and cell debris. After collecting supernatant, this conditioned media was concentrated in Centriprep 30 concentrator (Amicon, Danvers, MA). Protein concentration of samples were controlled by the Lowry method. Approximately 2 μg of total protein was loaded on each lane of substrate gel under non-reducing conditions. Gels were run at 15 mA/gel while stacking and at 20 mA/gel during the separating phase. The gels were soaked in 2.5% Triton X-100 with gentle shaking for 30 minutes at room temperature with one change of detergent solution. This was done to wash out the SDS from the gel,

to allow the proteinases to renature. The gels were rinsed and incubated for 16-18 hrs at 37°C in incubating buffer (50mM Tris-HCl buffer, 10 mM $CaCl_2$ and 0.02% NaN_3, pH 8). After incubation, the gels were stained for 15-30 minutes in 0.5% Coomassie Blue, 10% acetic acid and 30% isopropyl alcohol. Then, destained in 25% 2-propanol, 20% acetic acid and water and photographed. Gelatin degrading enzymes present were identified as clear zones in a blue background.

5. Immunoblotting

The identity of metalloproteinases secreted by CS-1 and BCS-1 cells was confirmed by Western blots. Conditioned media of both CS-1 and BCS-1 cells were analyzed on 10% gel and transferred to nitrocellulose. Nonspecific staining was blocked by incubating the transfers in 5% nonfat dried milk (Carnation™) in phosphate buffered saline for one hour at room temperature on a roller incubator. The transfers were then incubated for 48hrs with anti 72kD or 92kD gelatinase antibodies (sheep anti-porcine or sheep anti-human respectively from Dr J Reynold, Cambridge University) diluted 1:100 in the blocking buffer. The blots were washed once in non-detergent wash (50 mM Tris-HCl, 150mM NaCl, pH 7.5), twice in detergent wash (50mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholic acid and 0.1% SDS, pH 7.5) and twice in non-detergent wash again for 5 minutes each time. Then they were incubated with peroxidase-conjugated, affinity-purified donkey anti-sheep IgG diluted 1:100. The blots were washed again as described above. Bands were identified using the Enhanced Chemiluminescence Detection System and Hyperfilm (Amersham, Arlington Heights, Il).

6. Inhibitor gels

After the Triton X-100 step in 12% substrate gel zymography, the gels were incubated at room temperature for 10-20 minutes in APMA-activated rabbit skin conditioned media. This CM contains several proteinases that partially degrade the substrate within the gel under these conditions, except where inhibitors are located. The gel impregnated with rabbit metalloproteinases were then incubated overnight in incubating buffer, stained, and destained. This technique allowed for simultaneous visualization of both metalloproteinases and inhibitors.

RESULTS

The cell system we used was two hamster melanoma cell lines that exhibit unique patterns of adhesion to extracellular substrata and distinct levels of melanin synthesis (Farishian and Whittaker, 1979). An adherent hamster melanoma cell line, CM-1 is amelanotic and a nonadherent cell line, CS-1 cells that arose from CM-1 synthesize melanin. CS-1 can be induced to adhere in the presence of $2X10^{-6}M$ 5-bromodeoxyuridine (BrdU) (Avdalovic and Aden, 1978). This was called BCS-1.

1. Immunoprecipitation

Previous studies (Thomas and Damsky, 1989) had examined expression of several integrins in CS-1, CM-1 and these cells treated with BrdU (Table 1). All

cells expressed the specific FnR, α5/β1 and the specific LnR, α6/β1, although levels were greater in BrdU treated cells. CS-1 did not express αV subunits or α3 subunits and both were turned on by BrdU. Two potentially important integrins were not examined in those studies and were examined here for the following reasons. CM-1 and CS-1 cells showed poor attachment to collagen, only about one sixth as adhesive on collagen as on fibronectin. Treatment with BrdU increased attachment levels to 70% in both cell lines. Integrin receptors that might account for this difference are α1/β1, α2/β1, and α3/β1. Even though CM-1 expressed high levels of α3, they attached poorly to collagen. Also, no β1 associated α subunit with the high molecular weight of the α1 (180-200kD) was observed. The presence of α2 in a β1 immunoprecipitate would be difficult to resolve, because its migration is similar to α3, α5 and α6 which are present in BCS-1 cells.

α2/β1 integrin, also called GPIa/IIa in platelets has been shown to be a main receptor for type I collagen on platelets and for both laminin and collagen on endothelial cells (Kirchhofer, et al., 1990). They suggested the cell type-dependent regulation of the α2/β1 binding specificity. Different cell types receive different signals from the same type of extracellular matrix substrate. Since the previous studies by Thomas and Damsky (JCB 109 abstr:319a #1751) suggested that α1 and α3 were not likely to be involved in BrdU regulated adhesion to collagen, α2 was the most likely major collagen receptor in hamster melanoma CS-1 and CM-1 cells to be regulated by BrdU. Therefore, we tried anti-α2 antibodies obtained from Dr. L. Reichardt. Very faint bands in the appropriate molecular weight region were visible in the BCS-1 but not CS-1 (Figure 1). However, the signal was not strong enough to be certain of the result.

The α4/β1 integrin was also of interest. The presumed neural crest-derived precursor of melanocytes in embryos is likely to be highly migratory and invasive. Neural crest cells and their derivatives and lymphocytes are known to adhere to the IIICS domain in the variably spliced domain of fibronectin (Yamada, 1989), suggesting that this region of fibronectin might be involved in specific migratory events during embryonic development and lymphocyte trafficking (Mould, et al., 1990). Studies by Wayner, et al. (1989) showed that antibodies directed against the α4 subunit and the CS1 peptide from the IIICS domain block lymphocyte adhesion to high endothelial venules in frozen section assays. These results suggest a putative biological role for fibronectin and α4/β1 in lymphocyte homing. α4 is low or absent on most adherent cells, including fibroblasts, endothelial cells, and epithelial cells, all of which are apparently unable to recognize the IIICS, but is a major surface component of lymphocytes and is also found on RD rhabdomyosarcoma cells and some, but not all melanoma cell lines, each of which is IIICS responsive. Furthermore, in view of the ability of neural crest cells and their derivatives to recognize the IIICS region of fibronectin, we thought it would be instructive to examine the role of α4/β1 in migratory events during embryonic development.

Unfortunately, we didn't get the bands where the molecular weight of α4 should be. This may be because the antibodies we used didn't cross react with hamster cells. In future studies, it would be appropriate to determine if either cell line could attach to the CS1 peptide itself, which would reveal the presence of an α4/β1 receptor. Alternatively, the RT-PCR technique or Northern blotting could be used to determine whether RNA for the α2 and α4 subunits are expressed.

2. Invasion Assay

The previous data led us to suggest that BrdU suppresses the differentiated properties of CS-1 cells such that BCS-1 cells resemble a putative amelanotic, invasive melanocyte precursor. Melanocyte precursors must be migratory and capable of invading basement membrane because presumed neural crest derived melanocyte precursors migrate extensively during embryogenesis from neural tube to epidermis of the skin. Therefore we tested the ability of BrdU to regulate invasiveness of CS-1 cells. When we examined the upper surface of the filters, CS-1 cell and BCS-1 cell showed different behavior (Figure 2). CS-1 cells formed many small clusters distributed over the entire top surface of the filter. No CS-1 cells were observed on the bottom of the filter, even after 72 hours of culture. On the other hand, all BCS-1 cells aggregated into one large three dimensional nest-like structure in the center of the top surface of the filter within 24 hours. Also, BCS-1 cells were observed on the bottom of the filter beneath the nest within 24 hours. CM-1 cells formed one large or several small nest-like structures on the upper surface of the filter after 24 hours (Figure 3). Invasion by CM-1 cells was slower. A low level of invasion was evident within 24 hours. By 48 hours, CM-1 cells have covered the filter beneath the central aggregate.

Next, we investigated the idea whether conditioned medium from BCS-1 cells might contain factors that enhance the invasion of CS-1 cells or conditioned medium from CS-1 cells might contain factors that inhibit the invasion of BCS-1 cells. Therefore, we examined BCS-1 cells cultured in the presence of conditioned medium of non-invasive CS-1 cells to see if CS-1 conditioned medium could inhibit invasion of BCS-1 cells. The results showed that BCS-1

cells were unable to form well organized nest-like structures on the top of the filter. Very few BCS-1 cells were present on the bottom of the filter after 24 hours (Figure 5). Therefore, conditioned medium from the noninvasive CS-1 cells decreased invasion of BCS-1 cells. In contrast, BCS-1 conditioned medium did not promote invasion of CS-1 cells (Not shown). These results are consistent with the hypothesis that BrdU regulates the invasiveness of CS-1 cells, at least in part, by suppressing expression of a soluble factor that regulates the invasive phenotype. Proteinases and proteinase inhibitors are candidate molecules to be regulated by such a factor.

3. Proteinase Production

In order to invade in vivo or an artificial Matrigel^{TM} coated porous filter matrix barrier in vitro, cells require the proteinases necessary for local degradation of the matrix. Therefore we looked at whether the expression of plasminogen activators (PA) and matrix metalloproteinases (MMP) are also regulated by BrdU. Cell extracts and conditioned medium of both CS-1 cells and BCS-1 cells were loaded on a substrate gel to which gelatin, or casein and plasminogen were incorporated as the substrates. Lysis zones were revealed by Coomassie blue staining and destaining. Position of lysis zones suggests that BrdU upregulates expression of the 92kD type IV, type V collagenase (Figure 7). The multiple bands in the BCS-1 may represent activated forms of this proteinase. In order to confirm that BCS-1 cells express 72kD and 92kD gelatinase, we did a Western blot using CS-1 and BCS-1 conditioned media. Antibodies we used were sheep anti-human 72kD and sheep anti-porcine 92kD gelatinase Abs (Dr. J Reynold's, Cambridge University). Bands were observed

at the position of molecular weight 72kD on both cell lines and 92kD only on BCS-1 conditioned medium lane (Figure 8).

Both cell lines expressed multiple lysis zones (Figure 9) in casein-plasminogen gels. Bands at 52kD migrate like single chain uPA. Bands at 70-75 migrate like tPA. Lower molecular weight forms might represent activated forms of one or both enzymes. BrdU treatment of CS-1 cells enhanced expression of these putative activated forms of plasminogen activators.

4. Inhibitor Gel

Since net proteinase activity of the 2 cell lines depends on the balance of proteinase and proteinase inhibitors, and because a proteinase inhibitor was a good candidate for the invasion inhibition factor in CS-1 conditioned medium, we examined both metalloproteinases and their inhibitors on the same gel by the technique used by Herron et al (1986). Inhibition of the degradation of the gelatin substrate was observed in conditioned media from BCS-1 and CM-1 cells at the molecular weight 28kD, position of standard for TIMP-1 (Figure 10). No inhibitory zones were found with conditioned medium from CS-1 cells at this molecular weight. Very faint bands were observed at the molecular weight 19-21kD, position of standard for TIMP-2 on all lanes.

DISCUSSION

Data accumulated at the start of this study suggested the hypothesis that BrdU was regulating the differentiation of the CS-1 melanoma cell line. This line expresses melanin, and is responsive to α-melanocyte stimulating hormone (αMSH), which elevates melanin synthesis and reduces growth. Following BrdU treatment, CS-1 cells stop expressing melanin, are non responsive to αMSH and have an enhanced attachment to a wide variety of substrates. To test the hypothesis, we reasoned that the precursor of the melanocyte would be highly migratory and invasive in order to migrate from neural tube to epidermis.

Since the original data on the alterations in adhesion phenotype were incomplete, we first tried to answer the question ; How do hamster melanoma cells attach to collagen which is a major constituent of subendothelial matrix? It appeared that more than one receptor contributed to binding collagen. The BrdU-mediated induction of α3 in CS-1 cells could not account for the high level of collagen attachment exhibited by BCS-1 cells because CM-1 cells have lots of α3 and attach poorly to collagen. BrdU may induce a second collagen receptor, such as α2, which would explain the increased adhesion recorded for BrdU-treated CS-1 and CM-1 cells. Therefore we did immunoprecipitation using a polyclonal antiserum raised in a rabbit against the human cytoplasmic peptide of α2, obtained from Dr. L. Reichardt. Very faint bands in the appropriate molecular weight region were visible in the BCS-1 but not CS-1. Even though we didn't get the strong signal enough to be certain, maybe because of poor cross-reactivity, it still might be interesting to examine using

anti-α2 antibody which is more specific to hamster. If α2 expression turned out to be upregulated by BrdU, it might also need to further investigate whether this α2/β1 integrin binds only to type I collagen, like on platelets or both to laminin and fibronectin as well as type I collagen, as is the case with endothelial cells. Alternative ways to look for α2 expression would be to use RT-PCR or Northern blotting to see whether this subunit is expressed at the RNA level.

Next, we reasoned that the precursor of melanocyte would be highly migratory and invasive during embryonic development. Several neural crest derived cells express the α4/β1 integrin receptor which recognizes the CS1 peptide in the IIICS region of fibronectin. This interaction is thought to play an important role in migratory events (Mould, et al., 1990; Wayner, et al., 1989). Therefore we tried to investigate the expression of α4/β1 in BrdU treated cells by immunoprecipitations using anti α4 antibodies. We did not get the band where the molecular weight of α4 is going to be, maybe because of the poor cross-reactivity again. Cell attachment assays using the purified CS1 peptide or RT-PCR or Northern blotting to look for α4 RNA could both help to determine whether expression of this integrin is regulated by BrdU.

In the invasion assay, BCS-1 cells were able to actively invade reconstituted Matrigel^{TM}, while CS-1 cells could not. Our first expectation was that conditioned medium from BCS-1 cells might contain factors or enzymes that would enhance the invasion of CS-1 cells. However, when conditioned medium of non invasive CS-1 cells was added to BCS-1 cells, invasion of BCS-1 cells was decreased markedly. On the other hand, BCS-1 conditioned medium did not promote invasion of CS-1 cells. Therefore, it was reasonable for us to think

that certain molecules which inhibit invasion might be present in CS-1 conditioned medium.

In order for the tumor cells to migrate through the tissue, they must be able to degrade basement membrane and extracellular matrix. The results we got represents that BrdU treatment upregulated expression of 92kD and apparent activation of plasminogen activator system. These results are consistent with the thought that the more invasive cells would have to express more proteinases or more of their activated forms, in order to degrade the matrix. To test which enzyme system, PA's or MMP's is most critical in mediating BCS-1 cell invasion, it will be important to carry out invasion assays in the presence of inhibitors for each class of proteinase.

In order to test the idea that proteinase inhibitors in the conditioned medium of CS-1 cells might account for its invasion inhibitory activity, we ran inhibitor gels of the cell extracts and conditioned media of both cell lines. The result was that BCS-1 cells showed a strong inhibitory zone of molecular weight 28kD and a lighter band at 19-21kD while CS-1 cells had only a lighter band at 19-21kD. Thus the invasion inhibitory activity present in CS-1 conditioned medium was not a member of the TIMP family. Although it was at first surprising that TIMPs, as well as metalloproteinases were elevated in BCS-1 cells by BrdU, it is likely that the net proteolytic activity was enhanced by BrdU. This idea is now being tested directly using a sensitive total collagenase assay such as spectrophotometric assay using thiopeptolide substrate. We expect that the net collagenase activity will be greater in BCS-1 cells than CS-1. Further studies will also determine whether PA inhibitors might represent the invasion inhibitory activity in CS-1 conditioned medium.

In these studies, we showed that the well differentiated melanoma cell line CS-1 cells expressed melanin, responded to αMSH, suggesting they have αMSH receptors, displayed a restricted integrin adhesion receptor repertoire, expressed no detectable matrix metalloproteinases, and were not invasive in a Matrigel^{TM} invasion assay. Following treatment with BrdU, these BCS-1 cells became amelanotic, unresponsive to αMSH, highly invasive, and expressed high levels of the 92kD gelatinase and a broad integrin repertoire. Our results suggest that BrdU induces a net positive change in the proteolytic balance. A significant feature of metalloproteinase and TIMP action is that their expression can be regulated by growth factors and hormones. Collagenase has been reported to be induced by agents such as interleukin-1 (Meikle, et al., 1986), platelet-derived growth factor (Chua, et al., 1985) and phorbol esters (Brinckerhoff, et al., 1986). TIMP gene expression is also induced by growth regulatory agents such as epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), and phorbol esters (Murphy, et al., 1985). TGF-β also has been shown to have a direct inhibitory effect on endothelial cell invasion (Muller, et al., 1987; Mignatti, et al., 1989) by altering the balance of proteinase and proteinase inhibitor expression. Also, there is overexpression of receptor for TGF-α in several malignant tumors such as melanoma, squamous carcinomas, renal carcinomas, mammary carcinomas, hepatomas and glioblastomas (Herlyn, et al., 1990). Thus it was possible that the difference in invasiveness of the two cell lines in our hamster melanoma system resulted from differential expression of growth factors.

To determine whether conditioned medium from either cell line could affect invasive behavior of the other cell line, CS-1 cells were cultured in BCS-1 conditioned medium and BCS-1 cells were cultured in CS-1 conditioned medium. CS-1 conditioned medium inhibited BCS-1 cell invasion, while BCS-1

conditioned medium was not able to promote CS-1 invasion. Experiments are in progress to determine the nature of this product. Candidates include a proteinase inhibitor or one or more growth factors which affect proteinase expression and/or cell motility. The inhibitory factor in our cell system is not a TIMP because CS-1 cells do not express this inhibitor family, at least as detected by inhibitor gel zymography. It could be a plasminogen activator inhibitor (PAI). Therefore, we are now investigating the plasminogen activator inhibitor gels. Also, we are examining whether the presence of TGF-β in CS-1 conditioned medium might account for its invasion inhibitory activity. Experiments to test this idea include (1) can exogenous TGF-β mimic CS-1 conditioned medium, (2) can anti-TGF-β antibody stimulate invasion of CS-1 or block the ability of TGF-β to inhibit BCS-1 invasion. Recently, in our lab, it has been shown that both CS-1 and BCS-1 cells express TGF-β. Thus we are now investigating whether there is a difference in ability of the two cell lines to activate TGF-β.

During invasion, tumor cells must change their adhesive properties by selective expression of integrin receptors (Ruoslahti and Giancotti, 1989). It is reasonable that they would express multiple receptors that could bind to basement membrane-specific components, such as laminin and type IV collagen. On the other hand, interstitial tumor cells at the primary tumor site must be highly motile in order to reach neighboring vascular beds for subsequent dissemination. It would be reasonable to suggest that invasiveness would be enhanced by being able to adhere with low or moderate strength to a wide variety of ECM components. In support of this idea, BrdU enhances expression of 2 promiscuous receptors α3/β1 and αV/β3.

Relations among change in integrin profile, synthesis of proteinases and expression of growth factors are extremely complex and various dependent upon the cell lines being studied. However, it would be interesting to look at which molecules: adhesion receptors, growth factors and proteinases, play the most fundamental roles in regulating invasiveness in this system. This knowledge should be useful also in understanding normal differentiation.

Table 1

	>----------INVASIVENESS---------->			
	<----------DIFFERENTIATION----------<			
PROPERTY	**CS-1msh**	**CS-1**	**CM-1**	**BCS-1 BCM-1**
Adhesion				
Fn	+	+	+	+
Ln	+	+	+	+
Col	-	-	-/+	+
Vn	-	-	+	+
Fbg	-	-	+	+
Integrin Expression				
α3		-	++	++
α5		+	+	++
α6		+	+	++
αV		-	+	++
ß3		-	+	++
ß5		-	+	++
Melanin expression	++	+	-	-
αMSHr	+	+	-	-
Growth rate	+/-	++	++	++
Proteases				
72kD MMP		+/-	+	+
92 kD MMP		-	+	++
uPA		55kD	55, 22kD	22kD
tPA		+	+	+
Protease Inhibitors				
TIMP		-	+	++
Invasion		-72h	+ 48 h	++ 24 h

MMP: Matrix metalloproteinase
αMSH: Melanocyte stimulating hormone
TIMP: tissue inhibitor of metalloproteinase

Figure 1

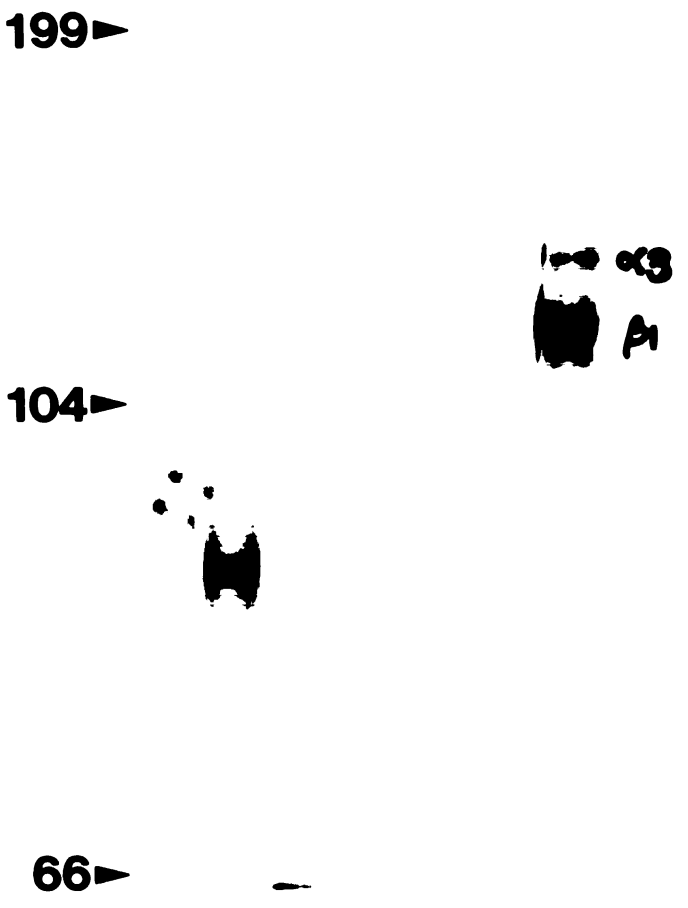

Figure 1 Immunoprecipitation with a rabbit polyclonal antiserum against human cytoplasmic peptide of α2

^{3}H labelled CS-1 and BCS-1 cell lysates were immunoprecipitated with an antibody raised against a human cytoplasmic peptide of α2 integrin subunit. Immunoprecipitates were analyzed by 7% SDS-PAGE followed by autoradiography. Lane 1; CS-1 for α2, Lane 2; BCS-1 for α2, Lane 3; CS-1 for α3, Lane 4; BCS-1 for α3. Very faint bands in the appropriate molecular weight region were visible in the BCS-1 lane but not CS-1. The identity of the 80-90kD in lane 1 is not clear.

Figure 2

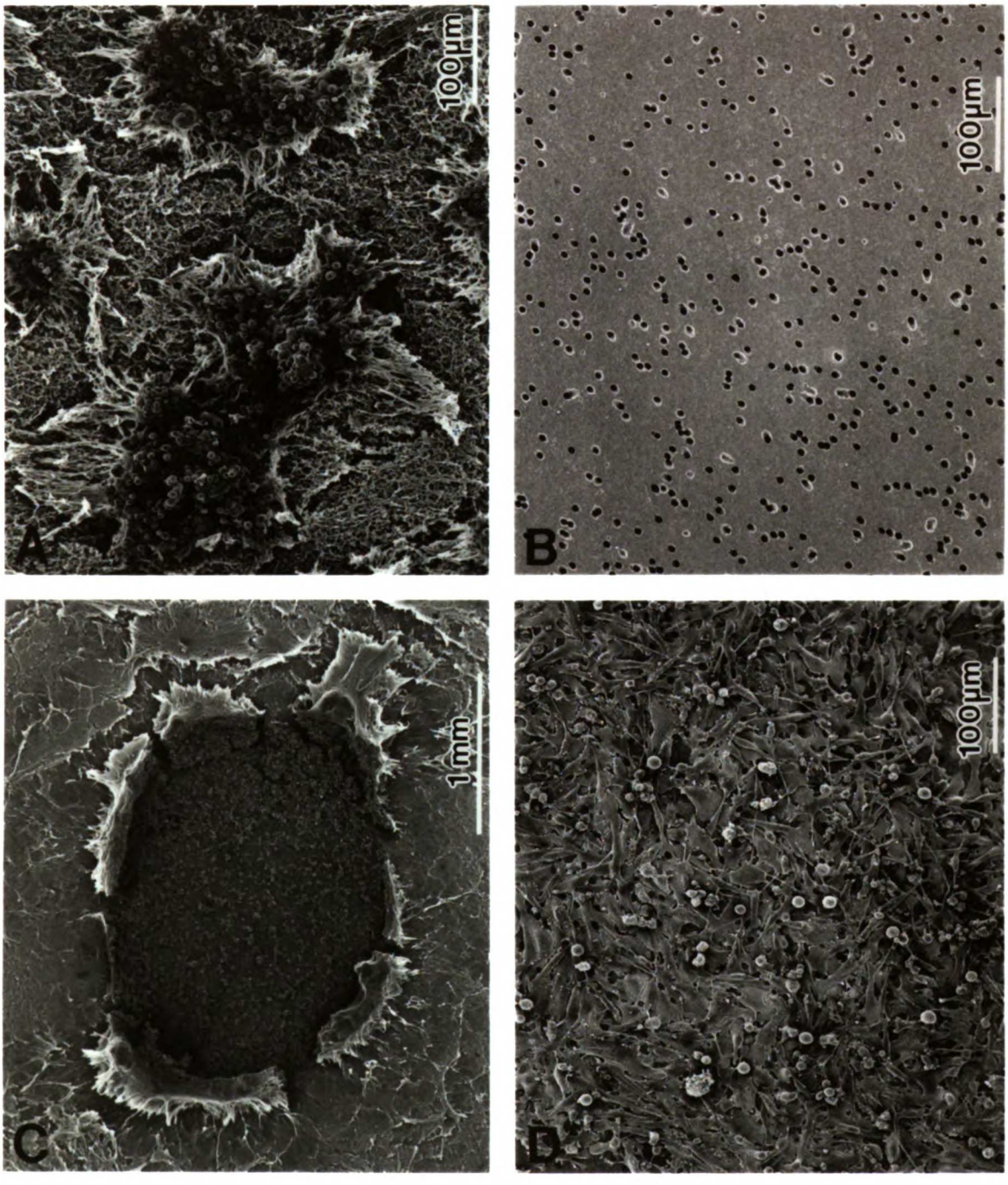

Figure 2 Scanning electron microscopy shows CS-1 and BCS-1 cell invasion on the Matrigel^{TM} coated polycarbonate filters

(A) CS-1 cells on the upper surface of the filter; After 48 hours, CS-1 cells formed several small clusters. (B) CS-1 cells on the bottom of the filter; None of cells penetrated to the underside of the filter. (C) BCS-1 cells on the upper surface of the filter; Within 24 hours, BCS-1 cells formed one large cell aggregate in the center of the filter. (D) BCS-1 cells on the bottom of the filter; Cells actively penetrated the Matrigel^{TM} and covered the undersurface of the filter beneath the large aggregate within 24 hours. BrdU treatment of CS-1 cells promoted their invasion of the reconstituted basement membrane matrix, Matrigel^{TM}.

Figure 3

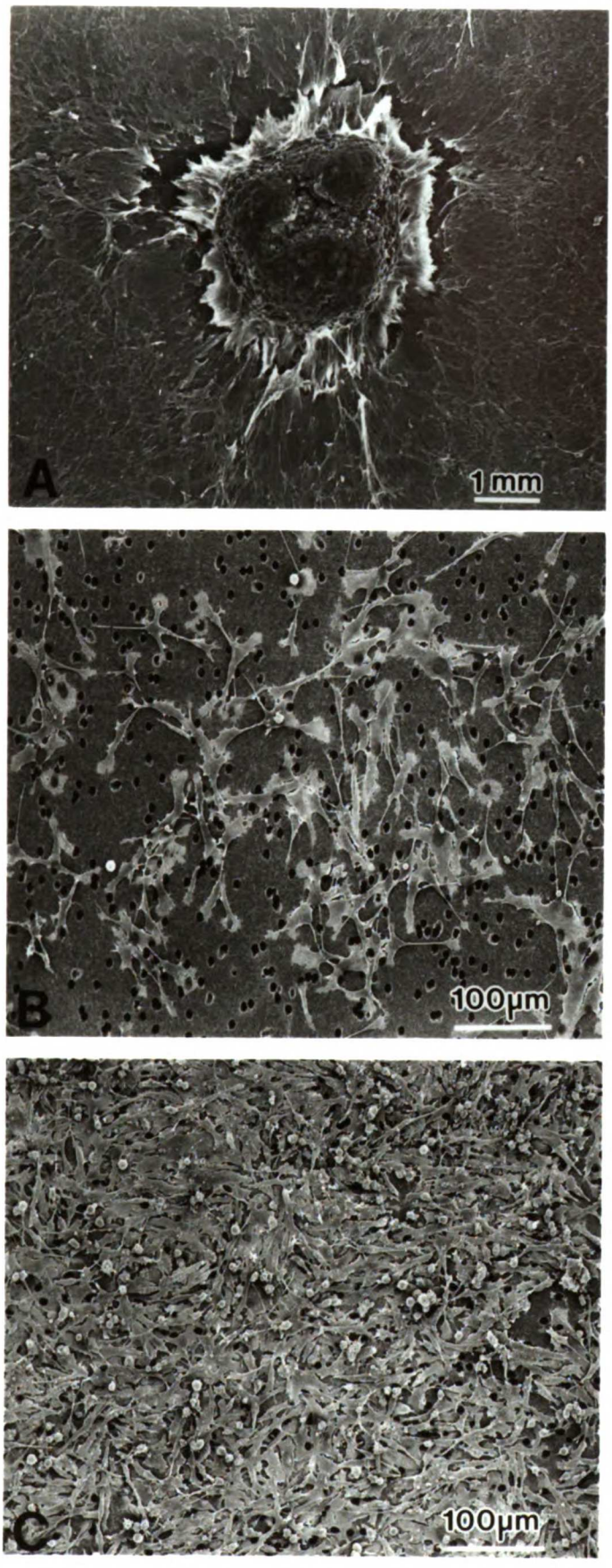

Figure 3 Scanning electron microscopy of CM-1 cell invasion on the Matrigel^{TM} coated polycarbonate filters.

(A) CM-1 cells formed nest-like structures on the upper surface of the filter after 24 hours. A small nest is shown here. (B) A low level of invasion is evident within 24 hours. (C) By 48 hours, CM-1 cells have covered the filter beneath the central aggregate.

Figure 4

Invasion Profile of Hamster Melanoma Cell Lines: CS-1, CM-1, BCS-1
% Surface Area of filter underside covered vs. Hours in Culture

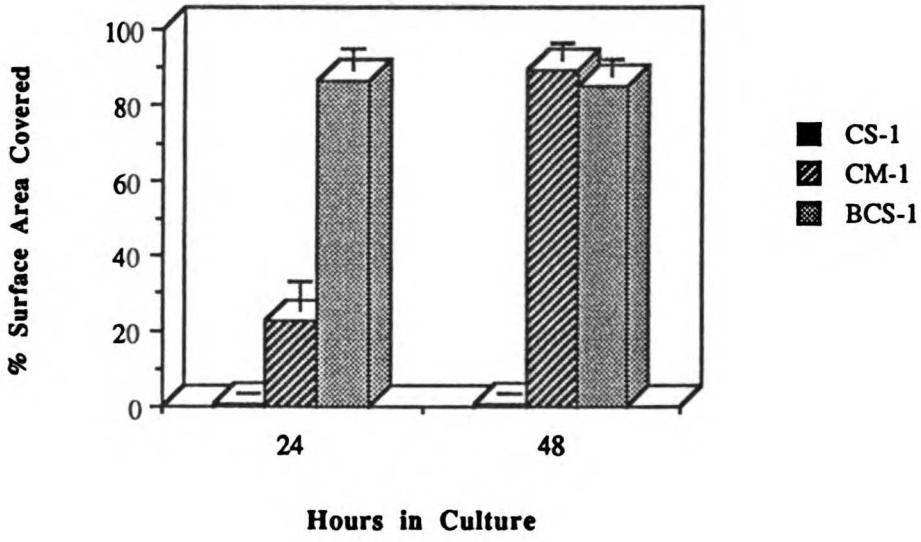

Figure 4 Invasion Profile of Hamster Melanoma Cell Lines: CS-1, CM-1, BCS-1.

The graph was plotted as % surface area of the filter underside covered versus hours in culture at two time points, 24 hours and 48 hours. % surface area covered by the 3 cell lines at 24 hours was significantly different ($p<.01$). At 48 hours, % surface area invasion between CM-1 and BCS-1 cells was not significantly different. Invasion of CM-1 cells was slower than BCS-1 cells. BCS-1 cells have covered the filter beneath the central aggregate within 24 hours, while CM-1 cells within 48 hours.

Figure 5

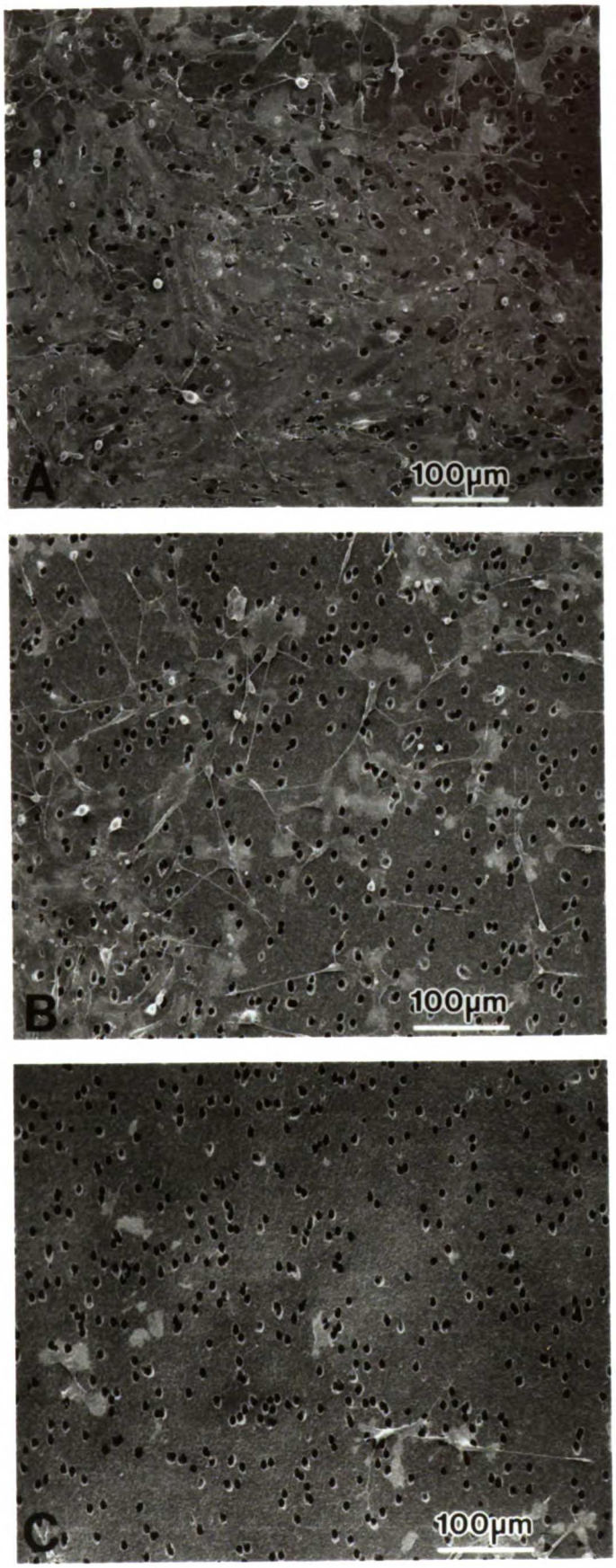

Figure 5 Effect of CS-1 conditioned medium on BCS-1 cell invasion at 24 hours.

(A) BCS-1 cells plated with BCS-1 conditioned medium as a control. (B) BCS-1 cells plated with CS-1 conditioned medium (1:4 dilution). (C) BCS-1 cells plated with CS-1 conditioned medium (1:8 dilution).

Conditioned medium from the noninvasive CS-1 cells decreased invasion of BCS-1 cells. BCS-1 conditioned medium did not affect invasion of either BCS-1 (A) or CS-1 (not shown).

Figure 6

Effect of CS-1 Conditioned Medium on BCS-1 Cell Invasion (11/24/91)

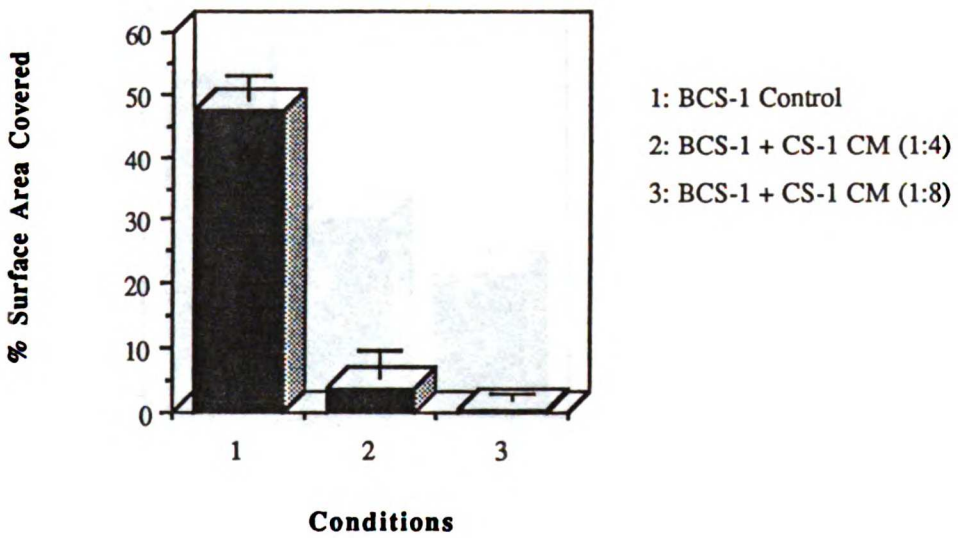

Figure 6

Effect of CS-1 Conditioned Medium on BCS-1 Cell Invasion (2/6/92)

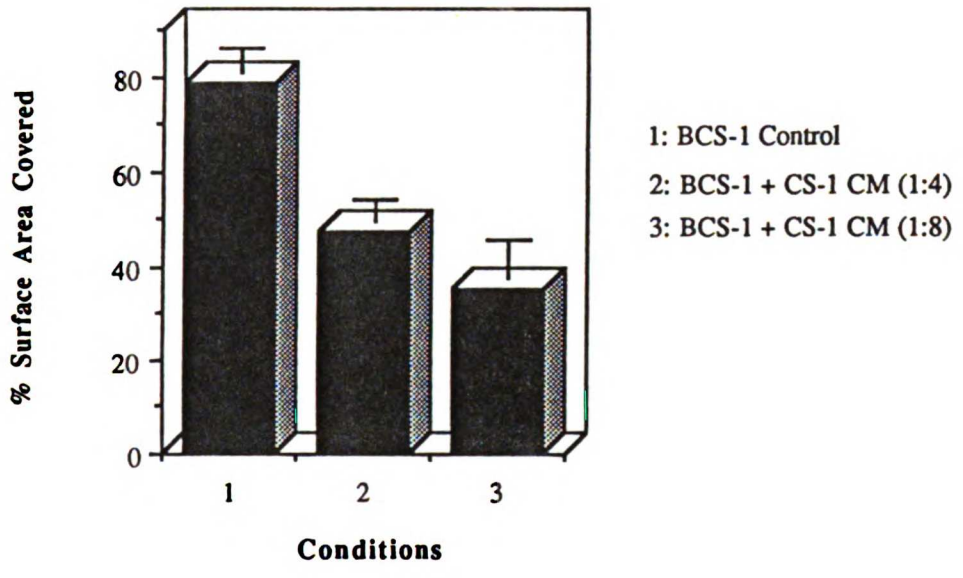

Figure 6 Effect of Conditioned Medium from CS-1 Cells on BCS-1 Cell Invasion at Two Different Dates.

The graph was plotted as % surface area covered versus medium added. Statistical analysis shows difference in surface area invasion between BCS-1 control and BCS-1 cells to which either a 1:4 or a 1:8 dilution of conditioned medium of CS-1 cells is significant ($p<.05$).

Figure 7

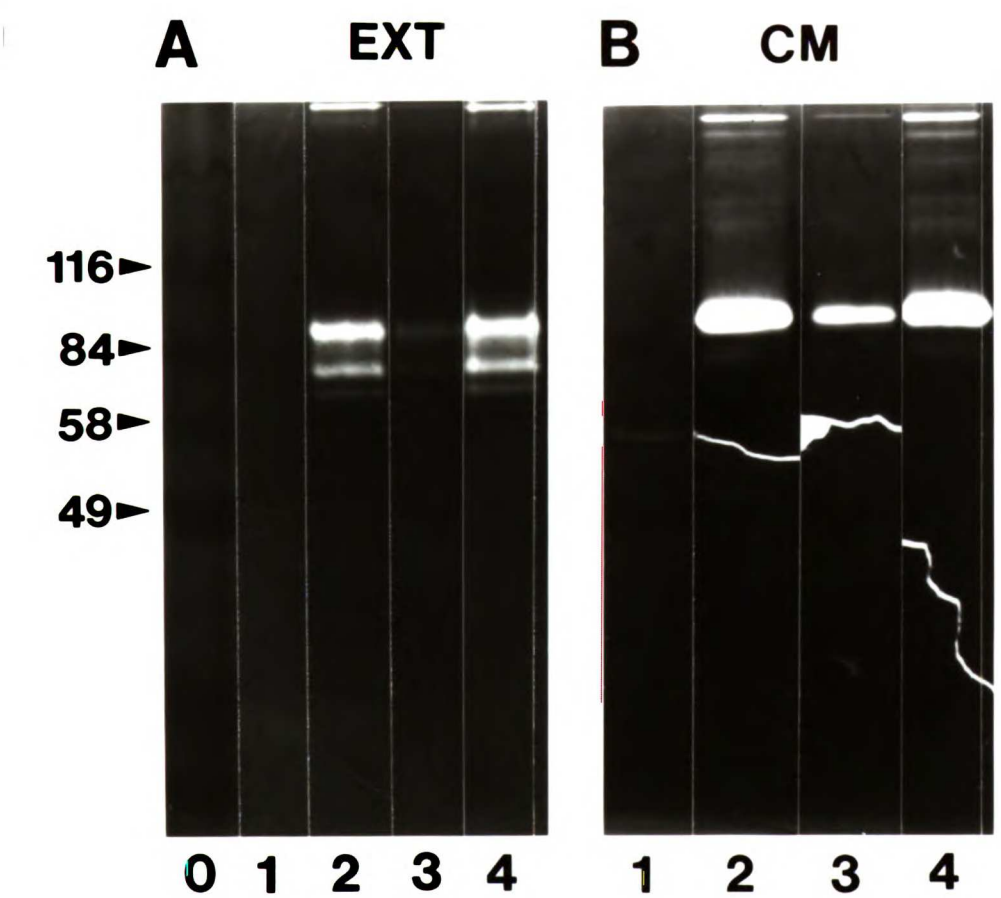

Figure 7 Substrate gel zymography for the detection of matrix metalloproteinases.

Samples of cell extract(EXT) and conditioned medium(CM) from cultures of hamster melanoma cells were analyzed on 10% SDS polyacrylamide gels containing 3mg/ml gelatin. (A) cell extract (B) conditioned medium. Lane 0; molecular weight standard, Lanes 1; CS-1 cells, Lanes 2; BCS-1 cells, Lanes 3; CM-1 cells, Lanes 4; BCM-1 cells

BrdU treatment upregulated expression of the 92kD collagenase.

Figure 8

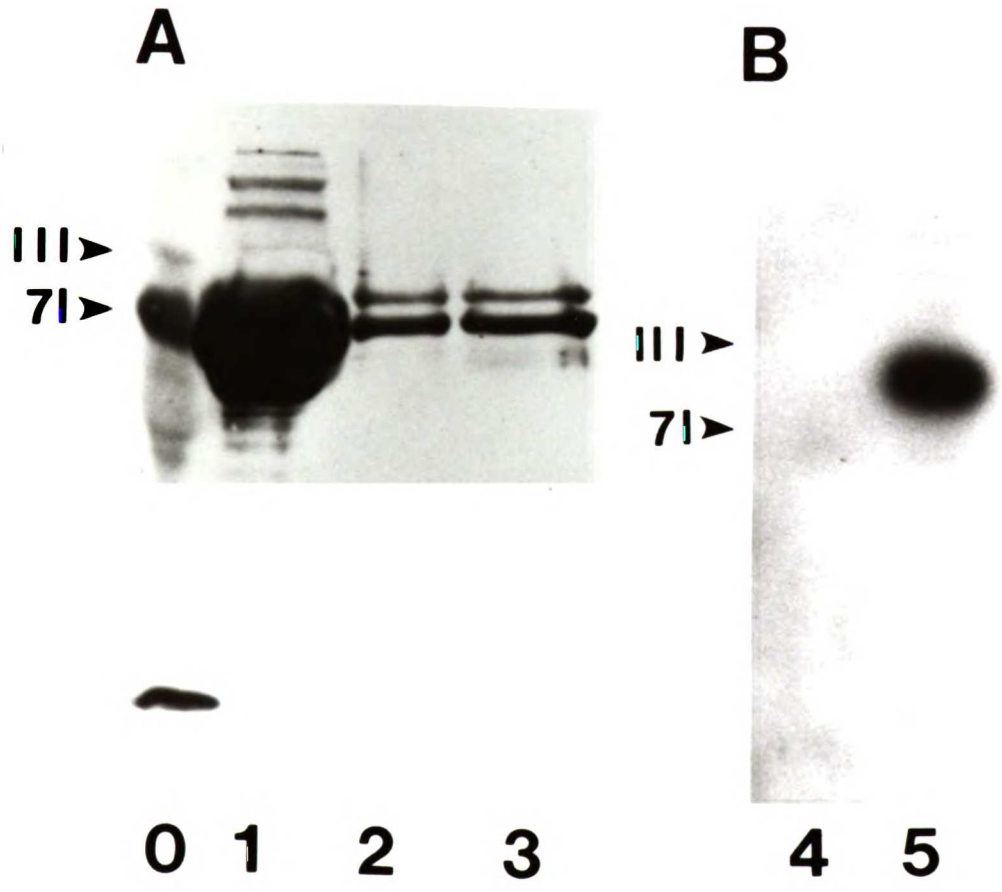

Figure 8 Visualization of 72kD and 92kD collagenase by Western blot.

Conditioned media from CS-1 and BCS-1 cells were electrophoretically separated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose. The blot was probed with anti 72kD (panel A) or 92kD (panel B) gelatinase antibody. Lane 0; molecular weight standards, 1; positive control for 72kD, 2; CS-1 conditioned medium, 3; BCS-1 conditioned medium, 4; CS-1 conditioned medium, 5; BCS-1 conditioned medium. 72kD gelatinase was expressed on both CS-1 and BCS-1 conditioned media. Only BCS-1 conditioned medium showed band at the position of molecular weight 92kD.

Figure 9

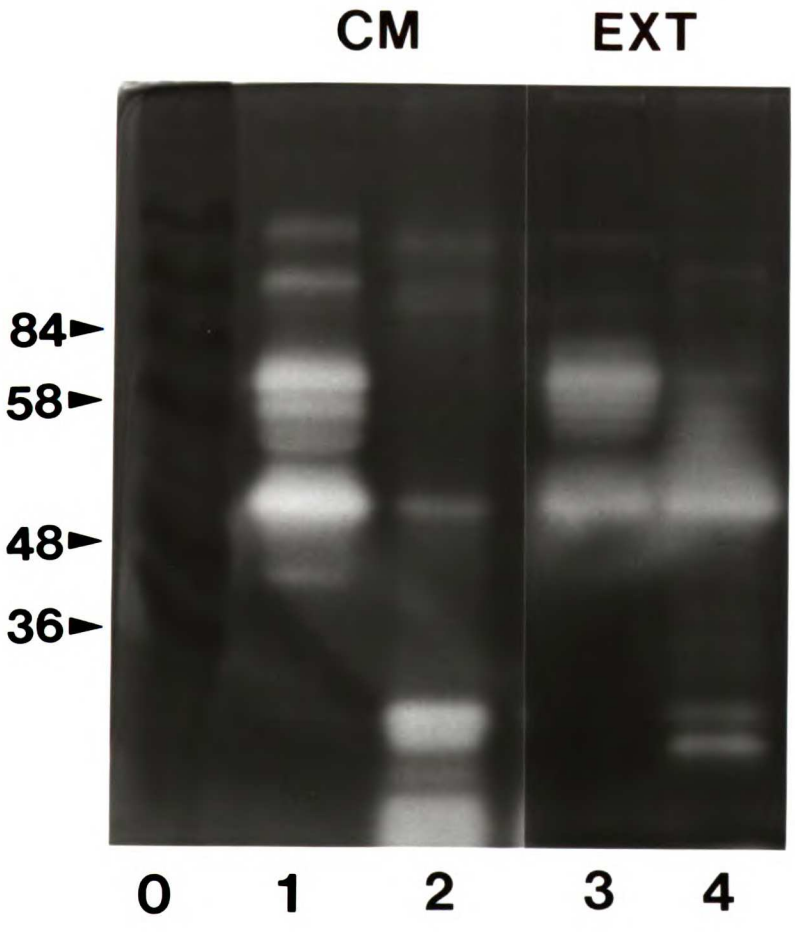

Figure 9 Substrate gel zymography for detection of plasminogen activator system.

Cell extract and conditioned medium were analyzed on 10% SDS polyacrylamide gels containing 3mg/ml casein and 2μg/ml plasminogen. Lane 0; molecular weight standards, 1; CS-1 conditioned medium, 2; BCS-1 conditioned medium, 3; CS-1 cell extract, 4; BCS-1 cell extract. Both cell lines expressed multiple lysis zones. Bands at 52kD migrate like single chain uPA. Bands at 70-75 migrate like tPA. Lower molecular weight forms might represent activated forms of one or both enzymes. BrdU treatment of CS-1 cells enhanced expression of putative activated forms of plasminogen activators.

Figure 10

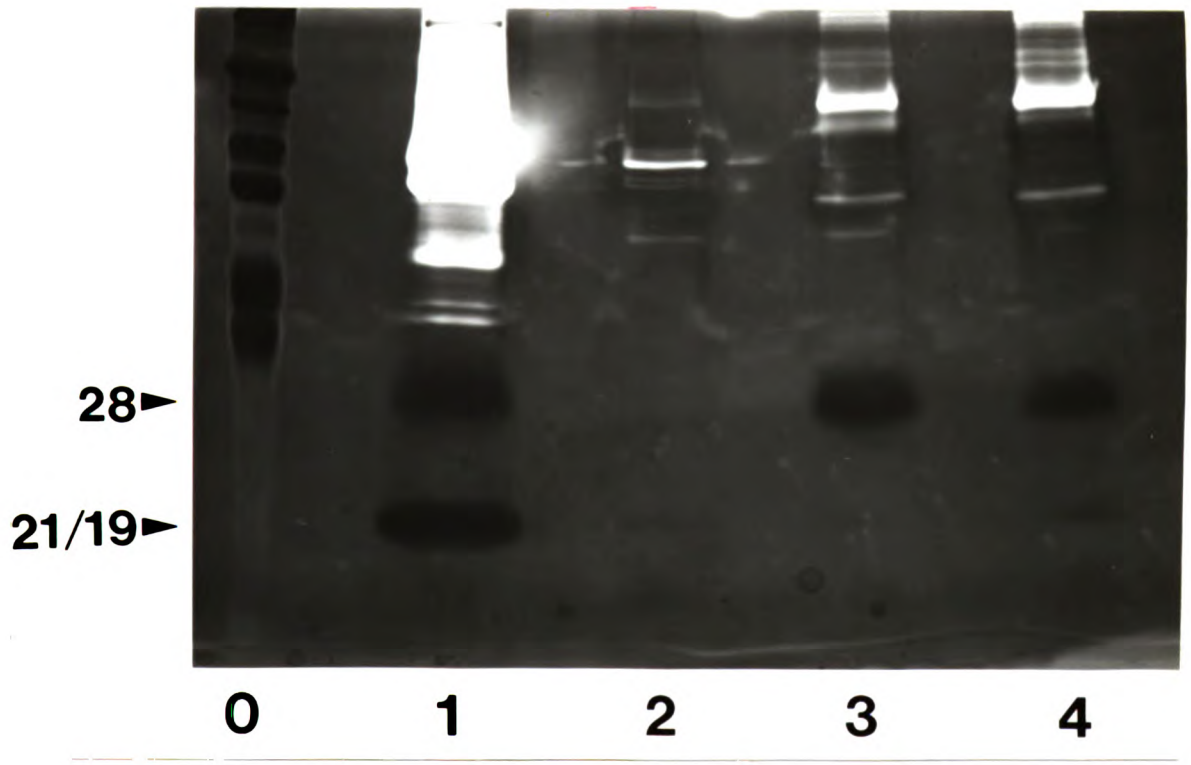

Figure 10 Identification of matrix metalloproteinase inhibitors from hamster melanoma cell lines by SDS-substrate gel analysis Conditioned media collected from CS-1 (lane 2), BCS-1 (lane 3), and CM-1 (lane 4) were separated on substrate gel prepared with 3mg/ml gelatin. After electrophoresis and wash with Triton X-100 to remove SDS, gel was treated with metalloproteinases from APMA-activated rabbit skin conditioned media. Then gel was incubated overnight in incubating media, stained, and destained. *Darkened* areas represent undigested substrate protected by metalloproteinase inhibitors and *cleared* areas represent proteinases resolved during electrophoresis. Lane 0; molecular weight markers, 1; TIMP 1 (28kD) and TIMP 2 (19-21kD) standard for control, 2; CS-1 conditioned medium, 3; BCS-1 conditioned medium, 4; CM-1 conditioned medium. The darkened bands were observed on BCS-1 and CM-1 conditioned medium lanes at the molecular weight 28kD. No inhibitory zone was found with conditioned medium from CS-1 cells at this molecular weight. Very faint bands were observed at the molecular weight 19-21kD on all lanes.

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