Title
The growth factor, TGF-beta 1, and the integrin, alpha V beta 3, regulate invasion of hamster melanoma cells

Author
Chan, Patti Walter

Publication Date
1992

Peer reviewed|Thesis/dissertation

The growth factor, TGF-beta 1, and the integrin, alpha V beta 3, regulate invasion of hamster melanoma cells.

by

Patti Walter Chan

THESIS

Submitted in partial satisfaction of the requirements for the degree of

MASTER OF SCIENCE

in

Oral Biology

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

San Francisco

Date University Librarian

Degree Conferred: 12-31-92

This work is lovingly dedicated to the memory of my father
John Alexander Walter
who died on December 15, 1991.
I hope he would have been proud.

Acknowledgements

I would like to thank the following people for their support during my graduate education:

My family, especially my mother, Joan Walter, and my husband, Raymond Chan, for their encouragement and unending support when things were tough.

Caroline Damsky for her guidance and friendship.

Everyone in the Damsky lab for the crazy and fun times we shared over the past three years.

Paul Riofski for his cheerful smile and helpful assistance with administrative procedures.

John McCulloch for his invaluable assistance and expertise with the scanning electron microscopy.

Most important, I would like thank God for directing my life and giving me the strength and wisdom to complete this phase of my graduate education.

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Introduction

Regulation of melanoma tumor cell invasion is a complex process which requires the interactions of many different molecules, including: extracellular matrix proteins (Terranova, et al 1984; Humphries, et al 1986; Saiki, et al 1989) and their adhesion receptors (Albelda, et al 1990; Nip and Brodt, 1990), growth factors (Kubota, et al 1991; reviewed by Rodeck and Herlyn, 1991), proteolytic enzymes (Liotta, et al 1980; Reich, et al 1988; Quax, et al 1991;), and protease inhibitors (Schultz, et al 1988; Knudsen and Nachman, 1988). Neoplasia may result from a set of alterations in the expression or activities of some of these factors such that the balance is tilted toward net tissue destruction and invasion by the tumor cells. Malignant tumor cells differ from other cancer cells in their ability to spread throughout the body, giving rise to new lesions at sites distant from the primary tumor. In order to invade and metastasize to other sites within the body, the tumor cell must interact with and migrate through a variety of ECM proteins. Thus, an altered adhesion phenotype may be an important feature of tumor cell progression to the malignant phenotype. Similarly, synthesis and secretion by tumor cells of proteolytic enzymes capable of degrading basement membranes and interstitial stroma is essential for the metastatic phenotype (Reich, et al 1988; Meissauer, et al 1991). On the other hand, invasion can be modulated by the presence of protease inhibitors which block the activity of matrix-degrading proteases (Knudsen and Nachman, 1988; Schultz, et al 1988). Decreased production of these inhibitors may favor the development of a more invasive tumor cell and result in matrix degradation.

Melanoma cells secrete a variety of growth factors and matrix-degrading proteases which could contribute to the regulation of metastatic behavior. In order to develop more effective treatment modalities and better diagnostic

techniques for this disease, it is necessary to understand the mechanisms regulating the invasive behavior of these cells. Specific growth factors, such as basic fibroblast growth factor (bFGF), may be necessary for the transformation of normal melanocytes and nevus cells to the invasive and metastatic phenotype of melanomas (Albino, et al 1991; Becker, et al 1989), while the activity of other growth factors, like transforming growth factor-β (TGF-β), may inhibit invasive behavior. TGF-β was originally defined as peptides that reversibly induced non-neoplastic cells to express a transformed phenotype (Roberts, et al 1985). Cells secrete TGF-β in a latent form which then undergoes complex proteolytic processing to become the mature, activated peptide (Lizonova, et al 1990). Once activated, TGF-β is a pluripotent growth factor exerting various biological effects in different cell culture systems. TGF-β inhibits the monolayer growth of both normal melanocytes and melanoma cells in culture, in addition to inhibiting the anchorage-independent growth of many human tumor cell lines including melanoma, lung, and breast carcinomas (reviewed by Rodeck and Herlyn, 1991). In some cell systems, TGF-β increases the synthesis of protease inhibitors (Edwards, et al 1987; Pepper, et al 1990), while decreasing the production of certain proteases (Edwards, et al 1987; Moscatelli and Rifkin, 1988; Overall, et al 1991). As well as modulating the activity of certain matrix-degrading proteases and their inhibitors, this growth factor may also serve to mediate the activation or release of other growth factors associated with the extracellular matrix or regulate expression of matrix components and their receptors, all of which may influence the stability of the matrix, and therefore melanoma cell invasiveness (reviewed by Flaumenhaft and Rifkin, 1991).

With this information in mind, we have been using a melanoma cell system, first described by Farishian and Whittaker (1979) and Knudsen, et al (1982), and further characterized by Thomas et al (submitted), to study the

regulation of tumor cell invasion and the role of specific molecules [one growth factor (TGF-β) and one integrin (αVβ3)] in this process. The three melanoma cell lines (CS-1, CM-1 and BCS-1) display distinct adhesive properties and integrin profiles, exhibit differences in their production of matrix-degrading proteases and their ability to invade a reconstituted basement membrane. The studies presented here were undertaken to identify molecules which are important in the regulation of invasion within our cell system and to investigate mechanisms by which regulation is accomplished.

Materials and Methods

Antibodies and Reagents: Fibronectin (Fn) was purchased from Telios Pharmaceuticals and Laminin (Ln) was purchased from Collaborative Research (Lexington, MA). Purified TGF-β1 was a kind gift of Dr. Rik Derynck, UCSF. Human PAI-1 was a kind gift of Dr. Robert Cohen, UCSF. Antibodies were from the following sources: 12H5 mouse anti-human TGF-β1 monoclonal antibody, 411A mouse anti-human TGF-β1 monoclonal antibody that specifically neutralizes TGF-β1, and 2G7 mouse anti-human TGF-β1 monoclonal antibody that neutralizes all three isoforms of TGF-β1 were kind gifts of Dr. Rik Derynck, UCSF. Rabbit anti-human polyclonal PAI-1 antibody was a kind gift of Dr. Robert Cohen, UCSF (Cohen, et al 1989). LM609, a complex-specific mouse anti-human αVβ3 antibody with adhesion-blocking function, was a kind gift of Dr. David Cheresh, Scripps Institute, La Jolla, CA (Cheresh and Spiro, 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 were derived from CM-1 by continuous passage of cells that did not adhere under the culture conditions used. CS-1 are melanotic and are nonadherent when grown in the presence of serum. CS-1 can be induced to adhere and to stop making melanin in response to the addition of $2x10^{-6}$M 5-bromodeoxyuridine (BrdU) to the culture medium. These cells are designated BCS-1. This effect is reversible: when BrdU is removed, BCS-1 revert to the original CS-1 phenotype. The four cell lines were routinely cultured in Dulbecco's Minimum Essential Medium (DMEM) as described previously (Farishian and Whittaker, 1979; Knudsen, et al 1982).

The human embryonic kidney cell line, 293, was transfected to express either latent TGF-β1 (B_6) or active TGF-β1 (C_{15}) (Arrick, et al 1992). To study the invasive properties of these cells, serum in the medium was replaced by 2% Nutridoma, Boehringer Mannheim (Indianapolis, IN), a supplement which does not contain growth or attachment factors (SFM-ND).

Transfection of CS-1 cells with cDNA for human αV and β3 subunits of the vitronectin receptor (VnR): Full-length cDNA encoding either the human β3 or the human αV integrin subunits ligated into the expression vector pcDNA1*neo* (kindly provided by Dr. David Cheresh, Scripps, La Jolla, CA) were transfected into CS-1 hamster melanoma cells using the lipofectin protocol (Leavesley, et al 1992). Briefly, sub-confluent nonadherent cells were incubated with 10 μg cDNA and 40 μg of lipofectin for 6 or 24 hr. The cells were allowed to recover for 48 hr before selection in neomycin for 2 wk at a concentration of 700 μg/ml. Cells which were adherent in serum and neomycin-resistant were expanded and the cell surface expression of αVβ3 was analyzed by flow cytometry using the αVβ3 complex-specific antibody, LM609. Immunoreactive cells were enriched by selecting for adherent colonies during cell culture. The CS-1:β3 cells are able to attach to plastic in the presence of serum and grow in a monolayer similar to the CM-1 and BCS-1 cell lines.

Collection of 48 hour serum-free conditioned medium: CS-1 cells were grown in 6 well (30 mm) tissue culture flasks (Costar) coated with either Fn or Ln in SFM-ND for 48 hr. CS-1 cells attach to both substrates, enabling all the cell lines tested to be adherent. CS-1 conditioned medium was collected after 48 hr in culture, centrifugated 2X at 1000 rpm to remove any cells, and filter

sterilized using syringe filters with 0.2μm pores (Nalgene). The conditioned medium was aliquoted and stored at -20°C until needed.

Activation of TGF-β present in serum-free conditioned medium: 48 hr conditioned media from CS-1 and BCS-1 cells was dialyzed in a Spectrapor-2 dialysis membrane (Spectrum, Houston, Tx) with a molecular weight cut-off range of 12-14 kD against 2-3 changes of 2 liters of 10mM acetic acid for 24 hr at 4°C. Acid dialysis results in the activation of any latent TGF-β present in media conditioned by these cells (Graycar, et al 1989). The dialysate was centrifugated to remove precipitates and the supernatant was stored at 4°C until needed. Acid-activated CS-1 and BCS-1 conditioned media were neutralized with 1 M Hepes and 1.44 N NaOH, pH 7.2 (mixed 1:1) and the pH of the conditioned media was checked with pH paper.

TGF-β Neutralization : For TGF-β antibody-neutralization, $2x10^4$ CS-1 or CS-1:β3 cells were plated in 6-well culture dishes (Costar) in DMEM containing 10% fetal calf serum for 24 hr. The cells were washed 1x in PBS to remove any serum and then serum-free DMEM-ND containing 50 μg/ml of 2G7, a mouse monoclonal antibody which neutralizes all isoforms of TGF-β (R. Derynck, UCSF) was added to the cells. Fresh medium and antibody was added to the cells every 48 hr for a total incubation of 7 days (Arteaga, et al 1990). Cells were then harvested and plated on Matrigel (lot #0270) in the presence of the 2G7 antibody in a 72 hr transwell invasion assay. Fresh antibody (50 μg/ml) was added to the invasion assay after 36 hr in culture. Cells were fixed and prepared for scanning electron microscopy as described previously.

Immunoblotting: Immunoblotting was used to establish the presence of TGF-β1 and PAI-1. Cells were plated on Fn or Ln in serum-free medium with 2% Nutridoma. Conditioned medium was collected after 48 hr and concentrated up to 6-fold using Centraprep concentrators with a 10kD exclusion filter, Amicon Corp. (Beverley, MA). Cells layers were washed with PBS and extracted with 2x Laemmli sample buffer. Protein content of all samples was determined (Lowry, et al 1951), and samples containing equal amounts of total protein were analyzed on 10% gels under reducing conditions then transferred to nitrocellulose using the Bio-rad protein transfer system (Towbin, et al 1979). The blots were blocked with 5% nonfat dried milk in 0.1% Tween-20 in PBS (T-PBS) as described previously (Librach, et al 1991) and incubated with primary antibodies against TGF-β1 and PAI-1 for 1.5 hr. 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 detected using the Enhanced Chemiluminescence Detection System (Amersham, Corp) in a 1:1 dilution.

Invasion Assays: Transwell culture chambers partitioned with a Nucleopore filter (8μM pore size; 0.28 cm^2 surface area; Costar, Cambridge, MA) were used in 24 well plates. The filters were coated with 30μl of a 1:1 mixture of Matrigel (lot #0270) and SFM-ND (final concentration of Matrigel was 6-8mg/ml protein). $1x10^5$ cells in 200μl SFM-ND were added to the top of the chamber and 0.8 ml of SFM-ND to the bottom. Cultures were incubated for 24, 48 or 72 hr at 37°C. When desired, 48 hr serum-free conditioned medium at several dilutions (either untreated or acid-activated) was added to the medium in the top and bottom of the filter. Serum-free conditioned medium was used from the following sources: CS-1, BCS-1, CS-1:β3, 293 (B_6), and 293 (C_6) cell

lines. The 293 cell line is a noninvasive human embryonic kidney cell line. To produce the 293 (B_6) cell line, 293 cells were transfected with a cDNA for normal TGF-β1 and produces elevated levels of latent TGF-β1. The 293 (C_{15}) cells were transfected with a mutated TGF-β1 construct such that the cell produces TGF-β1 that is constitutively active (Arrick, et al 1992). In some experiments, purified TGF-β1 was added to the medium at the start of the invasion assay at 2 ng/ml or 10 ng/ml concentration. In other experiments, 48 hr CS-1 conditioned medium pretreated with neutralizing TGF-β1 antibodies (411A or 2G7) for 2 hr on ice or overnight at 4°C before was added to the medium in the invasion assay.

Cultures were fixed with 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer, pH 7.2, and prepared for scanning electron microscopy by standard procedures. Samples were mounted so as to examine the underside of the filter, and observed in a JEOL JSM-840. The presence of cells on the underside of the filter indicated that the cells had penetrated the Matrigel barrier and migrated through the pores of the filter (Thomas, et al submitted; Librach, et al 1991). To quantify this assay, five representative photographs of the underside of each filter 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 percent surface area of the bottom of the filter covered by cells (Weibel and Bolender, 1974). The data are the average of two filters per experiment and at least two different experiments. The error bars represent the standard error of the mean (s.e.m.).

Detection of Secreted Protease Inhibitors: $5x10^5$ cells in 3 ml serum-free medium with 2% Nutridoma were plated for 48 hr in 6 well (30 mm) culture dishes coated with Ln so that all cell types would be adherent. The conditioned media were concentrated 6-9 fold as described above and protein content of all samples was analyzed (Lowry, et al 1951) so that equal amounts of conditioned medium protein were electrophoresed under nonreducing conditions on 10% gels containing 1 mg/ml gelatin. The gels were then incubated with 2% Triton X-100 for 30 min (2X) and 50 mM Tris-HCL, pH 8 with 5 mM $CaCl_2$ for 2 min (4-5X) to remove SDS. Upon removal of SDS, the gels were incubated for 15 min in rabbit synovial fibroblast conditioned medium in which all the proteinases have been activated with the organomercurial, APMA. The APMA-activated proteinases degrade the gelatin in the gel except for areas corresponding to protease inhibitors. Gels were incubated for 16-18 hr at 37°C in 50 mM Tris-Hcl, pH 8 with 5mM $CaCl_2$ and stained with Coomassie Blue. By timing the reaction properly, it was possible to visualize both protease inhibitors as dark blue bands and gelatin-degrading proteases as clear bands in a light blue background in the same gel (Fisher and Werb, 1992).

Results

Previous studies characterized a series of hamster melanoma cell lines (Thomas, et al submitted) **(Table 1)**. The first cell line, CM-1 (cell monolayer-1) is amelanotic, grows as a typical monolayer in serum-containing medium, and expresses a broad integrin repertoire including the αVβ3 and αVβ5 vitronectin receptors. CS-1 (cell suspension-1) cells arose as a subpopulation of CM-1: these cells are unable to attach in the presence of serum because they lack both αVβ3 and αVβ5 vitronectin receptors. These cells synthesize melanin, a melanocyte differentiation marker. BCS-1 cells were derived by treating CS-1 cells with the thymidine analog, 5-bromodeoxyuridine (BrdU). This treatment converted the CS-1 cells to an amelanotic cell line capable of growing in a monolayer similar to CM-1. Finally,CS-1 cells were transfected with either αV or β3 integrin subunits by lipofection and selected both by neomycin resistance and the ability to adhere to plastic in the presence of serum. Transfection of the αV subunit did not result in the expression of functional receptors for vitronectin. However, transfection of the β3 subunit into CS-1 cells resulted in the growth of neomycin-resistant cells that were adherent in the presence of serum. Analysis of the adherent cells by flow cytometry verified that they expressed complexes containing the human β3 integrin subunit, but not the human αV subunit **(Figure 1)**. The CS-1:β3 attached to fibrinogen and vitronectin (data not shown) but otherwise, displayed the same phenotype as the parental CS-1 cells which produce very limited amounts of proteases and are unable to invade matrigel-coated transwell filters in our in vitro invasion assay when cultured for up to 72 hr. In contrast, BrdU-treated CS-1 cells secrete high levels of proteases which degrade a variety of matrix proteins and extensively invade within 24 hr. The CM-1 cell line requires

48 hr to reach the same level of invasion as BCS-1 and displays an intermediate level of proteases which reflects their invasive behavior. Taken together, data up to this point indicates that these cell lines can be placed in a linear order of increasing invasiveness (BCS-1>>CM-1>CS-1:β3=CS-1).

Conditioned medium from noninvasive CS-1 melanoma cells inhibits tumor cell invasion.

To determine if either the noninvasive CS-1 or the highly invasive BCS-1 cells secreted soluble factor(s) which could regulate their invasive behavior, CS-1 and BCS-1 48 hr serum-free conditioned medium (SF-CM) was added to both CS-1 and BCS-1 cells in our transwell invasion assay. After incubating CS-1 cells with BCS-1 conditioned medium, the cells noninvasive phenotype remained unchanged; however, CS-1 conditioned medium inhibited the invasion of both BCS-1 and CM-1 cell lines in a concentration-dependent manner **(Figure 2).** This suggested that CS-1 melanoma cells secrete a soluble factor which suppressed their invasiveness in an autocrine fashion and to which the invasive BCS-1 cells were responsive. Possible candidates for the identity of the inhibitory activity in CS-1 conditioned medium include protease inhibitors, such as plasminogen activator inhibitors (e.g. PAI-1) or tissue inhibitors of metalloproteinase (TIMPs), or growth factors, such as transforming growth factor-β (TGF-β).

To determine if protease inhibitors were present in CS-1 conditioned medium, conditioned media from all three cell lines were blotted using a polyclonal antibody against human PAI-1 (Cohen, et al 1989). Both BCS-1 and CM-1 cells secreted PAI-1 as shown by bands at 50-55kD in their conditioned medium; in contrast, CS-1 conditioned medium was negative **(Figure 3).**

Furthermore, similar results were obtained when determining the expression of the matrix metalloproteinase inhibitors, TIMP and TIMP-2, using inhibitor gel zymography. Medium conditioned by either BCS-1 or CM-1 cells contained both TIMP (28kD) and TIMP-2 (21kD), while the noninvasive CS-1 cells did not appear to secrete either of these protease inhibitors **(Figure 4)**. Thus, the results so far indicate that the inhibitory activity in CS-1 conditioned medium is not due to the presence of protease inhibitors but to the presence of another factor which can inhibit invasion.

We then carried out a series of experiments designed to determine if TGF-β was invasion-suppressing ingredient in CS-1 conditioned medium. First, we determined that purified TGF-β1 added to the medium of the invasive cell lines, BCS-1 and CM-1, suppressed their invasion in our invasion assay for 24 hr or 48 hr respectively. Inhibition of invasion occurred when TGF-β1 was added at a concentration of 10 ng/ml **(Figure 5)**. Next, we determined that pretreatment of CS-1 conditioned medium overnight with a monoclonal antibody, 411A, which neutralizes TGF-β1, blocked the ability of the CS-1 conditioned medium to suppress BCS-1 or CM-1 invasion **(Figure 6)**. In fact, CM-1 cell invasion was slightly, but consistently stimulated when TGF-β1 was neutralized, suggesting that the invasive cells produce the TGF-β-like invasion-inhibiting activity at a low level.

CS-1 hamster melanoma cells secrete TGF-β1.

Data so far suggested that CS-1 cells produced more TGF-β than the invasive BCS-1 or CM-1 cells. Melanoma cells in culture produce TGF-β1 (Herlyn, et al 1990), as well as other growth factors. We wanted to determine if our cell lines differed in their synthesis and secretion of TGF-β1 and if it was

reflective of their distinct invasive phenotypes. To identify the presence of TGF-β1, we blotted both 48 hr conditioned media and cell extracts with a monoclonal antibody, 12H5, which specifically recognizes human TGF-β1. The conditioned media **(Figure 7)** and cell extracts (data not shown) of all cell lines contained a dimer which comigrated with the control (serum-free conditioned medium from a human embryonic kidney cell line which overexpresses constitutively active TGF-β1) at ~50kD. When TGF-β1 is present in high concentrations, it forms oligomers and may then migrate at a higher molecular weight than the activated 25kD form of TGF-β1 (R. Derynck, personal communication). Thus all the cell lines produced TGF-β1. Therefore, we hypothesized that although all cell lines secreted this growth factor, they differed in their ability to activate endogenous TGF-β1, resulting in their different patterns of invasion.

Hamster melanoma cells differ in their ability to activate TGF-β1.

Because CS-1 conditioned medium inhibited cell invasion in a dose-dependent manner, we endeavored to further confirm the identity of the inhibitory factor as TGF-β1 by activating all the latent TGF-β1 present in CS-1 conditioned medium through acid dialysis. If TGF-β1 is the inhibitory activity responsible for decreasing BCS-1 and CM-1 invasion, then higher dilutions of CS-1 conditioned medium should still inhibit invasion if all the latent TGF-β1 in the CS-1 conditioned medium is activated. To test this directly, we dialyzed 48 hr CS-1 conditioned medium against 10mM acetic acid for 24 hr; the acid-activated conditioned medium was neutralized using a hepes-based buffer and then added to either BCS-1 or CM-1 cells at several dilutions. Invasion was still inhibited at an 8 fold dilution less than the concentration of unactivated CS-1 conditioned medium which maximally inhibited cell invasion **(Figure 8)**.

BCS-1 and CM-1 invasion were still inhibited at a 1:64 dilution of acid-activated CS-1 conditioned medium, whereas unactivated CS-1 conditioned medium was inactive at a 1:16 dilution.

Since invasion of BCS-1 and CM-1 cells is inhibited by activated TGF-β1 in CS-1 conditioned medium, we next determined if these cell lines differed in their ability to activate latent TGF-β1 by adding serum-free conditioned medium from two 293 human embryonic kidney cell lines to the media of either BCS-1 or CM-1 cells in our transwell invasion assay: the 293 (B_6) cell line was transfected to overexpress latent TGF-β1 and the 293 (C_{15}) cell line was transfected to overexpress active TGF-β1 (Arrick, et al 1992). When BCS-1 or CM-1 cells were cultured in the presence of 293 (B_6) conditioned medium, the cells were still able to invade at levels similar to controls, but when conditioned medium from 293 (C_{15}) cells was added to the cells in our invasion assay, CM-1 cells were unable to invade while BCS-1 invasion was inhibited over 50% **(Figure 9)**. It was possible that the results from this experiment was reflective not of the difference between the cell lines in their ability to activate latent TGF-β1, but rather a difference in the amount of active or latent TGF-β1 present in the 293 (C_{15}) or 293 (B_6) conditioned media. Immunoblotting several dilutions of 48 hr conditioned media from either 293 cell line demonstrated that the conditioned media from 293 (C_{15}) or 293 (B_6) which was added to BCS-1 or CM-1 cells contained equivalent amounts of either activated or latent TGF-β1 respectively **(Figure 10)**. Taken together, these data suggest that the more invasive cell lines can bind and respond to pre-activated TGF-β1 but are unable to activate latent TGF-β1 in our assay.

Neutralization of TGF-β1 and the presence of the αVβ3 integrin are necessary for hamster melanoma cell invasion.

Since the cell lines differed in their ability to invade matrix and activate their TGF-β1, we hypothesized that CS-1 melanoma cells activate the TGF-β1 that they secrete, which then regulates invasion in an autocrine manner, while the more invasive cell lines fail to activate the TGF-β1 they secrete. If activated TGF-β is the only factor necessary to regulate CS-1 invasion, then antibody-neutralization of the TGF-β1 secreted by CS-1 should make them invasive. As the final test, CS-1 cells were incubated with a monoclonal antibody, 2G7, which neutralizes all isoforms of TGF-β. The cells were exposed to 50 μg/ml of the neutralizing antibody for 7 days before being plated on matrigel in the invasion assay for 72 hr in the presence of the neutralizing antibody. Upon treatment with the TGF-β neutralizing antibody, the CS-1 cells remained noninvasive **(Figure 11A, B; Figure 12)**. This was not surprising as CS-1 cells differ in many respects from both BCS-1 and CM-1 cells. One additional component that might also be very important for tumor cell invasion is expression of the appropriate adhesion phenotype. The studies of Albelda, et al (1990) and Nip and Brodt (1990) demonstrated this idea by showing that the presence of the αVβ3 cell surface adhesion receptor, along with α3β1 and αVβ5, was associated with the expression of more aggressive and invasive melanomas. BCS-1 and CM-1 cells express all three of these integrins while CS-1 cells do not express any of them. Therefore, we also tested the effect of TGF-β1 neutralization on the invasion of the CS-1 cells transfected with the integrin β3 subunit (CS-1:β3). The CS-1:β3 cell line was transfected with a full-length human cDNA for the β3 integrin subunit, which allowed these cells to express the αVβ3 complex at the cell surface, to attach to fibrinogen and

vitronectin and to grow as a monolayer in the presence of serum. By itself, the presence of the αVβ3 complex did not change the invasive **(Figure 11C and Figure 12)** and protease profiles (data not shown) from those of the parental CS-1 cells . However, exposure of the CS-1:β3 cells to the 2G7 neutralizing TGF-β antibody for 7 days in culture and for 3 days in the invasion assay enabled them to invade the matrigel-coated filters and migrate through the pores to the underside of the filter **(Figure 11D and Figure 12)**. The importance of the αVβ3 complex was confirmed by the observation that anti-TGF-β treated CS-1:β3 cells were no longer able to invade if the adhesion-blocking anti-αVβ3 antibody, LM609, was included in the medium during the invasion assay **(Figure 11E and Figure 12)**. LM609 also blocked the highly invasive BCS-1 cells which express the αVβ3 integrin, as well as αVβ5 and α3β1, from invading in vitro **(Figure 12)**. Thus, invasiveness in this system requires both a functional αVβ3 integrin and suppression of TGF-β function.

Discussion

Initially, we observed that a soluble factor (s) in medium conditioned by the noninvasive CS-1 cells was able to inhibit the invasive behavior of both the BCS-1 and CM-1 melanoma cell lines in a concentration-dependent manner. The data presented here support the hypothesis that the inhibitory factor in CS-1 conditioned medium is the growth factor, TGF-β1, and that this growth factor regulates invasion within this melanoma cell system. Purified TGF-β1 inhibits the invasion of both BCS-1 and CM-1 cells, while pretreating CS-1 conditioned medium with a neutralizing TGF-β1 antibody restores invasion to levels similar to controls.

Immunoblotting with an antibody that specifically recognizes TGF-β1 demonstrated that conditioned media and cell extracts from all cell lines contained a dimer which comigrated at 70 kD with controls. We next examined differences in the ability of the three cell lines to activate endogenous latent TGF-β1 by adding conditioned medium from two embryonic kidney cell lines that had been transfected to either overexpress latent [293 (B_6)] or active [293 (C_{15})] TGF-β1. The conditioned medium containing latent TGF-β1 was unable to block BCS-1 or CM-1 invasion; in contrast, medium conditioned by the 293 (C_{15}) cell line, which overexpresses active TGF-β1, inhibited invasion of both cell lines. Although the invasive BCS-1 and CM-1 cell lines as well as the noninvasive CS-1 secrete TGF-β1, the results suggest that only the CS-1 cells are capable of activating the secreted, latent form of this growth factor. Acid-activation of all the TGF-β1 present in CS-1 conditioned media inhibited BCS-1 and CM-1 invasion at even higher (greater) dilutions than observed for inhibition with unactivated CS-1 conditioned media, further establishing a role for this growth factor in the regulation of invasion within this cell system.

In order to demonstrate that CS-1 cell invasion is regulated by TGF-β1 in an autocrine manner, we pretreated the cells with an antibody that neutralizes all three isoforms of TGF-β before culturing them in our invasion assay. The CS-1 cells remained noninvasive. This result suggested that while the absence of functional TGF-β was important in permitting invasion of BCS-1 and CM-1 cells, it was not enough to convert the noninvasive CS-1 cells to an invasive phenotype. We then considered whether invasion also required a specific adhesion phenotype. Noninvasive CS-1 cells do not express matrix receptors which have been associated with the conversion of radial growth phase melanomas to the more invasive and metastatic vertical growth phase, specifically αVβ3, αVβ5, and α3β1 integrins (Albelda, et al 1990), while BCS-1 and CM-1 cells express all three of the receptors at levels which correspond to their distinct invasive behavior. Because integrins can regulate the expression of proteinases (Werb, et al 1990; Werb, et al 1989), we wanted to determine if the expression of these matrix receptors was linked to the regulation of protease production and invasion in our cell system. The CS-1 cells transfected with the human β3 subunit (CS-1:β3) express the vitronectin receptor, αVβ3. However, this single change was also insufficient to enable the CS-1 cells to invade Matrigel. These cells also expressed limited profiles of both matrix metalloproteinases and plasminogen activators similar, to the CS-1 parental cells (data not shown).

We then repeated the antibody neutralization experiment and included the CS-1:β3 cells, which were now capable of adhering to a wider variety of matrices. By neutralizing the endogenous TGF-β1, the CS-1:β3 cells invaded Matrigel and migrated to the underside of the filter, whereas the parental CS-1 cells did not invade. Therefore, TGF-β1 is an inhibitory factor in CS-1 conditioned medium and appears to regulate invasiveness in an autocrine

manner. This final experiment demonstrates as well, that the ability to adhere to a variety of matrices plays an important role in the invasion process. Since TGF-β1 is regulating invasion within this system, simply transfecting the CS-1 cells with a matrix receptor which could provide the appropriate adhesive machinery was not enough to overcome the negative regulatory effect of TGF-β1. The CS-1:β3 cells were capable of invading matrix but interference with the negative autocrine effects of the TGF-β1 was required before the cells could invade. The invasion of CS-1:β3 cells pretreated with the neutralizing TGF-β antibody could be blocked by adding the adhesion-blocking αVβ3 antibody, LM609, to the invasion assay, demonstrating the importance of this integrin in the invasion process. Seftor, et al (1992) showed the opposite effect in that monoclonal and polyclonal antibodies against the αVβ3 integrin, instead of inhibiting invasion, actually stimulated the invasion of a human melanoma cell line in vitro. These cells also upregulated their production of the 72 type IV collagenase at both the mRNA and protein levels. Since the protease profile of the CS-1 cell line did not change after transfecting in the β3 subunit, it will be important to determine if neutralization of TGF-β1 in these cells now stimulates the production of matrix-degrading proteases, demonstrating a role for the vitronectin receptor in the regulation of matrix degradation.

Growth factors are important in the regulation of the integrity of extracellular matrices by modulating the synthesis and activity of matrix-degrading proteinases and their inhibitors, the synthesis and deposition of matrix components, and the expression of adhesion molecules, like the integrin superfamily (reviewed by Rifkin, et al 1991 and Flaumenhaft and Rifkin, 1991). The growth factor, TGF-β, is a multi-functional regulatory protein which can affect growth, immune responses, angiogenesis, and formation of extracellular matrix. Among the many diverse effects of TGF-β, it increases the invasiveness

of human pulmonary (Mooradian, et al 1992) and mammary (Welch, et al 1990) adenocarcinoma, while decreasing invasion of a human fibrosarcoma cell line (Kubota, et al 1991). Tumor cell invasion involves complex interactions between proteases and their inhibitors, matrix proteins, adhesion molecules, and growth factors; each factor being capable of modulating the activity of the other. In order to understand the dynamics of this cellular behavior, it is important to uncover, first, the molecules which regulate invasion and, second, the mechanisms by which regulation is accomplished.

TGF-β inhibits the production of matrix-degrading proteases, such as matrix metalloproteinases (MMP) and plasminogen activators (PA) (Allan, et al 1991; Edwards, et al 1987; Overall, et al 1991). The invasive cell lines, BCS-1 and CM-1, synthesize and secrete significant levels of both MMP and activated forms of PA; in contrast to noninvasive CS-1 cells which produce low amounts of MMP and proenzyme forms of PA. Degradation of matrix proteins by cell-associated proteinases is an important component of the invasion process and would constitute one possible area for regulation by TGF-β. Preliminary experiments investigating this possibility suggest that TGF-β may regulate the proteinases in our system not at the level of synthesis or secretion but at the level of activation. Most proteinases are secreted in a proenzyme form and require activation. Substrate gel zymography suggests that incubating BCS-1 or CM-1 cells in the presence of CS-1 conditioned medium does not affect the production of MMP but appears to interfere with the activation of PA, resulting in the presence of only the proenzyme form of urokinase-PA (uPA) and not the 2-chain activated form of uPA (data not shown). The plasminogen activator system is an important component in the invasion of melanoma both in vitro and in vivo (Meissauer, et al 1991; Quax, et al 1991).

Proteinase activity may also be regulated by affecting the balance between proteinases and proteinase inhibitors produced by the tumor cell. TGF-β increases the production of tissue inhibitor of metalloproteinase (TIMP) which binds to MMP in a 1:1 molar ratio and inhibits the proteolytic activity (Edwards, et al 1987; Kubota, et al 1991). It also stimulates the synthesis of plasminogen activator inhibitor-1 (PAI-1), therefore regulating matrix degradation from the activity of PA (Allan, et al 1991). Both BCS-1 and CM-1 cells secrete the protease inhibitors, TIMP, TIMP-2 and PAI-1, as determined by inhibitor gel zymography and immunoblotting respectively. An increase in the production of any of these inhibitors through the actions of TGF-β could tip the balance toward maintaining tissue integrity and result in the inhibition of BCS-1 and CM-1 invasion. This does not appear to be the mechanism by which invasion is regulated in this cell system, as PAI-1 and TIMPS are not detected in extracts or conditioned medium of CS-1 cells but are detected in the invasive BCS-1 and CM-1 cells.

Another critical component of invasion is the ability of tumor cells to attach to matrix. Increased production of some matrix components as well as the induction of adhesion molecules, such as the integrin family, would favor increased adhesion and potentially, the inhibition of invasion. In some cell systems, TGF-β increases the synthesis of both matrix proteins (Andres, et al 1989) and integrins (Ruoslahti and Pierschbacher, 1987; Ruoslahti and Giancotti, 1989). From previous results (Thomas, et al), TGF-β does not appear to regulate invasion through this mechanism either, since both of our invasive cell lines express an extensive profile of integrins, with the capability of interacting with a wide variety of matrix proteins, and synthesize more fibronectin and vitronectin than CS-1 cells (data not shown).

The process of tumor cell invasion is dependent not only on the ability of the tumor cell to adhere to and degrade extracellular matrix but also to migrate into the areas which have been degraded. TGF-β may regulate invasion by inhibiting or interfering with the ability of CM-1 and BCS-1 cells to crawl or move on substrates through the synthesis of a factor that inhibits motility, such as Melanoma Inhibitory Activity (MIA) (Bogdahn, et al 1989). It is possible that TGF-β interferes with the function of other growth factors which may modulate the synthesis of motility factors critical for the locomotion of the tumor cell (Edwards, et al 1987). The ability of TGF-β to regulate motility within our cell system is currently under investigation.

The process of tumor cell invasion is complex and requires the interactions of many different molecules found in the intermediate environment of the tumor cell. Our cell lines represent a good system in which to study the invasion process, the underlying mechanisms that regulate invasion (like growth factors), and the components (such as the expression of the appropriate adhesion phenotype and the production of matrix-degrading proteases) that are necessary for invasion to occur. By elucidating the role of growth factors and/or adhesion molecules in the invasiveness of tumor cells, it may result in the development of more effective diagnostic and therapeutic regimens for the treatment of these invasive diseases.

Figure Legends and Figures

Figure 1. FACS analysis of αV-transfected CS-1 (CS-1:αV) and β3-transfected CS-1 (CS-1:β3) cells. CS-1 hamster melanoma cells were transfected with either a cDNA encoding full-length human αV or human β3 and neomycin-resistant genes. Cells which were neomycin-resistant and adherent in the presence of serum were analyzed for expression of human αV(LM142 antibody), human β3 (AP3 antibody), and αVβ3 (LM609 antibody). Neither CS-1:αV (A) nor CS-1:β3 (B) expressed the human αV subunit. CS-1:αV cells did not react with the anti-β3 antibody, AP3, (C) and also failed to react with the αVβ3 complex-specific antibody, LM609 (E). CS-1:β3 cells were positive for the expression of the human β3 subunit (D) as well as being able to express the αVβ3 complex (F).

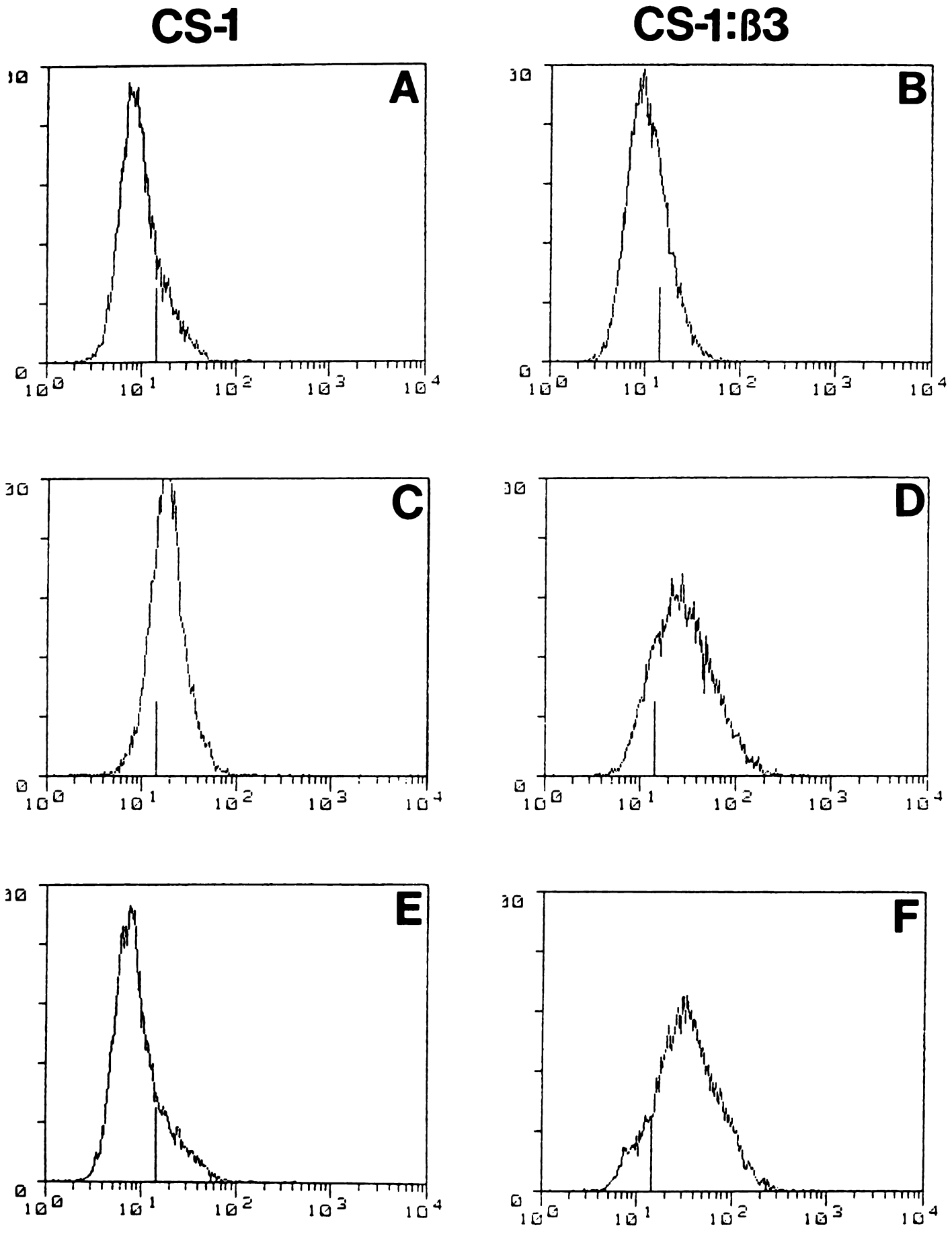

CS-1
CS-1:β3
A
B
C
D
E
F
0
10^0
10^1
10^2
10^3
10^4

Figure 2. CS-1 48 hr conditioned medium inhibits CM-1 or BCS-1 cell invasion in vitro. The undersides of matrigel-coated filters were photographed and surface area covered by invading cells calculated as described in the Methods. Two filters of each sample were scored in each experiment and the experiments were repeated twice. Error bars represent standard error of the mean (s.e.m.).

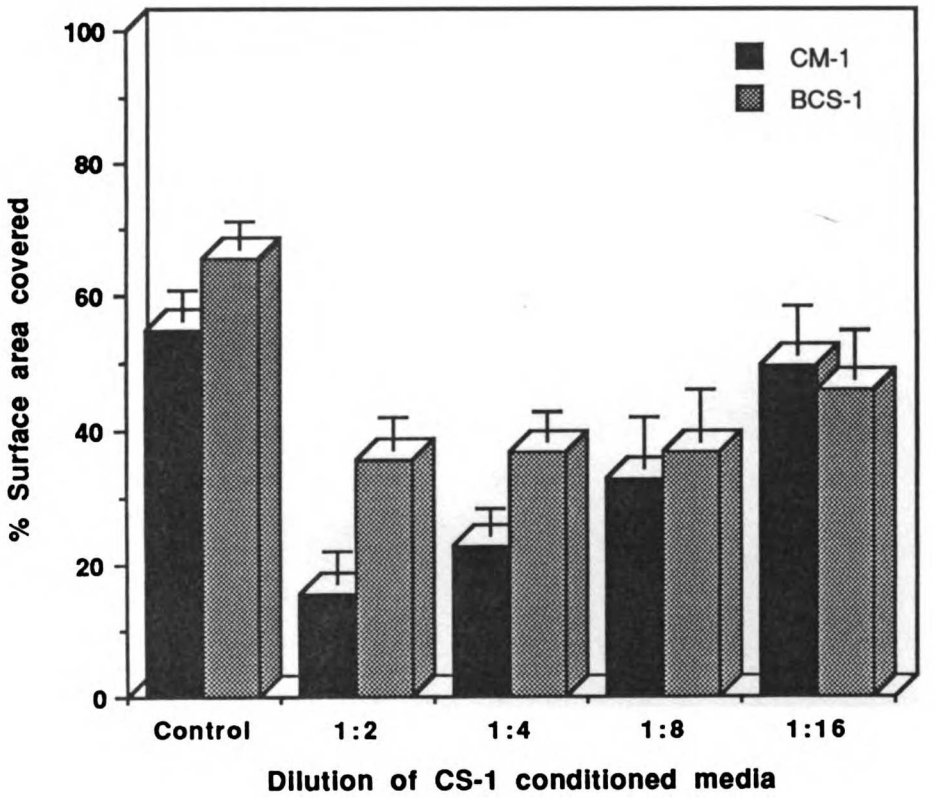
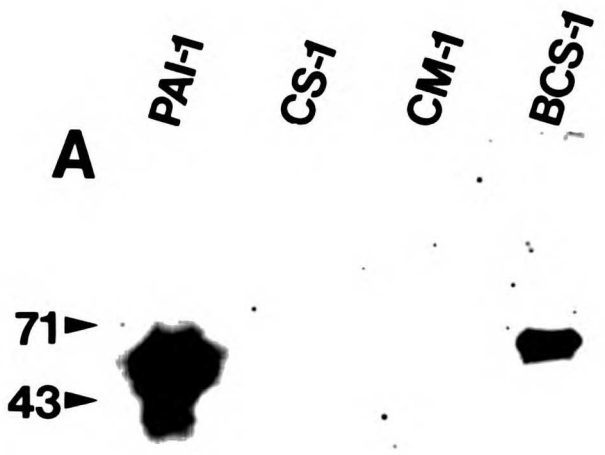
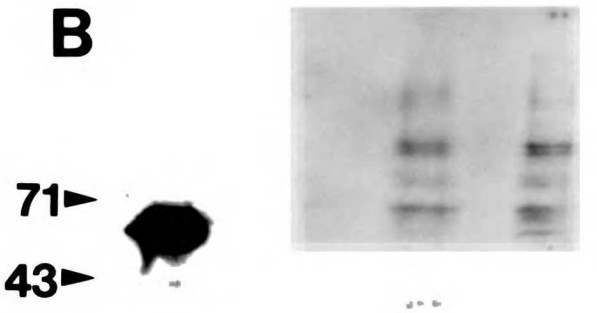

100
80
60
40
20
0
% Surface area covered
CM-1
BCS-1
Control
1:2
1:4
1:8
1:16
Dilution of CS-1 conditioned media

Figure 3. Detection of PAI-1 using Western Blot analysis of conditioned media (A) and cell extracts (B). CM-1 and BCS-1 melanoma cells reacted with a rabbit anti-human PAI-1 antibody, whereas CS-1 cells did not.



CONDITIONED MEDIUM



CELL EXTRACTS

Figure 4. Detection by inhibitor substrate gel zymography of the metalloproteinase inhibitors, TIMP and TIMP-1, in hamster melanoma cell conditioned media. Lane 0, molecular weight standards; Lane 1, TIMP standard; Lane 2, CS-1; Lane 3, CM-1; and, Lane 4, BCS-1. CM-1 and BCS-1 cells express both TIMP (28kD) and TIMP-2 (21/19 kD), while CS-1 conditioned media was negative. By timing the reaction properly, both inhibitor bands and proteolytic lysis zones are visualized in the same gel.

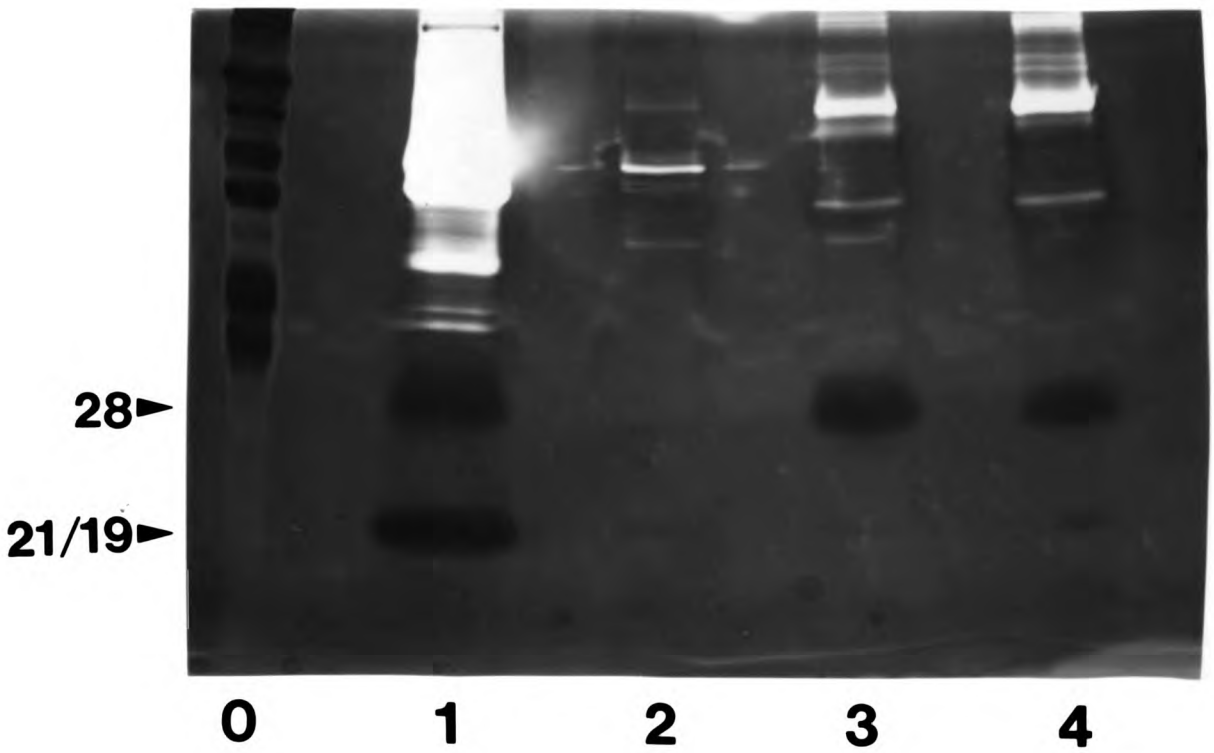

28
21/19
0
1
2
3
4

Figure 5. Purified exogenous TGF-β1 inhibits CM-1 and BCS-1 cell invasion in vitro in a concentration-dependent fashion. Values represent scoring of duplicate filters from two different experiments. Error bars represent the s.e.m.

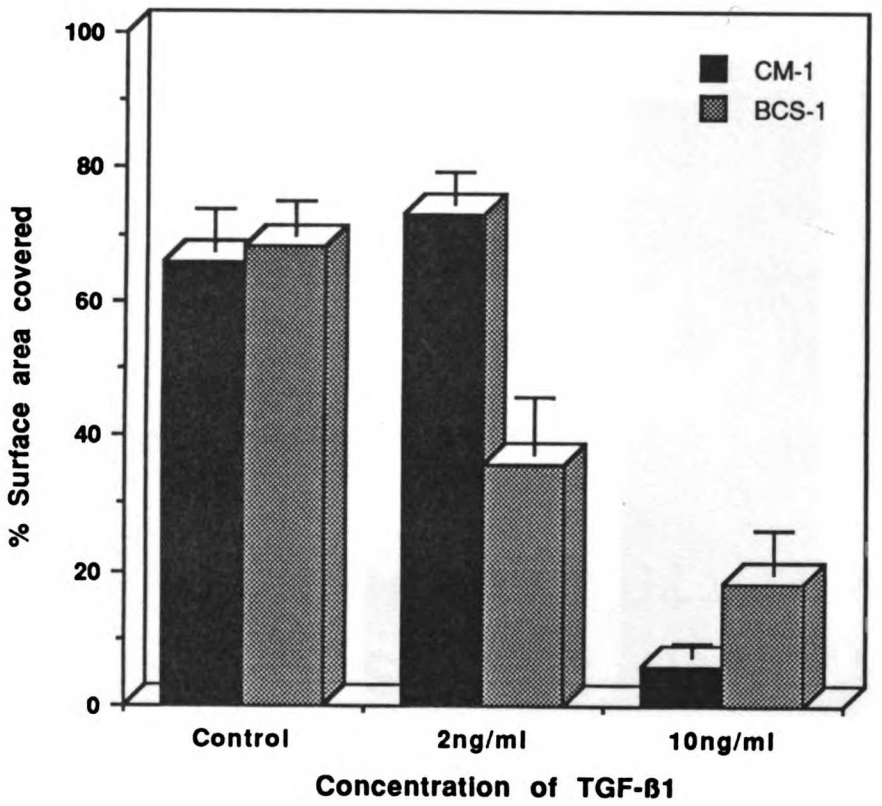

100
80
60
40
20
0
% Surface area covered
CM-1
BCS-1
Control
2ng/ml
10ng/ml
Concentration of TGF-ß1

Figure 6. Pretreatment of CS-1 48 hr conditioned media with the TGF-β1 neutralizing antibody, 411A, before adding the conditioned media to CM-1 or BCS-1 cells in a 1:4 dilution in the invasion assay reverses the inhibitory effect of CS-1 conditioned media. Error bars represent the s.e.m.

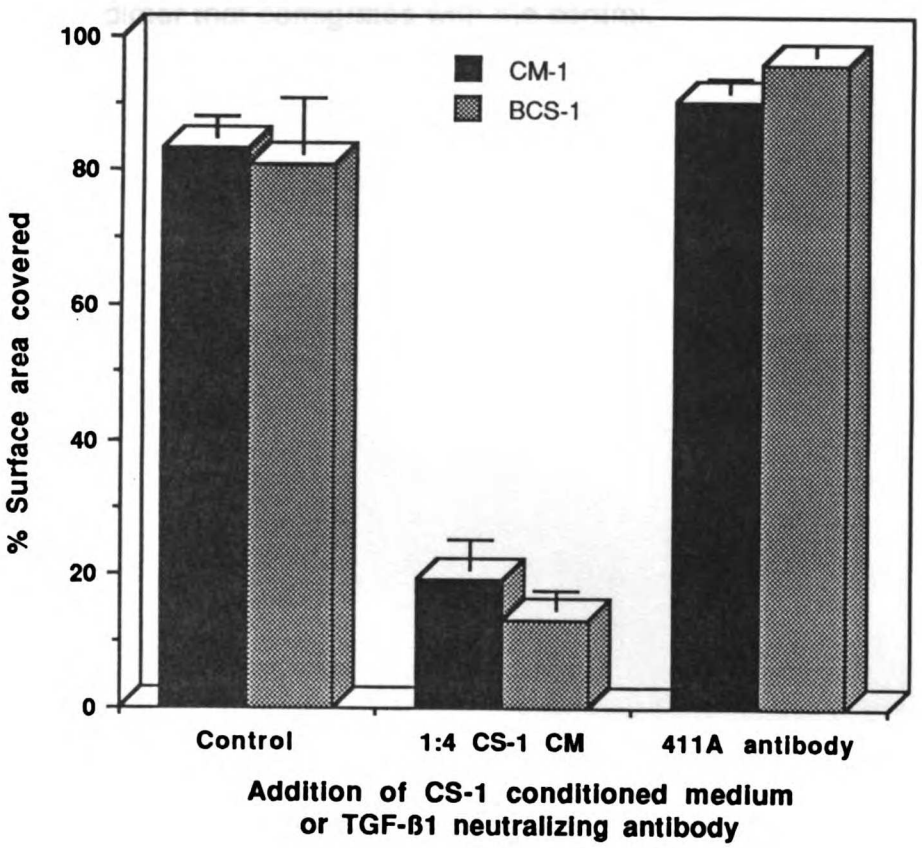

100
80
60
40
20
0
% Surface area covered
CM-1
BCS-1
Control
1:4 CS-1 CM
411A antibody
Addition of CS-1 conditioned medium
or TGF-ß1 neutralizing antibody

Figure 7. Detection of endogenous TGF-β1 in 48 hr conditioned media by immunoblotting with a monoclonal antibody that is specific for human TGF-β1. Conditioned media from the human embryonic kidney cell line, 293 (C_{15}), that was transfected such that it overexpresses activated TGF-β1 was used as a control. CS-1, CM-1, BCS-1, and β3-transfected CS-1 (CS-1:β3) cells express a dimer that comigrates with the control.

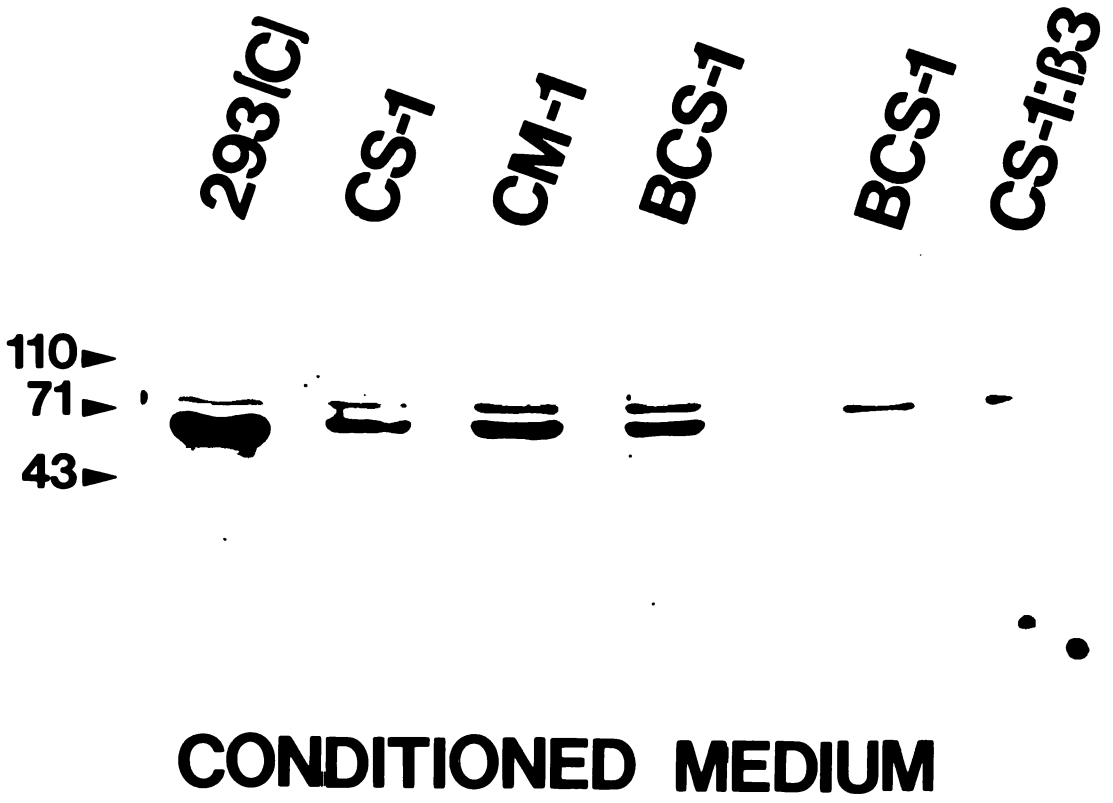

293(C)
CS-1
CM-1
BCS-1
BCS-1
CS-1:B3
110
71
43
CONDITIONED MEDIUM

Figure 8. Acid-activated CS-1 conditioned media inhibits CM-1 and BCS-1 invasion in vitro in a concentration-dependent manner. CS-1 48 hr conditioned media was dialyzed against 10 mM acetic acid to activate all latent TGF-β present. The activated conditioned media inhibits melanoma cell invasion at more dilute concentrations that unactivated conditioned media (see figure 2). Error bars represent s.e.m.

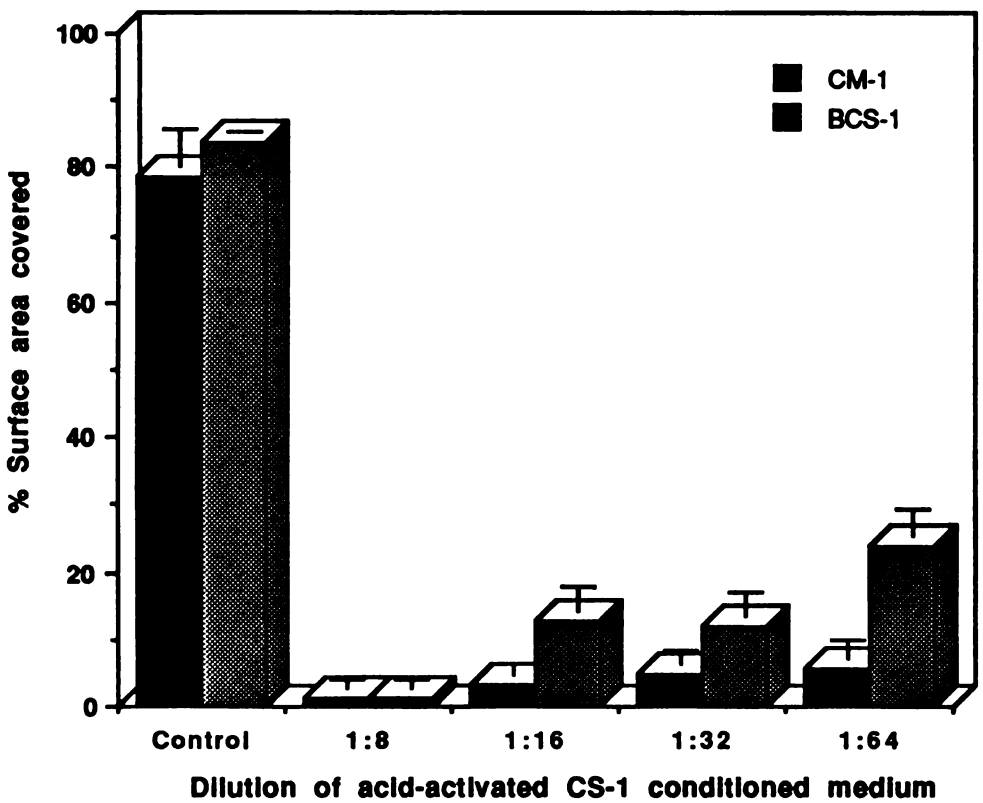

100
80
60
40
20
0
% Surface area covered
CM-1
BCS-1
Control
1:8
1:16
1:32
1:64
Dilution of acid-activated CS-1 conditioned medium

Figure 9. Conditioned medium from a human embryonic cell line, 293 (C_{15}), that overexpresses activated TGF-β1 inhibits CM-1 or BCS-1 invasion in vitro, while conditioned media from a human embryonic cell line, 293 (B_6), that overexpresses latent TGF-β1 does not block invasion. The 293 (C_{15}) conditioned media inhibits invasion at levels similar to the addition of a 1:4 dilution of CS-1 conditioned media to the invasion assay. Error bars represent s.e.m.

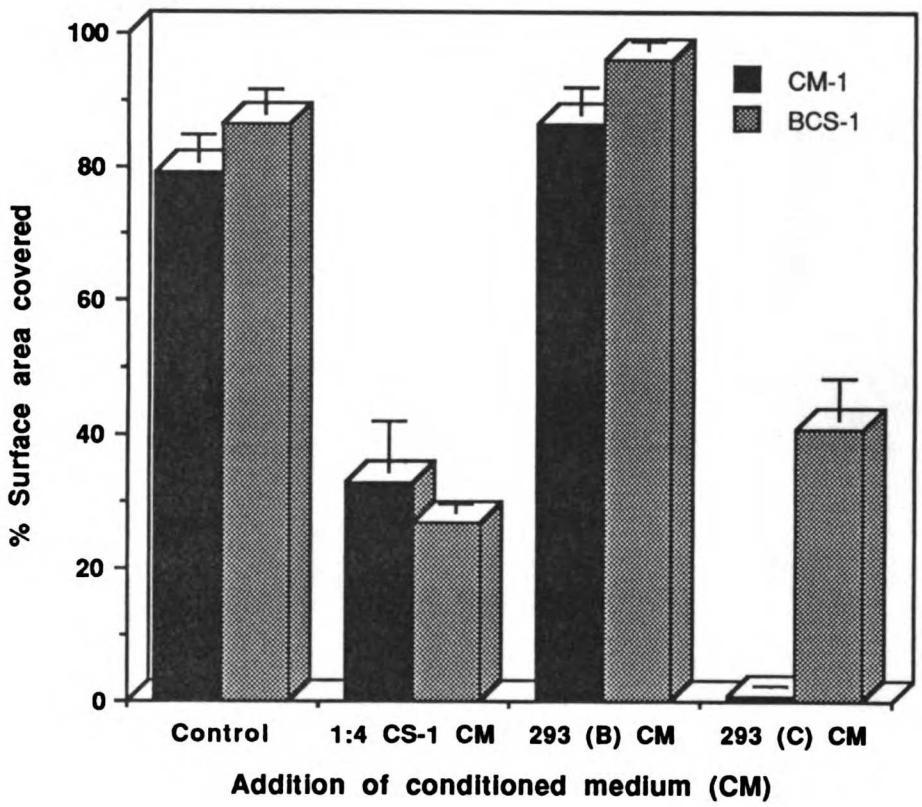

100
80
60
40
20
0
% Surface area covered
CM-1
BCS-1
Control
1:4 CS-1 CM
293 (B) CM
293 (C) CM
Addition of conditioned medium (CM)

Figure 10. Western Blot analysis of conditioned media from the 293 (C_{15}) and 293 (B_6) cell lines with the monoclonal antibody, 12H5, that recognizes human TGF-β1, demonstrates that the conditioned media contains similar levels of activated or latent (respectively) TGF-β1 and that the different effects of the conditioned media on melanoma cell invasion is due not to different levels of TGF-β1 present.

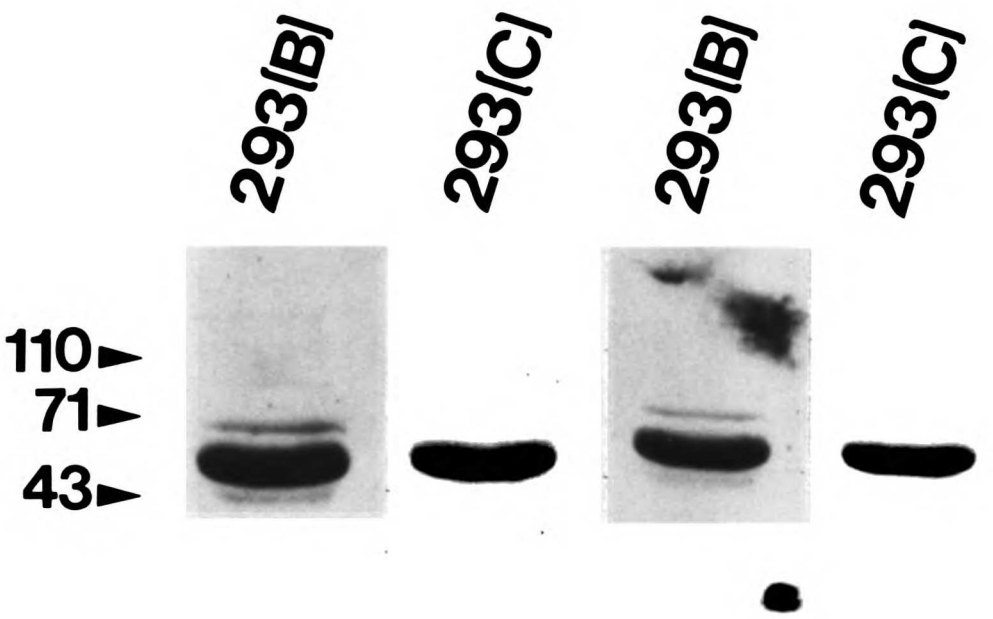

293/B/
293/C/
293/B/
293/C/
110
71
43
1:2
1:2
1:8
1:8
DILUTION OF COND MEDIUM

Figure 11. Scanning EM of the invasive activity of CS-1 (A, B) and CS-1:β3 (C-F) plated on matrigel-coated filters in serum-free media. Cultures were fixed after 72 hr. CS-1 cells did not invade under control conditions (A) or when treated with an antibody, 2G7, that neutralizes all isoforms of TGF-β, in culture for 7 days and then for 3 days in the invasion assay (B). CS-1:β3 cells did not invade under control conditions (C) but the cells did invade when treated continuously with the 2G7 antibody for 7 days in culture and 3 days in the invasion assay (D). Inset in D shows morphology of the CS-1:β3 cells that have invaded. Upon addition of LM609, an αVβ3-complex specific antibody with function-blocking ability, to CS-1:β3 treated with the TGF-β neutralizing antibody, 2G7, CS-1:β3 invasion was blocked (E). Non-specific mouse IgG had no effect on CS-1:β3 invasion (F).

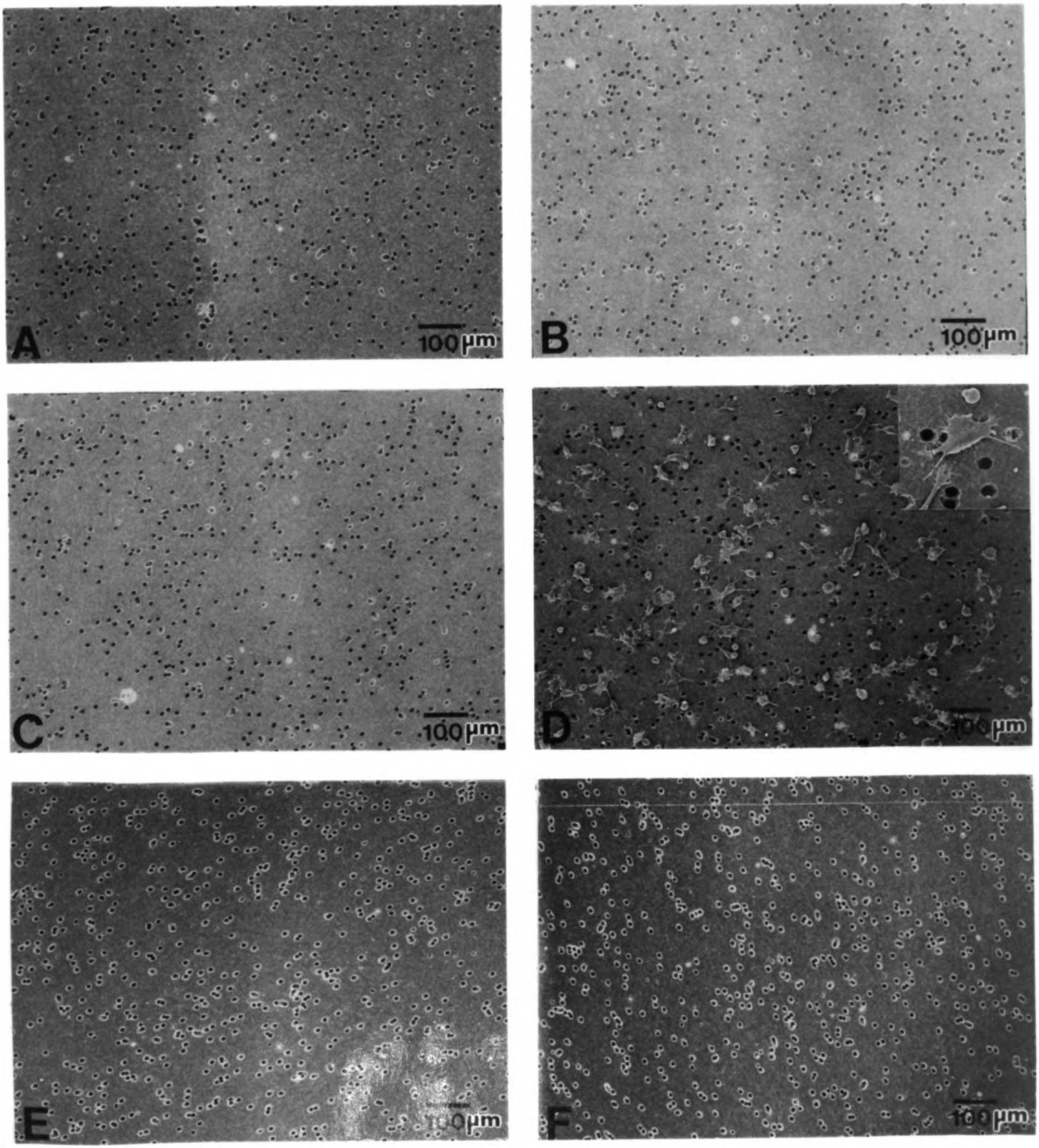

A
100 µm
B
100 µm
C
100 µm
D
100 µm
E
100 µm
F
100 µm

Figure 12. β3-transfected CS-1 cells (CS-1:β3) invade matrigel-coated filters when cultured in the presence of 2G7, a neutralizing TGF-β antibody, while parental CS-1 cells are unable to invade under the same conditions. LM609, a function-blocking αVβ3 specific antibody, blocks 2G7-treated CS-1:β3 and BCS-1 invasion in vitro. Error bars represent the s.e.m.

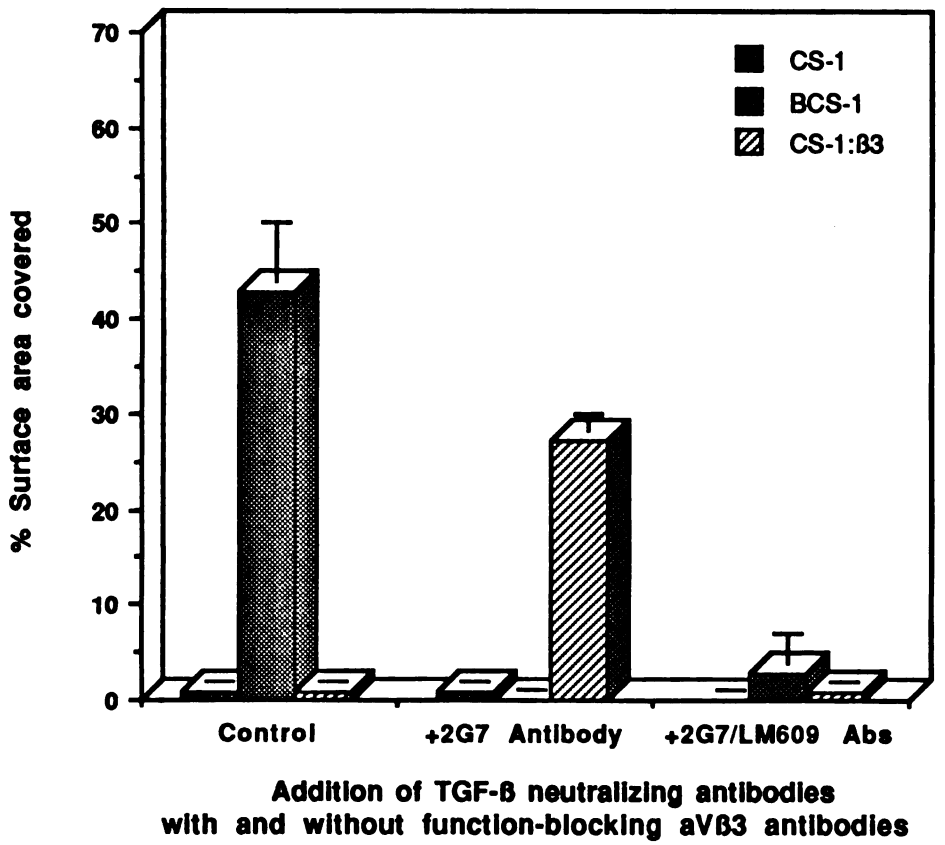

70
60
50
40
30
20
10
0
% Surface area covered
CS-1
BCS-1
CS-1:ß3
Control
+2G7 Antibody
+2G7/LM609 Abs
Addition of TGF-ß neutralizing antibodies
with and without function-blocking aVß3 antibodies

Table 1. Properties which may influence the invasive phenotype of control, transfected and 5-bromodeoxyuridine treated hamster melanoma cell lines

PATHWAY OF INVASION: ----------->Increasing Invasiveness----------->

PROPERTY	CS-1	CS-1:β3	CM-1	BCS-1
Adhesion Phenotype/ Integrin Profile	restricted	αvβ3	intermediate [integrins associated with metastatic melanomas]	broad-spectrum
Protease Profile	restricted	restricted	low	high
MMP	72kD	72kD	72kD, 92kD, stromelysin	72kD, 92kD stromelysin
PA	sc-uPA	sc-uPA	tc-uPA	tc-uPA
Invasion in Matrigel (hours)	No (72h)	No (72h)	Yes (48h)	Yes (24h)

CS-1:β3: CS-1 cells transfected with a cDNA for the human β3 integrin subunit; BCS-1: 5-bromodeoxyuridine-treated CS-1 cells; MMP: Matrix metalloproteinase; PA: Plasminogen activators; sc-uPA: single-chain urokinase type plasminogen activator; tc-uPA: two-chain urokinase type plasminogen activator.

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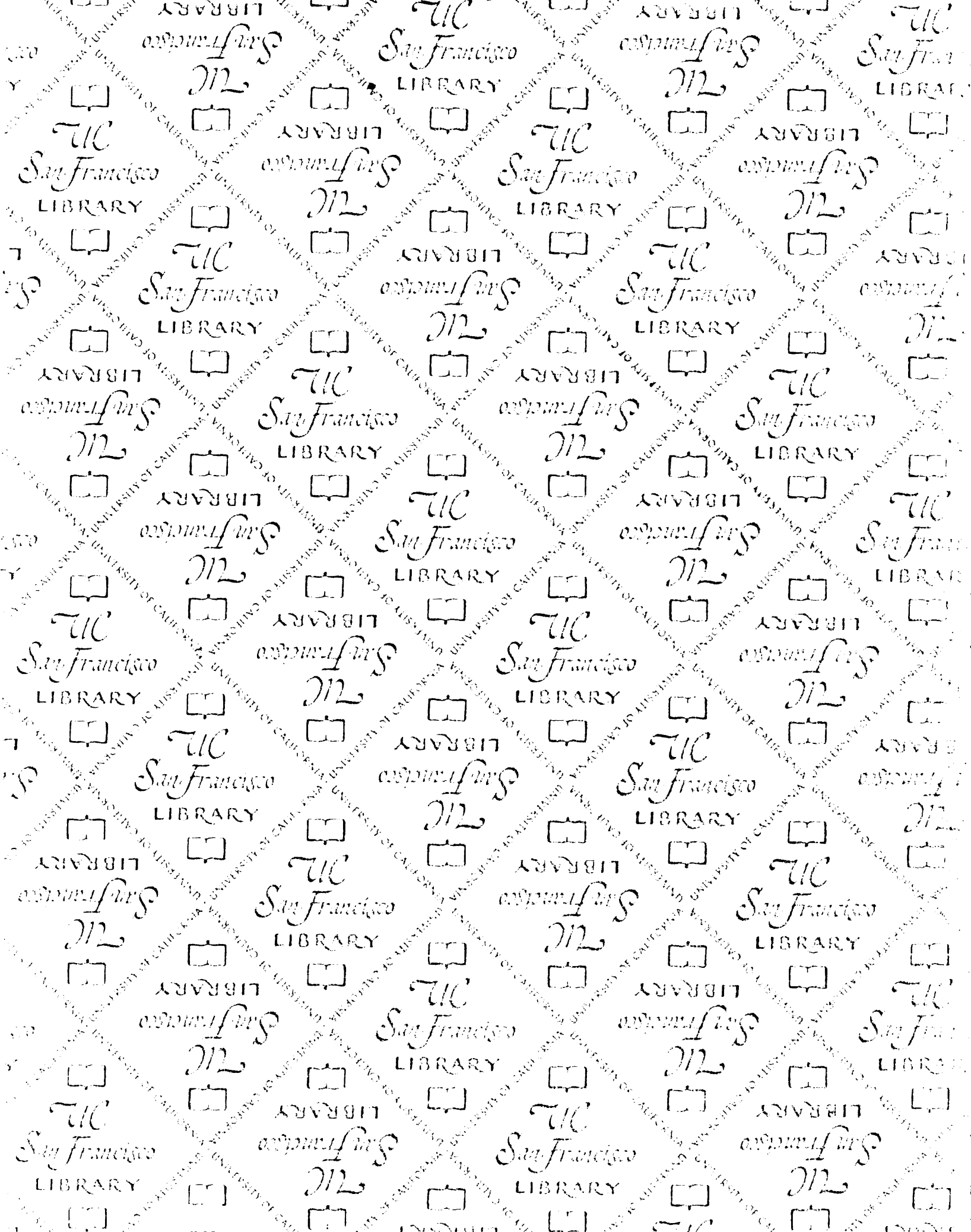
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