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Melanoma Cell Invasive and Metastatic Potential Correlates with Endothelial Cell Reorganization and Tenascin Expression

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Abstract

A direct correlation was observed between the invasive and metastatic potential of A375 melanoma cells and their expression of tenascin and ability to support endothelial cell adhesion and reorganization. Since the ability to metastasize and establish a neovasculature requires interaction of tumor cells with extracellular matrix and endothelial cells, we examined the potential of matrix proteins synthesized by three melanoma cell lines with low-A375P, medium-A375M and high-A375SM invasive and metastatic properties to induce adhesion and rearrangement of human umbilical vein endothelial cells (HUVECs) in vitro. HUVECs adhered to and reorganized into a network of connecting and aligned cells on wells conditioned by A375SM and A375M but not A375P cells. These changes in morphology suggested differences in matrix composition among the three melanoma cell lines. In comparison to low levels of substrate-bound fibronectin and laminin, increasingly higher levels of substrate-bound tenascin were synthesized by the A375P, A375M, A375SM cell lines. HUVEC adhesion and reorganization on A375-conditioned matrix was tenascin-dependent and could be inhibited with antibodies against human tenascin. HUVEC adhesion to A375SM-conditioned matrix and tenascin require $\alpha_v\beta_3$ while reorganization may require $\alpha_2\beta_1$ as well. Our results suggest that tenascin plays a role in integrin-dependent adhesion and reorganization of HUVECs in response to the extracellular matrix of metastatic melanoma cells.

Keywords

adhesion; angiogenesis; endothelial cells; integrins; tenascin

INTRODUCTION

The interaction of metastatic melanoma cells and endothelial cells plays an important role in the development of the tumor vasculature (angiogenesis). These interactions are mediated by cell-cell contacts, soluble cytokines and the extracellular matrix. Changes in the extracellular matrix components of metastatic melanoma appear to correlate with alterations in the adhesion of endothelial cells to these matrices. The interaction of endothelial cells in vitro with components of the extracellular matrix such as laminin (LN), fibronectin (FN), collagen (Col) and proteoglycans (Madri *et al.*, 1980) results in the modulation of shape and behavior of these cells (Madri *et al.*, 1988) resulting in changes which include cytoskeletal reorganization, cell spreading and migration (Basson *et al.*, 1990; Albelda *et al.*, 1989; Lampugnani *et al.*, 1991).

The purpose of this investigation was to determine whether adhesion and morphology of endothelial cells differed on matrix proteins synthesized by human melanoma cell lines with low (A375P), medium (A375M) and high (A375SM) metastatic and invasive properties (Kozlowski *et al.*, 1984, Gehlsen *et al.*, 1992). These studies revealed that endothelial cell substrate adhesion and morphology differed and correlated with the metastatic potential of the melanoma cell lines providing the adhesion matrix. Melanoma cells with the highest invasive and metastatic ability express elevated levels of tenascin (TN) and this directly correlates with the extent of reorganization of the adhered HUVECs. Furthermore, we have shown that adhesion of HUVECs, as well as their subsequent reorganization on the A375SM matrix is dependent on TN-integrin interactions. TN would appear to play a role in the ability of metastatic melanoma to alter endothelial cell adhesion and promote subsequent angiogenesis.

The role of TN in mediating endothelial cell-matrix interactions is particularly interesting in light of the expression of TN in the neovasculature of tumors (Bourdon *et al.*, 1983), and its possible role as an anti-adhesive or motility promoting component of the matrix (Erickson and Bourdon, 1989). TN was initially characterized as a novel extracellular matrix glycoprotein (Bourdon *et al.*, 1983, 1984, 1985) found in the neovasculature and stroma of tumors and has a restricted and transient expression in fetal tissues (reviewed in Erickson and Bourdon, 1989; Erickson and Lightner, 1988; Chiquet-Ehrismann, 1990; and Ekblom and Aufderheide, 1989; Sriramarao and Bourdon, 1993). Recent studies have revealed that human TN promotes not only the adhesion of endothelial cells but also their spreading and this interaction is dependent on $\alpha_2\beta_1$ (Sriramarao *et al.*, 1993) and $\alpha_v\beta_3$ integrins (Sriramarao *et al.*, 1993; Joshi *et al.*, 1993). TN also supports the adhesion of a variety of tumor cell lines including A375-derived melanoma cells. In the present investigation we demonstrate that TN-enriched A375 melanoma cell matrix can support endothelial cell adhesion and reorganization via interaction with $\alpha_v\beta_3$ and $\alpha_2\beta_1$ integrins.

MATERIALS AND METHODS

Cells

HUVECs were isolated and cultured essentially as previously described (Gimbrone *et al.*, 1976; Thornton *et al.*, 1983). Briefly, veins of fresh human umbilical cords from normal full term births were cannulated and were digested with collagenase. The cells were then harvested and cultured in Medium 199 with Earle's salts and L-glutamine and supplemented with 10% FBS, 30 $\mu\text{g/ml}$ endothelial cell growth supplement (Collaborative Research Inc., Bedford, MA) and 100 $\mu\text{g/ml}$ bovine lung heparin (Sigma Chemical Co., St. Louis, MO). The HUVECs were used between passages 1 and 3. Endothelial cell identity was established by the presence of Factor VIII and typical cobblestone morphology at confluence. The human melanoma cell lines A375P, A375M and A375SM derived from sequential selection for metastatic tumor formation (Kozlowski *et al.*, 1984) were obtained from Dr I. J. Fidler (MD Anderson Cancer Center, Houston, TX). The cells were cultured in DMEM supplemented with 10% FBS and 0.1% gentamicin. The cells were subcultured at a density of 2.5×10^6 cells per 100mm tissue culture plate.

Matrix Proteins

Soluble human TN was purified from the culture media of U251MG cells as previously described (Erickson and Bourdon, 1989). Human LN was isolated from a pepsin digest of placenta, while human FN was obtained from Collaborative Biomedical Products (Bedford, MA).

Antibodies

The monoclonal antibody (mAb) 81C6 directed against the twelfth FN type III repeat of human TN (Bourdon *et al.*, 1983, Murphy-Ullrich *et al.*, 1991) and rabbit polyclonal antibodies generated against soluble TN (Sriramarao *et al.*, 1993) were used in these studies. Polyclonal antibodies against human FN were purchased from Gibco BRL, while rabbit polyclonal antibodies against human LN were prepared against human placental LN. Immunoglobulin fractions of the polyclonal antisera were purified by ammonium sulfate precipitation and DEAE Sepharose chromatography. Purity of the IgG was established by SDS-PAGE and ELISA. In addition anti-integrin mAbs PIE6 (anti- α_2) and P1B5 (anti- α_3) were obtained from Chemicon (Temecula, CA), while LM609 (anti- $\alpha_v\beta_3$) was kindly provided by Dr. David Cheresh (Scripps Research Institute, La Jolla, CA).

Detection of A375-Derived ECM Proteins During In Vitro Culture

Ninety six well microtiter sterile plates (Linbro/Titertek plates, Flow Laboratories, McLean, VA) were plated with 50,000 cells of A375P, A375M and A375SM cell lines. The cell lines were cultured for 24, 48 or 72 hrs. The cultured wells were washed with cation free PBS and harvested with 2mM EDTA-PBS. The wells were observed microscopically to ascertain that all cells had been harvested. A375 melanoma cell conditioned wells were then assayed to determine the expression of substrate bound TN, FN or LN matrix. The wells were initially blocked with 100 μ l of PBS containing 1% BSA for 30 min at 37°C and then incubated with polyclonal antibodies raised against TN, LN or FN at an appropriate dilution and incubated for 30 min at 37°C. Dilutions of the different primary antibodies used for this assay were optimized by ELISA to obtain similar absorbance values when tested with 5 μ g/ml of immobilized extracellular matrix (ECM) protein (i.e., TN, FN or LN) in a direct binding assay.

The antibody bound wells were then washed and incubated with secondary antibody (goat anti-rabbit conjugated to horseradish peroxidase) diluted 1:2000 for 30 min at 37°C. After rinsing the wells, the reaction was developed with o-phenylenediamine and H₂O₂ and the absorbance measured at 492nm.

HUVEC Adhesion and Reorganization Assay

Tissue culture plates (100mm) (Costar) were cultured with A375P, A375M or A375SM melanoma cells in DMEM supplemented with 10% FBS and 0.1% gentamicin for 3 days until the cells were confluent. The cells were then harvested with sterile 2mM EDTA-PBS and washed extensively with sterile PBS and microscopically examined to ensure that all cells were harvested. Plates were stored in PBS until used. First or second passage HUVECs were then harvested with trypsin-EDTA, washed two times with M199 medium containing Earle's salts, L-glutamine and supplemented with 10% FBS, 30 μ g/ml endothelial cell growth supplement and 100 μ g/ml bovine lung heparin. HUVECs were also cultured in AIM-V serum free media (Gibco BRL) supplemented with endothelial cell growth supplement and heparin for certain experiments. HUVECs were then added to tissue culture plates that were previously conditioned with A375P, A375M or A375SM melanoma cells and microscopically examined at various time intervals (0–72 hrs). In some experiments tissue culture plates were coated with different concentrations (1–100 μ g/ml) of TN or FN and subsequently blocked with 1% BSA prior to plating with HUVECs.

RESULTS

Previous studies have shown that A375P, M and SM melanoma cell lines differ in their invasive and metastatic properties (Gehlsen *et al.*, 1992, Kozlowski *et al.*, 1984). A375P are poorly metastatic, A375M are moderately metastatic, and A375SM melanoma cells are

strongly metastatic. In this investigation we have examined the ability of substrate bound matrix synthesized by the A375-derived cell lines to support adhesion and reorganization of cultured HUVECs in vitro. Monolayers of confluent A375P, M and SM melanoma cells were treated with 0.2% EDTA-PBS to detach and remove cells. HUVECs were then cultured in the substrate conditioned plates for a period of 72 hrs. Although HUVECs were found to adhere to all three A375 conditioned matrices, significant differences in the morphology of HUVECs were observed when cultured on A375P, A375M and A375SM melanoma cell matrix (Fig. 1).

HUVECs adhere to A375P matrix as a cell monolayer and over a 72 hr time course HUVECs were confluent and maintained an adherent, spread morphology (Fig. 1). HUVECs also adhered to A375M matrix but during the next 72 hrs cell matrix-interactions were altered with cells gradually developing from a monolayer into a broad network of aligned adherent cells and intervening gaps. HUVEC cell adhesion and reorganization underwent striking changes on A375SM matrix. Endothelial cells adhered to A375SM matrix and initially formed a subconfluent monolayer of cells randomly distributed over the culture surface (Fig. 2). However, by three hours of culture endothelial cells were beginning to form groups of cells, some-times extended as aligned cells. At 72 hrs of culture endothelial cells had reorganized into extended network of cells with wide intervening areas devoid of cells. (Fig. 2). The appearance of the culture was consistent with a decrease in the adhesive ability of the matrix and the migration of cells to form a network (Fig. 2). No further change in morphology or reorganization of endothelial cells on A375P, M and SM conditioned matrices was observed over the next several days (data not shown). These two dimensional cultures do not support endothelial tubule formation, as is seen in three-dimensional matrigel culture. However the changes in cell culture are indicative of altered adhesion and increased motility which are necessary for vessel formation. The results demonstrate a direct correlation between increased melanoma metastatic potential and endothelial reorganization on the melanoma matrix. The correlation between metastatic melanoma cell matrix and endothelial cell adhesion and reorganization could be reflected in the composition of the matrix.

We examined whether differences in expression of TN, LN or FN by the different A375-derived cell lines could be correlated to the variations in HUVEC reorganization on A375 P, M and SM matrices. A375P, A375M and A375SM melanoma cell lines and HUVECs as control were cultured in 96 well tissues culture plates and the relative levels of matrix TN, LN or FN laid down by the different melanoma cells in vitro was determined by qualitative ELISA (Fig. 3). The melanoma cell lines were cultured for 24, 48 or 72 hrs at a concentration of 50,000 cells per well, harvested with 2 mM EDTA-PBS and the expression of immobilized matrix proteins determined. These experiments revealed that A375SM cells expressed the highest levels of TN, followed by A375M with moderate levels and low levels of expression by A375P cells. Expression of TN was found to increase in a time dependent manner and was maximally expressed after 72 hrs of culture (Fig. 3). In contrast, LN and FN were found to be expressed at background levels by the three melanoma cell lines at all time points (Fig. 3). In addition to immobilized TN, the three melanoma cell lines similarly secreted increased amounts of soluble TN into the culture media (data not shown). These results suggest that invasiveness and metastatic potential of the A375-derived cell lines directly correlates with the differential expression of substrate bound TN matrix but not LN or FN in vitro.

To determine the role of TN, LN, and FN in endothelial adhesion and reorganization on A375 matrices, A375-conditioned matrix plates were preincubated with polyclonal anti-TN, LN or FN rabbit antibodies for 1 hr prior to culturing with HUVECs and their effect on primary adhesion determined. At a concentration of 100 µg/ml anti-TN but not anti-FN or

LN antibodies resulted in a complete inhibition of adhesion of HUVECs to A375P, M, or SM plates demonstrating that TN is the major matrix protein promoting primary adhesion of HUVECs to the A375-derived matrix proteins in vitro (Fig. 4). We next examined if TN in the matrix is important for maintaining adhesion and reorganization of HUVECs on the A375SM matrix over the 72 hr culture period HUVECs were initially allowed to adhere to the A375SM matrix for 1–3 hrs and then cultured in the presence of anti-TN, LN or FN polyclonal IgG (100 µg/ml) for a period of 72 hrs. HUVECs were found to reorganize into a network of interconnecting and aligned cells in the presence of normal rabbit IgG, anti-LN or anti-FN IgG antibodies (Fig 5A–C). In contrast, co-culturing of HUVECs in the presence of anti-TN antibodies resulted in the detachment of endothelial cells from the A375SM conditioned matrix plates over the 72 hr culture period (Fig. 5D). Effects of anti-TN on HUVECs were also observed when the cells were allowed to adhere and undergo reorganization on the A375SM matrix for a period of 48–72 hrs before treatment. In contrast to normal rabbit IgG treated plates, treatment with anti-TN antibodies resulted in a progressive detachment of the adhered HUVECs requiring approximately 48–72 hrs for significant detachment (Fig. 6). Anti-TN IgG resulted in similar detachment of HUVECs not only from the A375SM matrix plates, but also from A375P and M conditioned plates (data not shown). These experiments and those described above for cell adhesion assays (Fig. 4.) suggest that both primary adhesion as well as continued adhesion during reorganization on the A375 conditioned matrix is TN dependent. The progressive detachment of HUVECs by anti-TN over 48 to 72 hr indicates HUVECs may be blocked in establishing new matrix adhesion sites upon cell migration and proliferation in the cultures. In the results described TN plays an essential role in the adhesion of HUVECs to the A375 conditioned matrix proteins. However it remains possible that TN acts in concert with other unknown components of the matrix to support endothelial reorganization.

To determine the effect of TN on HUVEC adhesion and cell morphology, endothelial cells were plated on wells coated with TN, FN, and BSA. Tissue culture 24 well plates were coated with increasing concentrations of soluble TN, FN or BSA (1–50 µg/ml) and cultured with HUVECs in a serum free media for 1–3 days. Both TN and FN supported rapid adhesion of HUVECs to coated wells, however, cell morphology on TN did not display the typical cobblestone appearance evident on FN. HUVECs on TN substrates appeared not to spread as extensively as on FN and were found to develop a patchwork of elongated cells reminiscent of the extensive reorganization on A375SM matrix (Fig. 7A–C 50 µg/ml). At a coating concentration of 1 µg/ml of TN, very few HUVECs attached, while at increasing concentrations of immobilized TN (>10 µg/ml), significantly higher number of cells attached and formed into a network of aligned cells (Fig. 7A; 50 µg/ml). At these concentrations tested (1–50 µg/ml), FN did not induce any reorganization of the cultured HUVECs and the cells remained adherent and eventually became confluent (Fig. 7B; 50µg/ml). Similar results were observed when HUVECs were plated on immobilized collagen. HUVECs attached and over a period of 72 hrs became a confluent monolayer of spread cells (data not shown). No attachment of HUVECs to BSA coated plates was observed (Fig. 7C). Although HUVECs were found to adhere and to a limited degree reorganize on TN, the pattern of reorganization was not as extensive as that observed on the A375SM matrix. The growth pattern of HUVECs on TN suggests it may be the minimally required matrix component for HUVEC adhesion and reorganization and may involve additional components of the melanoma matrix acting along with TN to promote the distinct pattern of endothelial cell reorganization observed on A375SM matrix.

Since adhesion and spreading of HUVECs on human TN is mediated by $\alpha_2\beta_1$ and $\alpha_v\beta_3$ integrins (Sriramarao *et al.*, 1993), we investigated whether adhesion and reorganization of HUVECs on the A375SM matrix was integrin dependent. HUVECs were allowed to adhere to the A375SM conditioned plates for 1–3 hrs and then cultured with mAbs to different

integrins for 24, 48 and 72 hrs. These studies revealed that treatment with mAbs to $\alpha_v\beta_3$ and α_2 (Figs. 8B,C), but not α_3 (mAb P1B5) (Fig. 8A), partially blocked endothelial cell adhesion and reorganization in vitro, respectively.

The LM609 (anti- $\alpha_v\beta_3$) treated culture showed significant detachment of cells from the A375SM matrix. This likely is a consequence of partially blocking $\alpha_v\beta_3$ integrin adhesion to TN (Sriramarao *et al.*, 1993), but HUVEC apoptosis triggered by anti- $\alpha_v\beta_3$ could also be involved (Brooks *et al.*, 1994). The α_2 blocking antibody PI E6 did not effect adhesion strongly, but the extent of HUVEC reorganization did appear to be inhibited. HUVEC adhesion and reorganization was however significantly effected when the cells were cultured in the presence of both LM609 and PIE6. These results suggest that blocking $\alpha_v\beta_3$ integrin could block the adhesive interaction of human endothelial cells with melanoma tumor matrix and that $\alpha_2\beta_1$ may play a role in mediating migration as has been shown for human glioma cell migration (Deryugina and Bourdon, unpublished observations).

DISCUSSION

In the present investigation we have demonstrated that the invasive and metastatic potential of three human melanoma cell lines correlates with changes in endothelial cell adhesion and monolayer morphology and expression of substrate bound TN. HUVECs when plated on the A375-derived matrix proteins initially adhered equally well on all three A375 melanoma cell lines. However, after 3hrs of adhesion, HUVECs started to reorganize on A375M and A375SM conditioned matrix proteins. In comparison to A375P and A375M matrix, endothelial cells exhibited a more motile phenotype on the A375SM matrix and displayed significant changes in cell distribution over 72 hrs in culture. HUVECs reorganized into a network of aligned cells with intervening areas devoid of adherent cells on A375SM matrix and to a lesser extent on A375M matrix. No significant change in HUVEC motility or redistribution on A375P matrix was observed. Endothelial cells grew into monolayers of confluent cells on A375P matrix with a cobblestone morphology typical of endothelial cultures grown on collagen coated substrates. These results suggest that the extracellular matrix of highly metastatic melanoma cells strongly effects the adhesion and morphology of normal endothelial cells. These effects of tumor matrices on endothelial cells may well be involved in the process of tumor angiogenesis.

Migration of endothelial cells is critical for not only normal development and wound repair but is also an important feature of tumor angiogenesis. Neovascularization or angiogenesis is characterized by the induction of endothelial cell migration and cell proliferation and the establishment of new blood vessels. These events appear to be dependent both on cell-cell and cell-matrix interactions. Previous studies have revealed that interaction of endothelial cells with components of extracellular matrix results in modulation of shape and behavior including cytoskeletal reorganization, cell spreading and migration (Madri *et al* 1988; Basson *et al.*, 1990; Albelda *et al.*, 1989; Lampugnani *et al.*, 1991). Since significant differences in the monolayer behavior of endothelial cells on the different A375-derived cell lines were observed, we compared the expression of immobilized TN, FN and LN by the A375P, M and SM cell lines. Although the migratory effects of endothelial cells in response to various ECM proteins has been investigated (Madri *et al* 1980; 1988; Augustin-Voss and Pauli, 1992) little has been known about the cellular behavior of endothelial cells in response to ECM containing TN. In view of the oncodevelopmental expression of TN and its association with angiogenesis (Bourdon *et al.*, 1983) it seemed possible that TN played a role in mediating cellular migration, and endothelial differentiation.

A375-derived melanoma cells were found to express elevated levels of substrate bound matrix TN (insoluble) as well as soluble TN (data not shown). While the expression of

substrate TN directly correlated with increased metastatic and invasive properties of the A375-derived cell lines, FN and LN were found to be minimally expressed as substrate bound protein by these cell lines. These results suggest that the increased endothelial reorganization on metastatic melanoma cell lines correlates with TN expression. Indeed it appears that TN is critical to endothelial adhesion to the melanoma matrix and would likely play a role in endothelial cell reorganization. Increased expression of TN has previously been correlated with migratory or actively proliferating cells during embryonic morphogenesis, wound healing tissues repair and oncogenesis (Bourdon *et al.*, 1983; Erickson and Bourdon, 1989; Chiquet-Ehrisman, 1990; Mackie *et al.*, 1988a; Daniloff *et al.*, 1989). In addition, a role for TN in substrate associated migration of neural crest cells and outgrowth of neurites of spinal cord explants has also been reported (Mackie *et al.*, 1988b; Chiquet-Ehrisman, 1990; Wehrle and Chiquet, 1990; 1993). More recently we have shown that TN can down regulate cell adhesion of A375C6 melanoma cells (Schwabe *et al.*, submitted, 1995) and promote tumor cell migration (Deryugina and Bourdon, unpublished observations). In the tumor migration assay TN promotes migration alone or with fibronectin through specific $\alpha_2\beta_1$ interactions.

Endothelial adhesion and redistribution on the A375SM derived matrix appears to be mediated by TN, as polyclonal antibodies to TN but not LN or FN inhibited this function. Such alterations and redistribution of HUVECs in vitro could possibly be related to the number and distribution of focal adhesions on the cell surface as well. Previous studies have revealed that treatment of endothelial cells with TN or other related proteins such as SPARC and thrombospondin (TSP) result in a diminished number of focal adhesions (Murphy-Ullrich and Hook, 1989; Vrucinic-Filipi and Chiquet-Ehrismann, 1993; Murphy-Ullrich *et al.*, 1991). Furthermore, these interactions might also result in modulation of adhesive contacts and cellular receptivity to motility factors leading to reorganization of the actin cytoskeleton (Goldblum *et al.*, 1994; Vogel *et al.*, 1993; Rosen *et al.*, 1990). The profound reorganization of HUVECs on A375SM matrix expressing TN and the role of TN in endothelium adhesion supports the hypothesis that TN might mediate tumor cell-endothelial cell interactions. These interactions are mediated by endothelial cell integrins.

Recent studies have demonstrated that endothelial adhesion and spreading on TN is mediated by $\alpha_v\beta_3$ and $\alpha_2\beta_1$ integrins (Sriramarao *et al.*, 1993; Joshi *et al.*, 1993). Endothelial adhesion and spreading on the TN rich A375SM matrix was found to be $\alpha_2\beta_1$ and $\alpha_v\beta_3$ integrin dependent, respectively. Since $\alpha_v\beta_3$ blocking results in cell detachment, it is not clear whether HUVEC reorganization on the A375-derived matrix components is completely integrin-dependent. It is also conceivable that TN could interact with other cellular components such as proteoglycans (Grumet *et al.*, 1994) and/or other ECM proteins to stabilize or destabilize cell-cell and cell-matrix interactions and promote in vitro endothelial cell reorganization. The multidomain structure of TN (Gulcher *et al.*, 1989; Sriramarao and Bourdon, 1993) is particularly suited to mediate a variety of cellular functions including adhesion and reorganization or migration through multiple interactions with cell surface receptors. It has been shown that neural crest cell migration and neurite extension may be mediated by separate TN domains (Halfter *et al.*, 1989; Lochter *et al.*, 1991).

In summary we have demonstrated that endothelial cells adhere and reorganize into a network of connecting cells with a motile phenotype on matrix proteins secreted by A375-derived melanoma cells. The extent of endothelial cell reorganization can be correlated to the invasive and metastatic potential of A375 melanoma cells and appears to be dependent on the presence of TN in the matrix. Likewise, a direct correlation between increased TN expression and the invasive and metastatic potential of A375-derived melanoma cell lines was also established. Further studies will be needed to determine if altering the extent of TN

synthesis by genetic manipulation will vary the phenotypic characteristics of the different metastatic cell lines and thereby affect endothelial cell adhesion and motility.

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FIGURE 1.

Endothelial cell reorganization on A375-derived matrix proteins. (A) A375P, (B) A375M and (C) A375SM cells were seeded in 100 mm tissue culture plates and the confluent cells harvested after 3 days after culture. HUVECs (1×10^6 cells/plate) were then cultured on the A375 P, M or SM conditioned plates for 72 hrs.

FIGURE 2.

Adhesion and reorganization of endothelial cells on A375SM conditioned plates.

Endothelial cells (1×10^6 cells/plate) were cultured on A375SM conditioned plates and their adhesion and subsequent reorganization at (A) 1 hrs, (B) 3 hrs, (C) 24 hrs, (D) 72 hrs.

FIGURE 3.

Differential expression of TN by A375-derived cell lines. A375P, M and SM melanoma cells were seeded into 96 tissue culture microtiter plates (50,000 cells/well) and cultured for 24,48 or 72 hrs. The cells were harvested and amount of TN, FN or LN laid down by the different cells determined by ELISA using polyclonal antibodies to the various ECM proteins. The expression of these ECM proteins by HUVECs was also determined. Background absorbance values (BSA binding) are indicated by vertical lines.

FIGURE 4.

Primary adhesion of HUVECs on A375-conditioned matrix is TN dependent. A375P, A375M and A375SM conditioned 96 well plates were initially blocked and then incubated with polyclonal rabbit antibodies against human TN, LN or FN (100 μ g/ml) for 1 hr. The wells were then washed with PBS and incubated with HUVECs for 1–2 hrs. The plates were then washed to remove unbound cells. The adherent HUVECs were fixed and stained with PBS containing 10% ethanol and 0.2% crystal violet and absorbance recorded on ELISA reader at 550_{NM}. The values are expressed at % bound.

FIGURE 5.

Effect of anti-TN, LN or FN polyclonal antibodies on HUVEC adhesion/reorganization on A375SM matrix proteins. HUVECs were cultured in 100 mm tissue culture dishes previously conditioned with A375SM cells. HUVECs were allowed to adhere for a period of 1–3 hrs and the media supplemented with (A) normal rabbit IgG or polyclonal antibodies (100 μ g/ml) against human (B) FN, (C) LN or (D) TN. Adhesion and reorganization of HUVECs in the presence of various antibodies was monitored for an additional 72 hrs.

FIGURE 6.

Effect of anti-TN IgG on the continued adhesion and reorganization of HUVECs on A375SM conditioned matrix. HUVECs were allowed to adhere and reorganize on A375SM cultured matrix dishes for 48–72 hrs and then incubated with 100 $\mu\text{g}/\text{ml}$ of (a) normal rabbit IgG and (b) anti-TN polyclonal rabbit IgG upto 72 hrs.

FIGURE 7.

TN promotes adhesion and reorganization of HUVECs in vitro. HUVECs were cultured in 100 mm tissue culture dishes that were precoated with 50 µg/ml of (A) soluble TN, (B) FN or (C) BSA. The effect of the immobilized TN or FN on HUVEC adhesion and reorganization was monitored for a period of 1–3 days.

FIGURE 8.

$\alpha_2\beta_1$ and $\alpha_v\beta_3$ integrins promote HUVEC continued adhesion on A375SM conditioned matrix. HUVECs were initially allowed to adhere to the A375SM conditioned matrix plates for 1–3 hrs and then cultured in the presence of (A) anti- α_3 (mAb P1B5); (B) anti- α_2 (mAb P1E6); (C) anti- $\alpha_v\beta_3$ (mAb LM 609) or (D) $\alpha_2 + \alpha_v\beta_3$ in combination, for a period of 72 hrs and the effect on continued adhesion and reorganization of HUVECs was determined.