A Potential Role for TGFβ in the Regulation of Uveal Melanoma Adhesive Interactions with the Hepatic Endothelium


PURPOSE. TGFβ has been shown to have a regulatory effect on uveal melanoma invasion, but it is not known which processes are specifically influenced. The purpose of this study was to analyze the effect of TGFβ stimulation on the adhesive interactions of uveal melanomas with the extracellular matrix (ECM) and endothelium and, in addition, its effect on the secretion of collagenases.

METHODS. Invasive and a noninvasive uveal melanoma cell lines, supported by short-term primary uveal melanoma cultures, were used to assess the effect of TGFβ on ECM and endothelial adhesion and degradation of the ECM. Changes in cell adhesion molecule expression were assessed by flow cytometry, and conditioned media were analyzed by gelatin zymography. Assays of adhesion to ECM substrates and endothelial cells were also performed.

RESULTS. Treatment with TGFβ increased low basal levels of adhesion molecule and latent MMP-2 expression, as well as adhesion to hepatic endothelial cells by the noninvasive cell line. Conversely, TGFβ reduced adhesion to laminin and a laminin-binding integrin by invasive cells but had no effect on their adhesion to the endothelium.

CONCLUSIONS. In this preliminary study, TGFβ was found to upregulate levels of MMP-2, reduce adhesion to laminin, and downregulate expression of laminin-binding integrins. Specifically, TGFβ was found to increase adhesion of noninvasive uveal melanoma cells to the hepatic, but not the dermal, endothelium and may therefore contribute to the preferential targeting of the liver by uveal melanomas. (Invest Ophthalmol Vis Sci. 2005;46:3473–3477) DOI:10.1167/iovs.04-1311

The transforming growth factor (TGF)-β family of proteins are potent regulatory factors known to inhibit the growth of several epithelial cell types and melanocytes, initiating signal transduction via a generic membrane-bound heterodimeric TGFβ receptor complex. During tumorigenesis, abnormal functioning of the intra- and extracellular components of the TGFβ pathway can occur, affecting the suppressive functions of these regulators and for some tumors, enhancing development and progression. In the early stages of tumor development TGFβ therefore acts as a tumor suppressor, but as the tumor progresses toward a more invasive phenotype, TGFβ can become pro-oncogenic. In addition to controlling cell growth, TGFβ also regulates other essential functions and thus when produced by tumor cells can enhance metastatic development, through direct autocrine effects or paracrine effects on the surrounding cells. In breast carcinoma for example, increased TGFβ expression by the tumor cells is associated with increased invasiveness. In melanoma, both paracrine and autocrine regulation of cutaneous melanocytes and tumor cells by all TGFβ isoforms has also been described, associating high levels of autocrine production with reduced sensitivity and a metastatic phenotype.

In common with cutaneous melanocytes, uveal melanocytes arising in the choroid, ciliary body, and iris are sensitive to the growth-inhibitory effects of TGFβ. Limited evidence in studies of uveal melanomas has suggested that abnormalities in the TGFβ pathway exist, implying that lack of growth control by TGFβ also plays an important role in the progression of uveal melanoma. Both melanoma types are derived from neural crest cells and, as such, share several common features, including the morphology and properties of the melanogenesis pathway. Substantial differences are nevertheless known to exist, as both variation in genes including those controlling apoptosis and expression of cell surface adhesion molecules have been reported. Metastasis also differs, as cutaneous melanomas are capable of both lymphatic and hematogenous spread, which is often less organ specific, whereas uveal melanomas almost exclusively disseminate through the blood and preferentially target the liver. This pattern of metastatic targeting by uveal melanomas suggests that factors associated with the liver, including cells of the hepatic vessels and hepatocytes themselves, could be involved with the favored colonization of this organ in preference to other anatomic sites.

Work previously performed in our laboratory using a Boyden chamber invasion assay, has shown that both TGFβ1 and -β2 inhibit invasion and migration of most uveal melanomas. In 20% of the cases studied, however, invasion was instead stimulated by both TGFβ isoforms. For these tumors, TGFβ actually appears to promote tumor invasion; and, as has been found in cutaneous melanoma, more advanced uveal melanomas could instead be stimulated by TGFβ. As TGFβ could have an influential role in several processes associated with invasion, including migratory, adhesive, and degradative steps, we explored the effect of both TGFβ1 and -β2 on adhesion to the extra cellular matrix (ECM), adhesion molecule expression, and gelatinase secretion, by using invasive and noninvasive uveal melanoma cell lines and short-term cultures (STCs). As endothelial cells lining the hepatic sinusoids may influence uveal melanoma metastasis, the effect of these factors on uveal...
Table 1. Source and Reference List for Monoclonal Antibodies (mAbs) Used in Immunohistochemistry and Flow Cytometry Experiments

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<tr>
<td>IgG1 isotype control</td>
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All mAbs were purchased from Chemicon International Inc., with the exception of the IgG1 isotype control (DakoCytomation, Ely, UK), TGFβ and TGFβRI (from Novocastra). —, details of the antibody clone are not appropriate (IgG isotype control, FITC-labeled goat anti-mouse and anti-rat IgG) or not given (TGFβ and TGFβRI).

melanoma adhesion to microvascular endothelial cells was also briefly investigated.

Materials and Methods

Cell Culture and Cytokines

Invasive (SOM 196B) and noninvasive (SOM 157d) cells were maintained as previously described within a 10-passage range.14,15 Human dermal microvascular endothelial cells (adult; HDMECAs) were obtained commercially (TCS Cellworks Ltd., Botolph Claydon, UK), and human liver sinusoidal endothelial cells (HuLSECs) were freshly extracted from liver resections and maintained as detailed earlier.16 STCs were derived from primary uveal melanomas, as previously detailed14-15 (SOM 365, -367a, -367b, -368, -376, -371, -377, -380, and -382) and were used within five passages of being established in culture. Because of the large number of cells needed to complete all aspects of this study, STCs of primary uveal melanoma could only be used to confirm findings related to collagenase secretion and adhesion to the ECM after treatment with TGFβ. Ethics committee approval had been obtained before the study, and protocols adhered to the principles of the Declaration of Helsinki. Human recombiant TGFβ (TGFβ1 and TGFβ2; Sigma-Aldrich, Inc., Dorset, UK) was used at a concentration of 0.1 ng/mL in all experiments, as it was found to be optimal in regulating invasion in vitro of uveal melanoma cultures.16 Stock TGFβ solutions were prepared according to the manufacturer’s instructions (reconstituting to 1 μg/mL with sterile 4 mM HCl with 0.1% bovine serum albumen [BSA], and storing at −20°C until required). In experiments involving TGFβ, serum-free RPMI-1640 with 0.1% BSA (assay medium) was used. In all cases, cells used in the experiments were at approximately 70% confluence.

Expression

Immunocytochemistry. Levels of expression of TGFβ1 and -β2 and the TGFβ receptor TGFβRI by uveal melanoma cell lines were assessed by immunocytochemistry. Cells from both SOM 196B and -157d were grown on sterile glass microscope slides and stained with a pan anti-TGFβ antibody and an anti-TGFβRI antibody (both from Novocastra, Newcastle-Upon-Tyne, UK; Table 1), according to a method previously detailed.16 Cells were also stained with a negative IgG, isotype control antibody for comparison. Analysis of expression levels was performed as recently detailed in Woodward et al.17 In brief, the percentage of positively stained cells was estimated, and the staining intensity was scored as high (+++), moderate (+), or weak (+).

Flow Cytometry. Integrin and intercellular adhesion molecule (ICAM)-1 expression was assessed by flow cytometry (FCATom; BD Bioscience, Franklin Lakes, NJ; Table 1) after incubation of SOM 196B and -157d with TGFβ1 or -β2 in assay medium for 24 hours. Expression was detected by use of appropriate FITC-labeled secondary antibodies (Table 1) with a band-pass filter (BP530/30) and compared with the untreated control. The data were collected and analyzed on computer (Cell Quest software; BD Biosciences), to assess the median levels of fluorescence. Test samples were run against an internal negative control sample, labeling cells with an IgG1, isotype-negative control antibody. Results are expressed as the relative median fluorescence intensity (MFI), comparing test with control samples stained with the negative control antibody. Relative MFIs greater than 2 were taken as positive, as expression levels were considered to have doubled. Experiments were repeated three times and a mean calculated from the results.

Adhesion Studies

ECM Adhesion Assays. Uveal melanoma adhesion to ECM proteins (collagen type I, collagen type IV, fibronectin, laminin, and vitronectin) was assessed with a screening kit (CytoMatrix; Chemicon International Harrow, UK), according to the protocol previously described.16 To investigate the effects of TGFβ isoforms on adhesion, cells were pretreated with TGFβ1 or -β2 for 24 hours in assay medium (SOM 196B and -157d). Cells were then washed, resuspended in assay medium and seeded at 2.5 × 10⁵ cells/well. They were allowed to adhere for 1 hour at 37°C, before nonadherent cells were removed by washing with PBS. Attached cells were stained with 0.2% cresyl violet in 10% ethanol before solubilization in equal volumes of 0.1 M NaH₂PO₄ (pH 4.5) and 50% ethanol. Levels of adhesion were determined by assessing the absorbance at 540 nm on a microplate reader (Dynex Technologies Inc., Chantilly, VA). The data were collected and analyzed on computer (Revelation software; Dynex Technologies Inc.). Triplicate wells were assessed for each treatment; experiments were repeated three times for cell lines, and the mean value calculated. Results were compared with untreated cells in which TGFβ was omitted. In all cases, adhesion to wells coated with BSA acted as the internal negative control, and levels of adhesion to ECM substrates were assessed relative to the control.

Endothelial Adhesion Assays. To study the involvement of TGFβ in attachment of SOM 196B and -157d cells to the endothelium, HDMECAs or HuLSECs (2 × 10⁵/well) were grown to confluence in 96-well plates, precoated with a gelatin attachment factor (TCS Cellworks Ltd.). Before setting up the adhesion assay, we removed the growth media from the wells and washed the cells twice with PBS. The adhesion assay was then performed as previously described.18 Briefly, 2.5 × 10⁴ cells, prelabeled with 5 μM carboxy-fluorescein diacetate succinimidyl ester (CFDA-SE; fluorescing at 492–517 nm; Molecular Probes Inc., Eugene, OR), and resuspended in assay medium with TGFβ1 or -β2 were added to each well. Plates were incubated at 37°C for 4 hours before nonadherent cells were removed. Adherent cells were fixed in 4% formaldehyde and PBS and rinsed in distilled water, before levels of adhesion were determined by assessing the absorbance at 510 nm on a microplate analyzer (fusion Universal; Perkin Elmer, Pangbourne, UK). The data were collected and analyzed for each condition, and experiments were repeated three times and the mean result calculated. Results were similarly compared with those from the untreated control cells, from which TGFβ isoforms were excluded. In all cases, adhesion to fibronectin (15 μg/mL) acted as the positive control. The negative control in which tumor cells were not added to endothelial monolayers was used to generate background levels of fluorescence, which was subsequently subtracted from all test values.

Gelatin Zymography

Gelatinase secretion was investigated by gelatin zymography as previously detailed19 using conditioned media (CM) produced by growing 5 × 10⁵/mL cells of the lines SOM 196B and -157d and STCs (SOM 365,
-367a, -367b, -368, -371, -380, and -382) in assay medium containing TGFβ1 or -β2 for 24 hours. Harvested CM was centrifuged to remove any residual cell debris and stored at −20°C until needed. Levels of gelatinase expression in CM were analyzed by separation on a non-denaturing gelatin/SDS polyacrylamide gel (100 V for 90 minutes; 3% acrylamide stacking gel, 7.5% acrylamide resolving gel; TV-100, Geneflow Ltd., Staffordshire, UK), according to the method of Laemmli,20 loading 10 μl/sample. Gels were subsequently washed in 2.5% Triton-X-100 before incubating in an appropriate substrate-developing buffer at 37°C overnight. Gelatin degradation was detected by staining gels with Coomassie brilliant blue R250; Sigma-Aldrich, Poole, UK). To improve the presence of metalloproteinases, 10 mM EDTA (acting as a chelating agent) was added to the developing buffer. A wide-range chelating agent) was added to the developing buffer. A wide-range molecular marker (Bio-Rad, Herts, UK) was used in each assay, and positive and negative controls, included in all instances, were a uveal melanoma cell line (SOM 177) known to secrete gelatinases and serum-free medium, respectively. Levels of secretion were analyzed by densitometry (Quality One software; Bio-Rad), and compared with that of the untreated control. Results were then expressed as the relative change in secretion, when normalizing equivalent untreated control levels to a value of 1. Untreated controls were also loaded at 2, 5, 10, and 20 μl, to generate a standard curve. Experiments with cell lines were repeated three times, and a mean result calculated.

**Statistical Analysis**

A Student’s t-test was used to compare population means of the ECM and endothelial adhesion assay and flow cytometry data, by analyzing TGFβ treatment against the untreated control. Because of the presence of low fluorescence units, the variance in the data was found to be heterogeneous, and consequently a square root transformation was applied: √(x + 0.5). In all cases P < 0.05 was taken as significant and was used to establish that cellular adhesion or adhesion molecule expression was significantly increased or decreased, compared with the respective untreated control level.

**RESULTS**

**TGFβ and TGFβR1 Expression**

Both SOM 157d and -196B cells stained positively for TGFβ and part of the TGFβ receptor complex TGFβR1, with >90% of cells staining positively in all samples. Staining intensity of TGFβR1 was high in both samples (+ + +). For TGFβ, however, the staining intensity of SOM 157d cells was much greater than that for SOM 196B (+ + + for SOM 157d, and + for SOM 196B), but due to the nature of the antibody, no information regarding the isoform of TGFβ could be gained.

**Regulation of Adhesion Molecule Expression and Adhesion to ECM Substrates by TGFβ**

Before treatment with TGFβ, SOM 196B cells expressed higher levels of α1, α2, α3, α4, α5- and α6-integrins than did SOM 157d cells.18 Neither cell type expressed ICAM-1, and levels of αvβ3 were low in both cultures.18 For invasive SOM 196B, treatment with TGFβ2 significantly downregulated expression of α5-integrins (laminin-binding; P < 0.05; Fig. 1), but although adhesion of SOM 196B to laminin decreased, the change was not significant (P > 0.05). Neither TGFβ isoforms had a significant effect on adhesion to other ECM proteins or changes in adhesion molecule expression (P > 0.05).

**Modulation of Gelatinase Secretion by TGFβ**

SOM 157d and -196B secreted high levels of gelatinases (type IV collagenases) of approximately 70 kDa, assumed to be latent MMP-2 (Fig. 2). Treatment of a duplicate gel with EDTA, prevented activity of the 70-kDa protein on the gel, suggesting that this protein was indeed a metalloproteinase, as these enzymes require a high level of cations for activity. Levels of latent MMP-2 were upregulated by both TGFβ isoforms (Fig. 2); TGFβ1 had a greater effect on SOM 157d, increasing levels by 1.71 ± 0.194 when normalized to the control, whereas TGFβ2 had a more noticeable effect on SOM 196B (Fig. 2), increasing levels by 2.02 ± 0.0757 when compared with the control. Secretion of proteins indicative of active MMP-2 (58 kDa), latent or active MMP-9 (120 or 92 kDa, respectively) was not observed in either culture. These results were supported by five of the seven STCs (SOM 365, 367a, 367b, 371, and 380) in which TGFβ1 and -β2 treatment upregulated levels of the 70-kDa gelatinase (data not shown). In the SOM 365 cells, TGFβ treatment also upregulated the level of the 92-kDa gelatinase (data not shown).

**Regulation of Tumor Cell Adhesion to Endothelial Cells by TGFβ**

SOM 196B cells have been shown to adhere in significantly higher levels than SOM 157d cells to both dermal (HDMECs) and hepatic (HuLiSECs) cells (P < 0.05).18 To investigate briefly whether TGFβ affects the adhesive interactions of uveal melanomas cells to the endothelium, TGFβ1 or -β2 was added to the adhesion assay, as previously described.18 Adhesion of noninvasive SOM 157d to HuLiSECs but not to HDMECs was significantly increased (P < 0.001) by TGFβ2, but not by TGFβ1 (Fig. 3). A similar effect was not observed for invasive SOM 196B cells (P > 0.05; Fig. 3). It is unclear from these results, however, whether the tumor or the endothelial cells were affected by the TGFβ treatment.

**DISCUSSION**

It is evident that TGFβ can act as both a tumor suppressor and a tumor promoter in several cancers and that this action is dependent on the point at which the cells are regulated during tumor progression.3 Previously, using a Boyden chamber inva-

**FIGURE 1.** Effect of TGFβ isoforms on SOM 196B adhesion molecule expression. SOM 196B cells were treated with TGFβ (0.1ng/mL) for 24 hours before cell adhesion molecule expression was assessed by flow cytometry. In all cases, test samples were run against an internal negative control sample of cells labeled with an IgG1, isotype negative control antibody. Results are expressed as the relative MFI, comparing test with control samples stained with the negative control antibody. Relative MFIs of greater than 2 were taken as positive, as expression levels were considered to have doubled. Experiments were repeated three times and a mean calculated. Data are the mean ± SEM percentage change in MFI, comparing TGFβ-treated cells with untreated controls, in three experiments *P < 0.05 when comparing levels of expression by TGFβ-treated cells with the untreated control.
expression than did TGF-1. As a part of a dual regulatory role, TGF-1 inhibits the growth of tumor cells, but otherwise TGF-1 stimulates them. For example, TGF-1 inhibits the growth of HBMEC and induces the expression of type IV collagenase, and stimulates the migration of cultured fibrovascular tissue into the retina. In contrast, TGF-1 induces the expression of type IV collagenase, and stimulates the migration of cultured fibrovascular tissue into the retina. In this current investigation, regulation by TGF-1 appears to be specific and may contribute to the targeting of TGF-1 receptors to the liver, but whether this role is inhibitory or stimulatory is unclear. To investigate, we studied the effect of TGF-1 inclusion in adhesion assays between uveal melanoma cells and both dermal and hepatic endothelial cells. Previously, we had shown that TGF-1 expression is higher in noninvasive than in invasive cancers. In this investigation, regulation by TGF-1 did not affect adhesion of SOM 196B to endothelial cells, and the noninvasive cells were similarly unaffected in adhesion assays with dermal endothelial cells. Of importance, however, adhesion of SOM 157d to hepatic endothelial cells was significantly increased with stimulation by TGF-2. Whether it is the melanoma or the endothelial cells that are specifically targeted is not known at this time, but these initial findings suggest that TGF-2 is capable of positively enhancing uveal melanoma attachment in the hepatic environment and that this regulation is specific and may contribute to the targeting of the liver by uveal melanomas. A similar effect was not seen for the invasive SOM 196B cells, and it is possible that the more invasive uveal melanomas are capable of naturally stimulating adhesive interactions with the hepatic endothelium through release of TGF-2 themselves. TGF-2 stimulation during extravasation within the liver could therefore be vital to successful colonization, again reflecting a positive stimulatory role in regulating these melano-

![Figure 2](image2.png)

**Figure 2.** Effect of TGFβ isoforms on SOM 196B cell gelatinase secretion, measured by gelatin zymography. SOM 196B cells were treated with TGFβ (0.1 ng/mL) for 24 hours, before the conditioned medium (CM) was harvested. The CM was then run on a nondenaturing SDS polyacrylamide gel (10 µL/sample). A wide-range molecular marker was included as indicated. Positive and negative controls, included in all instances, were a uveal melanoma cell line known to secrete gelatinases and serum-free medium, respectively. Untreated CM samples were loaded at 2, 5, 10, and 20 µL to generate a standard curve. When normalized to the equivalent control levels of secretion (10 µL), TGFβ2 had a greater effect on SOM 196B cell 70-kDa gelatinase expression than did TGFβ1.

In this study, both the invasive and the noninvasive cell lines expressed TGFβ and part of the TGFβ receptor complex TGFβR1. In common with other cell types, uveal melanoma cells could therefore use TGFβ to stimulate themselves and surrounding cells and are therefore open to autocrine and paracrine regulation. Because TGFβ1 and -β2 altered cell behavior in most of the assays, it seems that a functional receptor complex to which TGFβ can bind is expressed by uveal melanomas, although, as expression of only one part of the receptor complex was assessed, this awaits confirmation. No information regarding the isoform of TGFβ expressed could be deduced, because of the nature of the anti-TGFβ antibody used and detection of TGFβ 1, -β2, or -β3 was therefore possible. Further studies in additional tumors would assist in clarifying this point.

As a part of a dual regulatory role, TGFβ suppresses the development of early-stage tumors while promoting the more advanced. In this current investigation, the findings suggest that TGFβ has the potential to be inhibitory in the earlier stages of tumor progression, reflecting reports of other cancers. Previously, we found that TGFβ isoforms reduced invasion of SOM 196B, and in this study TGFβ2 downregulated adhesion of SOM 196B to laminin and the corresponding laminin-binding α3-integrins (Fig. 1), correlating with a proposed inhibitory role. In contrast to this negative role, pretreatment by TGFβ1 and -β2 was found in this study consistently to upregulate proteins indicative of latent MMP-2, but not active MMP-2 or -9, by both cell lines (Fig. 2) and most of the STCs studied (SOM 365, 367a, 367b, 371, and 380). As these proteolytic enzymes are secreted by malignant tumors to assist migration by breaking down the matrix proteins and providing chemotactic fragments for the cells to migrate toward their upregulation by TGF-β would more closely fit a stimulatory role in uveal melanoma invasion.

Perhaps the most interesting findings of this present study are those relating to the interaction of uveal melanomas with endothelial cells. Uveal melanomas preferentially spread to the liver, and, once within the hepatic microenvironment, could be open to regulation by TGFβ, but whether this role is inhibitory or stimulatory is unclear. To investigate, we studied the effect of TGFβ inclusion in adhesion assays between uveal melanoma cells and both dermal and hepatic endothelial cells. Previously, we had shown that TGFβ expression is higher in noninvasive than in invasive cancers. In this investigation, regulation by TGFβ did not affect adhesion of SOM 196B to endothelial cells, and the noninvasive cells were similarly unaffected in adhesion assays with dermal endothelial cells. Of importance, however, adhesion of SOM 157d to hepatic endothelial cells was significantly increased with stimulation by TGFβ2. Whether it is the melanoma or the endothelial cells that are specifically targeted is not known at this time, but these initial findings suggest that TGFβ2 is capable of positively enhancing uveal melanoma attachment in the hepatic environment and that this regulation is specific and may contribute to the targeting of the liver by uveal melanomas. A similar effect was not seen for the invasive SOM 196B cells, and it is possible that the more invasive uveal melanomas are capable of naturally stimulating adhesive interactions with the hepatic endothelium through release of TGFβ2 themselves. TGFβ2 stimulation during extravasation within the liver could therefore be vital to successful colonization, again reflecting a positive stimulatory role in regulating these melano-

![Figure 3](image3.png)

**Figure 3.** Effect of TGFβ treatment on SOM 196B and -157d cell adhesion to HuLiSECs. Shown is the mean (±SEM) number of tumor cells adhering to fibronectin (used as a positive control) or HuLiSECs in the presence or absence of TGFβ1 or -β2 (0.1 ng/mL) for 4 hours. Data represent the mean results in three experiments. Units of fluorescence at 510 nm are measured on the y-axis. Inclusion of TGFβ2 in the adhesion assay significantly increased SOM 157d cell adhesion to HuLiSECs, when compared with the untreated control (P < 0.001). In contrast, no significant difference in adhesion of SOM 196B cells to HuLiSECs was observed after TGFβ treatment (P > 0.05) when compared with the untreated control.
mas. A theory that has recently gained support from the work of Siegel et al.,22 who found that TGFβ signaling in the tumor cells actually increases extravasation, and it may be that, as has been found in this study, increased adherence to the endothelium is a factor.

In summary, we have shown in these preliminary studies of uveal melanoma that TGFβ has a potential dual role in controlling uveal melanoma invasion: downregulation of adhesion and upregulation of protease secretion. The evidence presented herein also suggests that uveal melanomas could be regulated by both autocrine and paracrine stimulation of TGFβ, and that the tumor, its microenvironment, and the stage of the tumor could therefore affect how TGFβ modulates uveal melanoma invasion. In particular, TGFβ may specifically regulate interaction of uveal melanoma cells with the hepatic endothelium by promoting adhesion and thus may play a key role in uveal melanoma metastasis. Further studies of additional melanomas are needed to confirm these interesting findings.

Acknowledgments

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References