Expression and structural features of endoglin (CD105), a transforming growth factor \( \beta \)1 and \( \beta \)3 binding protein, in human melanoma

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Summary Human endoglin (CD105) is a member of the transforming growth factor beta (TGF-\( \beta \)) receptor family that binds TGF-\( \beta \)1 and -\( \beta \)3, but not TGF-\( \beta \)2, on human endothelial cells. Immunohistochemical analyses demonstrated that CD105 is expressed on normal and neoplastic cells of the melanocytic lineage. The anti-CD105 MAb, MAEND3, stained 50, 25 and 34% of intradermal naevi, primary and metastatic melanomas investigated, respectively, and nine out of 12 melanoma cell lines. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE) analysis revealed that CD105 expressed by melanoma cells consists of a homodimeric protein with an apparent molecular weight of 180 and 95 kDa under non-reducing and reducing conditions. Cross-linking of \(^{125}\)I-labelled TGF-\( \beta \)1 to melanoma cells, Mel 97, by disuccinimidyl suberate (DSS) demonstrated that CD105 expressed on pigmented cells binds TGF-\( \beta \); the pattern of binding of TGF-\( \beta \)1 to melanoma cells was found to be similar to that of human umbilical vein endothelial cells. The addition of exogenous, bioactive TGF-\( \beta \)1 significantly (\( P < 0.05 \)) inhibited the growth of CD105-positive melanoma cells, Mel 97, but did not affect that of CD105-negative melanoma cells, F0-1. These data, altogether, demonstrate that CD105 is expressed on pigmented cells and might play a functionally relevant role in the biology of human melanoma cells by regulating their sensitivity to TGF-\( \beta \)-ds.

Keywords: endoglin; melanoma; transforming growth factor \( \beta \); transforming growth factor \( \beta \) receptor(s)

Human endoglin is a homodimeric membrane glycoprotein of about 180 kDa, composed of disulphide-linked subunits of 95 kDa (Gougos and Letarte, 1988a). Endoglin is a type I integral membrane protein with an extracellular region, a hydrophobic transmembrane domain and a short cytoplasmic tail (Gougos and Letarte, 1990). The amino acid sequence of human endoglin, but not that of murine (Ge and Butcher, 1994) and porcine (Yamashita et al., 1994) species, contains the tripeptide arginine-glycine-aspartic acid (RGD), which is located in an exposed region of the extracellular domain (Gougos and Letarte, 1990). In the course of the fifth International Workshop on Leukocyte Differentiation Antigens, endoglin was assigned the cluster of differentiation (CD) number 105 (Letarte et al., 1995).

CD105 was first identified and characterised on the pre-B leukaemic cell line HOON (Gougos and Letarte, 1988a,b) by the murine monoclonal antibody (MAb) 44G4. Additional studies demonstrated the expression of CD105 on normal and neoplastic cells from different hematopoietic lineages (Quackenbush and Letarte, 1985; Kreindler et al., 1990; Buhring et al., 1991; Gougos et al., 1992). In addition, by using MAb 44G4, CD105 was found to be highly expressed on vascular endothelium from normal tissues and on cultured human umbilical vein endothelial cells (HUVECs) (Gougos and Letarte, 1988a). Immunohistochemical analyses performed with the anti-CD105 MAb, PN-E2, on normal, inflammatory and neoplastic tissues demonstrated that CD105 is weakly expressed on endothelia from capillary, venous and arterial blood vessels (Westphal et al., 1993a). The intensity of staining of endothelia was up-regulated under proliferative and inflammatory conditions and was

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melanoma cells binds TGF-β1, and (4) exogenous, bioactive TGF-β1 inhibits the growth of CD105-positive, but not that of CD105-negative, melanoma cells.

Material and methods

Monoclonal antibodies and conventional antisera

The anti-CD105 MAb, MAEND3 (IgG), was generated by immunising BALB/c mice with TNF-α-treated HUVECs and characterised for reactivity with human CD105 by sequential immunodepletion of HUVEC lysates with the anti-CD105 MAb, 44G4 (Gougoussis and Letarte, 1988a), followed by immunoprecipitation with MAb, MAEND3, and SDS-PAGE analysis (M Altomonte, personal communication). The specificity of MAb, MAEND3, was confirmed during the fifth International Workshop on Human Leukocyte Antigens (Letarte et al., 1995). MAb, TS1/22 (mouse IgG1), directed to leucocyte function-associated antigen-1 (LFA-1), which is not expressed in human melanomas (Altomonte et al., 1993), was purchased from the American Type Culture Collection (Rockville, MD, USA). MAbS were purified from ascitic fluid by sequential precipitation with caprylic acid and ammonium sulphate (Temponi et al., 1989). The purity of MAb was tested by SDS-PAGE (Laemmli, 1970) under reducing and non-reducing conditions.

Dichlorotriazinylaminofluorescein (DTAF)-conjugated F(ab)2 fragments of goat anti-mouse IgG antibodies, Fc fragment-specific and AffiniPure rabbit anti-mouse IgG, Fc fragment-specific were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA).

Tissue samples

Surgical biopsies of benign naevi were obtained from patients who had undergone reconstructive surgery. Primary and metastatic lesions were removed from patients who had not received treatment in the previous 2 months. Each sample was divided into two portions. One was processed for routine histopathology and the other one was snap frozen in liquid nitrogen. From each specimen, 4 μm cryostat sections were obtained and fixed in absolute acetone for 10 min. Fixed sections were either used immediately in immunohistochemical assays or kept frozen at −20°C with no loss of immune reactivity. Sections stained with 1% toluidine blue were used to evaluate the histological features of the lesions. Histological diagnosis was done according to Clark et al. (1972). Tumour thickness was assessed according to Breslow (1975).

Cells

The human melanoma cell lines, 70-W, Colo 38, F0-1, MeWo and its highly metastatic variant MeM 50-10, Mel 90, Mel 91, Mel 97, Mel 99, Mel 100, Mel 109, Mel 116 and the EBV-B lymphoblastoid cell line JY that lacks CD105 expression (data not shown), were grown in RPMI-1640 medium (Flow Laboratories, McLean, VA, USA), supplemented with 10% heat-inactivated fetal calf serum (FCS) (Flow) and 2 mM L-glutamine. HUVECs were grown in umbilical vein medium (Rodeck et al., 1989), supplemented with 2% FCS, 1% Nutridoma-SR (Boehringer Mannheim, Milan, Italy) and 2 mM L-glutamine. HUVECs were obtained by treating umbilical veins with 0.1% DNAase and collagenase (Sigma Chemical Co., St Louis, MO, USA) for 30 min at 37°C and grown in TC199 medium (Flow), supplemented with 15% FCS, 2 mM L-glutamine, 24 IU ml−1 sodium heparin (Roche, Milan, Italy), 100 μg ml−1 bovine endothelial cell growth supplement (Sigma), 8% pooled AB serum (Flow) and 100 μg ml−1 gentamycin (Sigma).

Cytokines and reagents

Ultrapure bioactive natural TGF-β1 was purchased from Genzyme (Milan, Italy). 125I-labelled TGF-β1 and 125I-labelled TGF-β1 were purchased from Amersham International (Amersham, UK). Disuccinimidyl suberate (DSS) was purchased from Pierce Chemical Co. (Rockford, IL, USA).

Serological assays

Indirect immunofluorescence (IF) was performed as previously described (Maio et al., 1990). Samples were analysed for cell surface fluorescence using a FACScan flow cytometer (Becton Dickinson Immunocytometry System, Mountain View, CA, USA) equipped with a model 9135C Hewlett-Packard computer (Hewlett-Packard, Palo Alto, CA, USA). Fluorescence was collected by using a four-decade logarithmic amplifier. Viable cells (1 × 10⁴, volume gated) were collected in a list mode fashion for data analysis. The latter was performed with Consort C32 software (Becton-Dickinson). Results are expressed as percentage of positive cells and mean values of fluorescence intensity on a logarithmic scale. A sample was classified as positive when more than 10% of the cells were stained with the relevant MAb.

Indirect immunoperoxidase stain was performed using primary MAb at concentrations ranging from 10−30 μg ml−1 and a commercially available avidin–biotin kit (Vector, Burlingame, CA, USA). Negative controls consisted of tissue sections on which the incubation with the primary antibody was omitted. The stain of endothelial cells of the vascular wall observable with MAB, MAEND3, provided a positive control in each specimen studied; in contrast, smooth muscle cells were not stained by MAB, MAEND3. The immunoenzymatic reaction was detected using 3-amino-9-ethylcarbazole as a chromogenic substrate and Mayer’s haemtoxylin as nuclear counterstain. Specimens were scored positive when a specific plasma membrane and/or cytoplasmic staining pattern could be detected on melanocytic cells, either with a homogeneous or a heterogeneous distribution.

Radiolabelling of cells, indirect immunoprecipitation and SDS-PAGE

These were performed as described elsewhere (Maio et al., 1990). Briefly, cells (2 × 10⁵) were labelled with 125I (Amersham) using the lactoperoxidase method (Zwieg et al., 1983). Then, cells were solubilised by incubation for 60 min at 4°C in lysis buffer containing 1% Nonidet P-40 (NP-40) (Sigma), 10 mM Tris-HCl (pH 8.2), 0.5 mM sodium chloride, 1 mM EDTA, 1 mg ml−1 BSA, and 1 mM phenylmethylsulphonyl fluoride (PMSF) (Sigma), and incubated for 12 h with the anti-CD105 MAb, MAEND3, bound to Protein A-Sepharose (Pharmacia), precoated with rabbit anti-mouse Ig antibody (Fc fragment specific). One dime of 10% polyclayamide slabs were incubated for 10% polyclayamide slabs under reducing and non-reducing conditions using the buffer system described by Laemmli (1970). Gels were processed for autoradiography using a Kodak XAR-5 film (Eastman Kodak Company, Rochester, NY, USA).

Affinity cross-linking of 125I-labeled TGF-β1 to melanoma cells

Cross-linking of 125I-labelled TGF-β1 to whole melanoma cells was carried out as described (Massagué, 1987) with the following modifications. Semi-confluent monolayers of melanoma cells Mel 97 and HUVECs were washed twice with cold binding buffer (128 mM sodium chloride, 5 mM potassium chloride, 5 mM magnesium sulphate, 1.2 mM calcium chloride, 50 mM Hepes, pH 7.5, 2 mg ml−1 BSA) and then cells were detached with 1 mM EDTA in PBS for 30 min at 37°C, washed twice with cold binding buffer and resuspended (1 × 10⁶) in 500 μl of cold binding buffer. Cells suspensions of JY cells were washed twice with binding buffer and resuspended (1 × 10⁶) in 500 μl of cold binding buffer. Cells were then incubated at 4°C with 100 pm 125I-labeled TGF-β1 in binding buffer in the presence or absence of 10 nM (100-fold excess) of cold ultrapure natural TGF-β1 on an orbital.
shaker. After a 3 h incubation, cells were washed once with cold binding buffer and incubated with 0.3 mM (final concentration) DSS in binding buffer lacking BSA at room temperature. Following a 15 min incubation, cells were washed twice with cold binding buffer, resuspended in lysis buffer and CD105 was immunoprecipitated as described above. One-dimensional SDS-PAGE was performed on 7.5% polyacrylamide gels under non-reducing conditions; then gels were processed as described above.

Cell proliferation assays

Melanoma cell proliferation assays were performed as described previously (Rodeck et al., 1987). Briefly, melanoma cells ($5 \times 10^3$), Mel 97 and F0-1, grown in complete W489 medium for at least 2 weeks, were cultured in 96-well flat-bottom plates (Falcon, Lincoln Park, NJ, USA) in 200 μl of complete W489 medium. After 24 h, cultures were added with 25 ng ml$^{-1}$ bioactive TFG-β1. Control cultures were incubated under the same experimental conditions but without TGF-β1. After a 16 h incubation at 37°C in a carbon dioxide humidified atmosphere, cultures were pulsed with 1 μCi per well of $[^3]H$TdT for 12 h, harvested on glass fibre strips, and $[^3]H$TdT incorporation was measured by a MATRIX 96 Direct Beta Counter (Packard, Meriden, CT, USA).

Statistical analysis

Data were analysed by the Student’s paired t-test using the StatWorks statistical package from Cricket Software (Philadelphia, PA, USA). Differences with $P < 0.05$ were considered statistically significant.

Results

Immunohistochemical analysis of CD105 expression in benign and malignant lesions of melanocytic origin

Benign lesions (50%), primary (25%) and metastatic melanomas (34%) were stained by the anti-CD105 MAb, MAEND3 (Table I). Within the CD105-positive benign melanocytic lesions, staining was detected on A-type naevic cells (Figure 1a) and junctional areas. Staining of primary melanomas did not correlate with tumour thickness. Metastatic melanoma lesions were also reactive with the anti-CD105 MAb, MAEND3 (Figure 1b). In seven lesions the staining outlined the cell boundaries, while in the remaining lesions, the antibody reactivity appeared to be confined to the cytoplasm (Table I). Except for one lesion, which was homogeneously stained, the majority of the metastatic cells displayed a variable extent of immunoreactivity ranging from 30–70% (data not shown). Among the lesions tested no correlation was found between the expression of CD105 and the anatomical site of malignant (primary and/or metastatic) lesions (data not shown). Endothelial cells, but not smooth muscle cells, of blood

| Type of lesion       | Positive aborted | Staining pattern |
|----------------------|------------------|------------------|
| Intradermal naevi    | 5/10             | Mostly A-type cells and junctional areas |
| Primary melanomas$^a$| 3/12             | Weak cell membrane (7) and cytoplasmic stain (3) |
| Metastatic melanomas | 10/29            | Weak cell membrane (7) and cytoplasmic stain (3) |

$^a$Four-μm-thick cryostat sections were tested in immunoperoxidase.

$^b$Endothelial cells, but not smooth muscle cells, from blood vessels within all tested tissue sections were strongly stained by MAb MAEND3. $^c$Thickness between 0.4 and 6 mm.

Figure 1 Immunohistochemical detection of CD105 by indirect avidin–biotin immunoperoxidase stain of 4 μm acetone-fixed cryostat sections by MAb MAEND3. Variable levels of CD105 are expressed by A-type naevic cells (a) (arrows mark cells more strongly stained). Melanoma cells from a metastatic melanoma (b) display variable degree of expression of CD105 (arrow marks vessels, arrowheads mark melanin-filled macrophages). Ep, epidermis. Original magnification × 240.

Figure 2 Expression of CD105 on human melanoma cell lines. Melanoma cells ($1 \times 10^5$) were suspended in PBS–bovine serum albumin–0.01% sodium azide, and sequentially incubated with anti-CD105 MAB MAEND3 or the anti-LFA-1 MAB TS1/22 and with DTAF-conjugated F(ab')$	ext{2}$ fragments of goat anti-mouse IgG xenoantibodies. Then cells ($1 \times 10^5$, volume gated) were analysed by flow cytometry. Data represent the percentage of cells stained by MAB MAEND3 (●), and the values of mean fluorescence intensity obtained with MAB MAEND3 (○), and with the isotype-matched MAB TS1/22 (□), used as negative control primary antibody.
vessels within all lesions investigated were strongly stained by the anti-CD105 MAb, MAEND3 (data not shown).

### IIF analysis of CD105 expression on melanoma cell lines

IIF staining with the anti-CD105 MAb, MAEND3, showed a heterogeneous expression of CD105 on the melanoma cell lines, 70-W, Colo 38, F0-1, MeWo, MeM 50-10, Mel 90, Mel 91, Mel 97, Mel 99, Mel 100, Mel 109 and Mel 116 (Figure 2). The percentage of stained cells ranged from 36–89% for Colo 38 and Mel 91 melanoma cells respectively. The values of mean fluorescence intensity, which are an indirect expression of antigen density, ranged from 7 to 25 for melanoma cells, Colo 38 and Mel 97, respectively. The anti-CD105 MAb, MAEND3, stained less than 10% of cells from the melanoma cell lines, F0-1, Mel 90 and Mel 99 (Figure 2). In contrast, MAb, MAEND3, stained 100% of HUVECs with a value of mean fluorescence intensity of 313 (Figure 2).

### Molecular profile of CD105 expressed by melanoma cells

SDS-PAGE analysis demonstrated that the anti-CD105 MAb, MAEND3, immunoprecipitated components with a molecular weight of about 180 kDa and 95 kDa under non-reducing and reducing conditions, respectively, from radiolabelled melanoma cell lines, Mel 91, Mel 97 and 70-W (Figure 3). Components with a similar molecular weight were immunoprecipitated by MAb, MAEND3, from radiolabelled HUVECs (Figure 3). SDS-PAGE analysis of immunoprecipitates from melanoma cells, F0-1, did not identify components with a molecular weight similar to those obtained for the other melanoma cell lines investigated (Figure 3).

### Binding of 125I-labelled TGF-β1 to CD105 expressed on melanoma cells

Cross-linking by DSS of 125I-labelled TGF-β1 to melanoma cells, Mel 97, and HUVECs followed by immunoprecipitation with an anti-CD105 MAb, MAEND3, demonstrated that CD105 expressed on pigmented cells binds TGF-β1. Figure 4 shows that the molecular weight of the complex 125I-labelled TGF-β1–CD105 immunoprecipitated by MAb, MAEND3, from melanoma cells was about 200 kDa (lane 4) and similar to that immunoprecipitated from HUVECs (lane 2). The binding of 125I-labelled TGF-β1 to melanoma cells was completely inhibited by the addition of a 100-fold excess of cold TGF-β1 (lane 5); the latter concentration of cold TGF-β1 did not completely inhibit the binding of radiolabelled TGF-β1 to HUVECs (lane 3). An additional band of about 300 kDa was detectable following cross-linking of 125I-labelled TGF-β1 to melanoma cells (lane 4) and HUVECs (lane 2) by DSS. The intensity of the latter component decreased for both melanoma cells (lane 5) and HUVECs (lane 3) in the presence of cold TGF-β1. Opposite to melanoma cells and HUVECs, 125I-labelled TGF-β1 did not bind to EBV-B cells, JY (Figure 4, lanes 6 and 7), that do not express CD105 (data not shown).

### Growth inhibition of melanoma cells by exogenous bioactive TGF-β1

The addition of bioactive TGF-β1 (25 ng ml−1) to cultures of melanoma cells significantly (P<0.05) inhibited the growth of CD105-positive melanoma cells, Mel 97, but did not affect that of CD105-negative melanoma cells, F0-1. Figure 5 shows the mean value of inhibition of [3H]TdR uptake by melanoma cells from three experiments.

### Discussion

Immunohistochemical analyses demonstrated that CD105 is expressed in benign and malignant lesions of melanocytic origin and on cultured melanoma cells. This observation represents the first report demonstrating the expression of CD105 on pigmented cells and the presence of an RGD-containing
molecule on the cell surface of human cells of the melanocytic lineage. The expression of CD105 that we observed with the anti-CD105 MAb, MAEND3, is at variance with a previous report in which melanocytic cells were not stained by the anti-
CD105, MAb, PN-E2 (Westphal et al., 1993b). This discrepancy is probably due to a different affinity of the two anti-CD105 MAb, since MAb, PN-E2, weakly stained endothelial cells (Westphal et al., 1993a,b) that were strongly stained by the anti-CD105 MAb, 44G4 (Gougou and Letarte, 1988a) and by our MAb, MAEND3 (Figure 2).

SDS-PAGE performed under non-reducing conditions demonstrated that the molecular weight of CD105 expressed on melanoma cells is identical to that observed for endothelial cells. In addition, as previously reported for endothelial and lymphoid cells (Gougou and Letarte, 1988a), the analysis of the components immunoprecipitated by the anti-CD105 MAb, MAEND3, under reducing conditions revealed that CD105 expressed by melanoma cells consists of two subunits with an identical molecular weight of 95 kDa. The structural similarity of the components immunoprecipitated from the melanoma cell lines, Mel 91, Mel 97 and 70-
W, suggests the absence of molecular heterogeneity for CD105 expressed by different melanoma cells. In addition, the similarity of CD105 expressed on HUVECs and melanoma cells suggests that malignant transformation does not affect the molecular structure of the molecule.

Several studies have shown that melanocytes secrete TGF-
β1 and TGF-β2, that melanoma cells secrete all three isoforms of TGF-β (Le-Ming and Herlyn, 1993; Rodeck, 1993; Filmus and Kerbel, 1993; Reed et al., 1994) and that both cell types can be growth-inhibited by TGF-β1 (Le-Ming and Herlyn, 1993; Rodeck, 1993; Filmus and Kerbel, 1993). Nevertheless, little is known about the expression of the TGF-β receptor complex on melanocytes and melanoma cells. Rodeck et al. (1994) have recently shown by cross-
linking of radiolabelled 125I-TGF-β1 to cells that the melanoma cell line WM164 co-expresses TGF-β type I and type III receptors. In this study we report that melanocytic cells express CD105, which has been shown to present TGF-
βs to TGF-β type I and/or type II receptors (ten Dijke et al., 1994, Yamashita et al., 1994). In addition, we demonstrate that CD105 expressed by melanoma cells binds TGF-β1. In fact, following cross-linking of 125I-labelled TGF-β1 to melanoma cells by DSS, MAb, MAEND3, immunoprecipitated a component with a molecular weight of about 200 kDa. This shift in the molecular weight of CD105 is compatible with its binding to TGF-β1 and the molecular weight of the complex is similar to that reported for the complex CD105-TGF-β1 immunoprecipitated from HUVECs using the anti-CD105 MAb 44G4 (Letarte et al., 1995) and MAB MAEND3 in this study (Figure 4, lane 2). This 200 kDa band was completely abolished in immunoprecipitates of melanoma cells (Figure 4, lane 5), but not of HUVECs (Figure 4, lane 3), when cells were cross-linked with 125I-labelled TGF-β1 by DSS in the absence of competing cold TGF-β1. The latter discrepancy is probably caused by the lower amounts of CD105 expressed on melanoma cells compared with HUVECs (Figure 2).

SDS-PAGE analysis of the components immunoprecipitated by MAB MAEND3 following cross-linking of 125I-
labelled TGF-β1 to melanoma cells by DSS identified an additional band of about 300 kDa. The latter is likely to represent an oligomer of CD105 being similar to that reported for HUVECs using MAB 44G4 (Letarte et al., 1995) and MAB MAEND3 (Figure 4).

Our cross-linking studies did not identify additional components suggestive for the presence of TGF-β type I and type II receptors in the immunoprecipitates performed with MAB MAEND3, either in melanoma or in HUVECs. This finding is at variance with the demonstration provided by Yamashita et al. (1994) that an antiserum raised to the intracytoplasmic domain of porcine CD105 immunoprecipitates TGF-β type I and type II receptors from porcine aortic endothelial cells cross-linked to 125I-labelled TGF-β1 by dithiothreitol. Nevertheless, the intensity of the components corresponding to TGF-β and type I and type II receptors was much stronger when immunoprecipitation was performed with an antiserum to TGF-β type II receptor. In addition to differences in experimental conditions, several factors may explain the discrepancy between the two studies including: the cell types analysed by Yamashita et al. (1994) and ourselves; the type of anti-CD105 antibodies used in the two studies; and their recognition of intracellular or extracellular domains of the molecule. The lack of detection of TGF-β type I and type II receptors in immunoprecipitates of melanoma cells may also be explained by their low expression of CD105, and by the suggestion that only a fraction of CD105 is associated with TGF-β type I and type II receptors (Zhang et al., 1996). The lack of detection of TGF-β type I and type II receptors in immunoprecipitates of HUVECs cross-linked to 125I-labelled TGF-β1 by DSS (Figure 4) is consistent with the data reported with HUVECs using different MAb to human CD105 (Letarte et al., 1995), and suggests that the complex composed of CD105 and TGF-β type I and type II receptors is not readily detectable in HUVECs as compared with porcine endothelial cells (Yamashita et al., 1994). A similar finding has recently been reported by HUVECs as compared with pre-B cells by Zhang et al. (1996).

The differential effect of exogenous, bioactive TGF-β1 in the growth inhibition of CD105-positive melanoma cells, Mel 97, as compared with CD105-negative melanoma cells, F0-1 suggests that CD105 contributes to the regulation of the antiproliferative effect of TGF-β1 on melanoma cells.

The identification of CD105 on normal and neoplastic cells of the melanocytic lineage helps to dissect the complexity of the TGF-β receptor system on pigmented cells. The ability of CD105 to bind TGF-β1 further supports the functional role of TGF-βs in pigmented cells.

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