

The human melanoma associated protein melanotransferrin promotes endothelial cell migration and angiogenesis in vivo

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Melanotransferrin is a member of the transferrin family, which is comprised of serum transferrin, lactoferrin and ovotransferrin, and is highly expressed on melanoma cells compared to normal melanocytes. Since melanoma is an highly vascularized tumour that expresses melanotransferrin at high levels, we tested purified recombinant melanotransferrin for its capability to induce angiogenesis in the chick chorioallantoic membrane. Macroscopic and microscopic evaluation of the vascular density demonstrated that melanotransferrin exerts an angiogenic response quantitatively similar to that elicited by fibroblast growth factor-2. Overexpression of vascular endothelial growth factor-receptor-2 was observed in newly formed vessels, suggesting that the angiogenic activity of melanotransferrin may depend on activation of endogenous vascular endothelial growth factor. In addition, when antibodies against vascular endothelial growth factor were included in the assay, the angiogenic response was inhibited by 50%. In a Boyden chamber assay purified recombinant melanotransferrin induced chemotactic migration of vascular cells, which was decreased in the the presence of anti-vascular endothelial growth factor antibodies suggesting an involvement of vascular endothelial growth factor present in endothelial cells also in this

assay. However, melanotransferrin was found not to directly bind to integrin $\alpha_v\beta_3$ or the vascular endothelial growth factor-receptor-2 as assessed in a BIAcore assay. A possible correlation between vascularization occurring during melanoma progression and the expression of melanotransferrin and vascular endothelial growth factor was established by immunolocalization of the two factors in sections of melanoma at different clinical steps of melanoma progression. These latter data strongly imply that melanotransferrin may participate in the vascularization of solid tumours and that inhibition of melanotransferrin could form the basis for intervention in tumours which use this pathway.

Introduction

Melanotransferrin (MTf), also called p97, is a member of the transferrin family, which is comprised of serum transferrin (TF), lactoferrin and ovotransferrin. There are two forms of the MTf, a membrane-bound form, which is anchored to the cell surface by a glycosylphosphatidylinositol (GPI)-anchor and a soluble secreted form (Food et al., 1994). MTf was initially found to be highly expressed on melanoma cells compared to normal melanocytes (Brown et al., 1981). However, subsequent studies have shown that MTf is expressed in fetal intestine (Alemany et al., 1993), sweat gland ducts (Brown et al., 1981), and liver sinusoidal lining cells (Sciot et al., 1989; Alemany et al., 1993). Immunostaining of human brain sections shows

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that MTF and the Tf receptor are highly localized to capillary endothelium, while Tf itself is mainly localized to glial cells (Rothenberger et al., 1996). Metal binding studies on a cell line transfected with human MTF show that MTF-anchored p97 can mediate iron uptake and the process is both temperature dependent and saturable (Kennard et al., 1995).

The MTF protein shares 40% protein sequence identity with Tf and lactoferrin (Rose et al., 1986). We have previously shown that Tf, expressed by hypertrophic chondrocytes in culture, represents in cell culture medium a chemotactic factor promoting in vitro transmigration and basement membrane invasion of endothelial cells (Carlevaro et al., 1997). Endothelial migration is strongly decreased by polyclonal antibodies directed against Tf or by blocking antibodies directed against the Tf receptor (Carlevaro et al., 1997). Furthermore, we have demonstrated that purified Tf induces an angiogenic response in the chick embryo chorioallantoic membrane (CAM), comparable to that obtained with fibroblast growth factor-2 (FGF-2), a well-known angiogenic molecule (Carlevaro et al., 1997).

Human melanoma progresses through different steps: nevocellular nevi, dysplastic nevi, in situ melanoma, radial growth phase melanoma (Breslow index < 0.75 mm), vertical growth phase melanoma (Breslow index > 0.75 mm) and metastatic melanoma. As the metastatic melanoma progresses, it acquires a rich vascular network (Barnhill et al., 1992; Ribatti et al., 1992), whereas an increasing proportion of tumour cells express the laminin receptor, which enables their adhesion to the vascular wall (Vacca et al., 1993). In the tumour several angiogenic cytokines are expressed, including FGF-2, vascular endothelial growth factor (VEGF), interleukin-8 (IL-8) and granulocyte/macrophage-colony stimulating factor (GM-CSF) (Lazar-Molinar et al., 2000).

In this study, we investigate the ability of human recombinant MTF to promote endothelial cell migration in vitro and stimulate angiogenesis in the CAM assay. Furthermore, we correlate the extent of angiogenesis with the number of tumour cells expressing MTF and VEGF in tissues from proliferative lesions of the melanocytic lineage. This also closely correlates with the stages of melanoma progression.

Materials and methods

Cells

HMEC-1 immortalized endothelial cells, kindly supplied by Dr. F. J. Candal (National Center for Infectious Diseases, Center for Disease Control, Atlanta, Georgia), were maintained in MCDB 131 medium (Life Technologies, Grand Island, NY), containing 10% fetal calf serum (FCS), dexamethasone 1 µg/ml (Sigma Chemical Co., St. Louis, MO), and human recombinant epidermal growth factor (EGF) 10 ng/ml (Peprotech Inc., Rocky Hill, NJ, USA), and cultured according to published procedures (Ades et al., 1992). SkMel 28 were maintained in Dulbecco's modified minimal essential medium (DMEM) supplemented with 10% FCS and non-essential amino acids (Sigma).

Production and purification of recombinant secreted human MTF

The recombinant secreted human MTF cDNA was generated by introducing a STOP codon immediately following the glycine residue at position #711 (J. Yang, Masters Thesis, University of British Columbia). This was achieved using the polymerase chain reaction (PCR) with the primers WJ67 (5'-GGCATAAGCTTGGCCCAGC-CAGCCCCGACGGCGCC-3') and WJ68 (3'-CAGCAGAGTCGT-

CACGAGCCCCGACTATTTCGAATACGG-5'). The PCR product was cloned into the pNUT vector and transfected into baby hamster kidney cells, BHK TK⁻ ts13 (ATCC CRL 1638). The cells were cultured in 1-l roller bottles with 600 ml of DMEM supplemented with 10% FCS, 20 mM HEPES, 2 mM L-glutamine, 0.08 mM zinc sulphate and 500 µM methotrexate. Cells were seeded at a density of $1-2 \times 10^5$ cells/ml and incubated at 37 °C in a 5% CO₂ humidified atmosphere. Melanotransferrin production was recorded every 2 days using an immunofluorescence assay (Kennard et al., 1993). When MTF production leveled off which occurred after 10 to 12 days of incubation, the supernatant was recovered and centrifuged at 3000g for 20 min at 4 °C.

The Mtf was affinity purified using a 10-ml column of anti-MTF MAb L235 (ATCC HB8446) immobilized on AffiGel-10. The p97 protein was eluted with 0.1 M citric acid, pH 2.5 ± 0.4, and subsequently neutralized with 1 M HEPES to pH 7.4 ± 0.4. Purified Mtf was concentrated to approximately 1.5 mg/ml using 10 000-MW ultrafiltration membranes (Centricon-10, Centriplus, Amicon, Beverly, MA). Melanotransferrin concentration was determined using the immunofluorescence assay, and Mtf purity was determined by SDS-PAGE analysis.

Antibodies

The hybridoma cell line L235, a mouse anti-human MTF mAb (ATCC) was used. Antibodies against VEGF and VEGFR-2 were used for immunostaining (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Specificity of the antibodies was examined. Anti-VEGF antibodies were rabbit anti-human polyclonal antibodies against the N-terminal peptide 1-140 (Santa Cruz catalog SC-507) and anti-VEGFR-2 (Flk 1) were rabbit anti-mouse polyclonal antibodies against the C-terminal peptide 1158-1345 (Santa Cruz catalog SC-504). Cross reaction with chicken was established as described in by Carlevaro et al. (2000). Rabbit anti-human von Willebrand factor antibody was obtained from DAKO (Glostrup, Denmark) (catalog A0082).

Tissues studied

Tissues were selected according to the six clinical steps of melanoma progression described by Clark et al. (1984) (Table I). Step 1 tissue included 14 common acquired nevi. Steps 2 and 3, which usually coexist, included 11 nevi with Architectural Disorder with varying degrees of Melanocytic Atypia (termed "nevi with ADMA"), as revised by the National Institutes of Health Consensus Conference on Early Melanoma (1992). Step 4 included 16 early primary melanomas, i.e., tumours in radial growth phase with limited (0.75 mm or less) Breslow vertical thickness (Breslow, 1970). Step 5 included 30 advanced primary melanomas, i.e., those in vertical growth phase with greater thickness. These tumours were subdivided into three groups according to their thickness (< 0.75 mm, 0.76-1.5 mm, greater than 1.5 mm); these groups define steps in progression in terms of its linear relation with the competence for and the incidence of metastasis (Clark et al., 1984; Breslow, 1970). Step 6 included 21 metastases from regional subcutaneous/skin, regional lymph nodes, and distant visceral (jejunum) sites. Tissues were divided into two portions immediately after surgical removal and were formalin fixed. One part was used for immunohistochemistry (see below). The other was used for diagnosis and classification. Biopsies of metastases were obtained before chemotherapy or immunotherapy was initiated.

Chemotaxis assays

Following described methods (Albini et al., 1987), Boyden chamber chemotaxis assays were performed with some modifications. Polycarbonate filters (12 µm pore size; PVP-free, Nucleopore, Concorezzo, Italy) were coated with gelatin (5 µg/ml, type A, Sigma Chemical Co.) Solutions of recombinant MTF (10, 1, 0.1, and 0.01 µg/ml) and human Tf (Sigma) (10, 1, 0.1 µg/ml) were used as chemoattractants in the lower compartment of the Boyden chamber.

HMEC-1 cells are an immortalized human microvascular endothelial cell line retaining morphologic, phenotypic and functional characteristics of the corresponding normal human type (Ades et al., 1992). Based on this, HMEC-1 cells were used for the experiments reported in the present work. HMEC-1 (1.3×10^5 cells per chamber) were placed in the

Tab. I. Clinical and histologic information on patients studied and extent of staining to VEGF and MTf.

		Number	Staining to VEGF ^a	Staining to MTf ^a
Overall		92		
Common nevi	Average age: 35 years Men/women: 6/18	14	+	+
Nevi with architectural disorder with varying degrees of melanocytic atypia	Average age: 38 years Men/women: 5/6	11	++	++
Primary skin melanomas ^b	Average age: 54 years Men/women: 22/32 Histologic type ^c	46	+++	+++
	SSM LMM NM ALM			
	33 7 13 2			
	Clark level			
	I II III IV V			
	2 19 12 19 3			
	Tumour thickness (mm)			
	≤0.75 0.76–1.5 >1.5			
	16 12 18			
	Erosion or ulceration			
	Absent/present: 44/10			
Metastatic melanomas	Average age: 44 years Men/women: 8/13 Location	21	++++	++++
	Subcutaneous/ skin: 13 Lymph node: 7 Visceral site: 1			

^a Both the extent of staining (relative number of VEGF- and MTf-positive cells) and the intensity of the reaction product were taken into account: –, not detected; +, < 1% positive cells; ++, 1–10% positive cells with slight to moderate staining or 10–50% positive cells with slight staining; +++, 10–50% positive cells with moderate to marked staining; +++++, >50% positive cells with moderate to marked staining. Assessment was performed in a blinded fashion, independently, by two investigators.

^b All primary melanoma were Stage I, according to the ITNM classification (UICC, 1987).

^c Established according to Clark et al. (1986). SSM: superficial spreading melanoma; LMM: lentigo malignant melanoma; NM: nodular melanoma; ALM: acral lentiginous melanoma.

upper compartment of the Boyden chamber after harvesting with trypsin and washing with serum-free medium.

Migration assays were prolonged for 6 hours, at 37 °C in 5% CO₂. Cells adhering on the upper surface of the filter were mechanically removed, whereas those that migrated beyond the filter and located on the lower surface were stained (Toluidine Blue, Sigma Chemical Co.) to allow microscopic quantitation. Each condition was tested in triplicate with at least one repetition of each experiment performed. Standard significance was based on counting five to ten random fields on each filter. Anti-VEGF antibodies 200 ng/ml were added to recombinant MTf 10 µg/ml and to F12 control medium and incubated overnight at 4 °C before the assay.

Northern hybridization

For Northern analysis cellular RNA was extracted from HMEC-1 cells incubated for 6 hours with melanotransferrin in serum-free medium using TRIzol Reagent (Life Technologies). Ten µg of total RNA was size fractionated through 1% agarose formaldehyde gels and transferred to Hybond XL membranes (Amersham Pharmacia Biotech, Italy). Blots were prehybridized at 42 °C for 3 hours in 50% formamide, 0.75 M NaCl, 25 mM NaH₂PO₄/Na₂HPO₄, pH 7.2, 5 mM EDTA, 0.5% SDS and 0.25 mg/ml carrier DNA (heat-denatured). Hybridization was performed overnight at 42 °C adding to the above mixture 10% dextran sulphate and ³²P-labelled VEGFR-2 riboprobe (Promega-Riboprobe in vitro Transcription System). The membranes were extensively washed in 2× SSC (1× SSC is 0.3 M NaCl, 0.03 M sodium citrate pH 7.2), 2% SDS for 30 minutes at 65 °C, and the radioactive bands were visualized by

autoradiography using a Kodak X-Omat film and intensifying screens (Eastman Kodak Co., Rochester, N.Y.).

BIAcore assay

A BIAcore 2000 biosensor system (Pharmacia Biosensor, Uppsala, Sweden) was used to assay the interaction of MTf with α_vβ₃ integrin (kind gift of Alistair Henry of Celltech plc.) and vascular endothelial growth factor receptor 2 (VEGFR-2) (R&D Systems) at 25 °C. The extracellular domains of the α_v and β₃ subunits were linked at the C-terminal end to the Fc of mouse IgG1 to enforce heterodimerization (Stephens et al., 2000). The BIAcore measures surface plasmon resonance, an optical, label-free measurement of binding interactions between a ligand (fixed to the surface of a dextran-coated chip) and an analyte (which flows over the surface of the chip and binds to the ligand). The binding of an analyte to the ligand results in changes in surface plasmon resonance that are directly proportional to the binding.

The carboxymethylated dextran matrix (Sensor Chip CM5, Research Grade, BIAcore) was preactivated with N-hydroxysuccinimide/N-ethyl-N'-[3-(diethylamino)propyl] carbodiimide according to the manufacturer's recommendations. Random amine coupling of the MTf or α_vβ₃ integrin to the chip was achieved by subsequent injection of 10 µg/ml MTf in 10 mM sodium acetate at pH 4.5, and α_vβ₃ integrin at 50 µg/ml at pH 4.5. One cell on the chip was left blank to serve as a control, and the sensorgram generated was subtracted as a baseline from the experimental sensorgrams. To perform binding assays, samples of MTf, α_vβ₃ integrin, or VEGFR2 were injected in Hepes buffer (10 mM Hepes, 150 mM NaCl, 0.005% polysorbate 20, 2 mM MgCl₂, 0.6 mM CaCl₂, pH 7.4).

Immunohistochemistry

Immunohistochemical analysis was carried out on formalin-fixed, paraffin-embedded sections of five randomly selected biopsies from each clinical step of melanoma progression (Table I). Sections were deparaffinized and treated with methanol:H₂O₂ (49:1) for 20 min, to inhibit endogenous peroxidase. They were then washed in PBS. Non-specific sites were saturated with normal goat serum, for 20 min, at room temperature. Sections were incubated with the specific antisera (L235, anti-VEGF, anti-VEGFR-2, anti-factor VIII diluted 1:10, 1:25, 1:25, 1:250, respectively for 3 h at room temperature), washed several times in PBS, incubated with biotinylated goat anti-rabbit IgG (1:200, 30 min, room temperature) (Jackson Laboratories Inc., West Grove, PA) and, after additional washing, reacted with peroxidase-conjugated egg white avidin (Jackson Laboratories, Inc.) (1:500, 30 min, at room temperature). After washes in PBS and sodium acetate buffer (50 mM, pH 5), antibody-binding sites were detected through the enzymatic activity on 3-amino-9-ethyl-carbazole substratum (0.4% in dimethylformamide, 1 ml; sodium acetate, 50 mM, pH 5, 9 ml; H₂O₂ 30%, 0.01 ml). After 10 min, in the dark at room temperature, sections were counterstained with Harris' hematoxylin, mounted (Glycergel, Dako) and photographed in a Zeiss Axiophot microscope (Zeiss, Oberkochen, Germany).

Morphometric analysis of VEGF and MTF immunoreactivity in malignant melanoma

Some sections of different clinical steps of melanoma progression, immunostained using anti-VEGF and anti-MTf antibodies, were analyzed on a Zeiss Axioplan 2 microscope at 250× magnification connected via a digital video camera TK-C 1381 (JVC, Yokohama, Germany) to an automatic image analyzer KS 300 (Zeiss). The whole image was captured under contrast conditions of stabilized light source, camera settings, and white balance reference. The digital image consisted of 850 × 530 pixels. The image was binary processed, and the cells marked by the antibodies were coloured in yellow. Tumour cell immunoreactivity was detected according to the cytoplasmic staining to VEGF and MTF. Both the extent (relative number of VEGF- and MTF-positive cells) and the intensity of staining were taken into account: –, not detected; +, <1% positive cells; ++, 1–10% positive cells with slight to moderate staining or 10–50% positive cells with slight staining; +++, 10–50% positive cells with moderate to marked staining; +++++, >50% positive cells with moderate to marked staining. Assessment was performed in a blinded fashion, independently, by two investigators.

Chick embryo chorioallantoic membrane assay

Fertilized White Leghorn chicken eggs (30/group) were incubated under constant humidity at 37°C. On the third day of incubation, a square window was opened in the shell and 2–3 ml of albumen were removed so as to detach the developing CAM from the shell. The window was sealed with a glass of the same dimension, and the eggs were returned to the incubator.

CAMs were treated at day 8 with 1.0 µg/embryo of recombinant human MTF, dissolved in 2 µl PBS and adsorbed on 1 mm³ sterilized gelatin sponges (Gelfoam, Upjohn Company, Kalamazoo, MI). Sponges containing vehicle alone (PBS) were used as negative controls, while sponges containing 1.0 µg/embryo of recombinant human FGF-2 or VEGF₁₆₅ (R & D Systems, Abingdon, UK) were used as positive controls. All procedures were performed under sterile conditions. In some experiments, MTF was mixed with 400 ng/embryo of anti-FGF-2 antibody before implantation (Santa Cruz, catalogue SC-79-G) or with 400 ng/embryo of anti-VEGF antibody against N-terminal peptide 1–140 (Santa Cruz, catalogue SC-507) or with a mixture of the two antibodies.

CAMs were examined daily until day 12 and photographed in ovo with a stereomicroscope SR equipped with the Zeiss camera system MC63. In some experiments, blood vessels entering the sponge within the focal plane of the CAM were counted by two observers in a double-blind fashion at 50× magnification (Brooks et al., 1994). At day 12, CAMs were processed for light microscopy. Briefly, the embryos and their membranes were fixed in ovo in Bouin's fluid, dehydrated in

graded ethanols, embedded in paraffin, serially sectioned at 7 µm, according to a plane perpendicular to their free surface and stained with a 0.5% aqueous solution of toluidine blue and observed under a light microscope.

At day 12, some CAMs were also injected intravascularly with India ink according to Olivo et al. (1992).

In some sections, CAM blood vessels were marked by using an anti-VEGFR-2 polyclonal antibody (Santa Cruz catalog SC-504) against the C-terminal peptide 1158–1345. Homology of the peptide recognized by the antibodies and the avian corresponding sequences was previously described (Carlevaro et al., 2000). Eight-micrometer histological sections collected on poly-L-lysine-coated slides (Sigma Chemical, St. Louis, Mo., USA) were deparaffinized and stained with a three-layer avidin-biotin-immunoperoxidase system technique. The sections were rehydrated in a xylene-graded alcohol series and then rinsed for 10 min in 0.1 M phosphate-buffered saline (PBS). Intrinsic peroxidase activity was blocked with 3% H₂O₂ for 10 min in the dark. Thereafter, the sections were treated with 0.1% trypsin (Sigma) in CaCl₂ (0.01 M) for 30 min at room temperature and then sequentially exposed to primary antibody anti-VEGFR-2 diluted 1:100 in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS) overnight at 4°C, and to biotinylated swine anti-rabbit Ig (Dako, Glostrup, Denmark) diluted 1:300 in RPMI-1640 supplemented with 10% heat-inactivated FCS for 15 min at room temperature. Following the first procedure, the sections were then exposed to a streptavidin-peroxidase conjugate (Vector, Burlingame, CA, USA) diluted 1:250 in PBS for 15 min at room temperature, and the developing reaction was performed using 0.05 M acetate buffer, pH 5.1, 0.02% 3-amino-9-ethylcarbazole grade II (Sigma), and 0.05% H₂O₂ for 20 min at room temperature. Following the second procedure, the immunodetection was performed with alkaline-phosphatase-anti-alkaline-phosphatase (APAAP, Dako) for 30 min and fast Red as chromogen for 20 min. In both cases the sections were counterstained with Gill's hematoxylin number 2 (Polysciences, Warrington, PA, USA), and mounted in buffered glycerin. A preimmune rabbit serum (Dako) replacing the primary antibody served as a negative control. Staining intensity of VEGFR-2 immunoreactivity was graded on a scale of 0 to 3; 0, no detectable staining; 1, traces of staining; 2, moderate amount of diffuse staining; 3, a large amount of diffuse staining. This grading is a modification of that of Tahakashi et al. (1995).

Quantitation of the angiogenic response

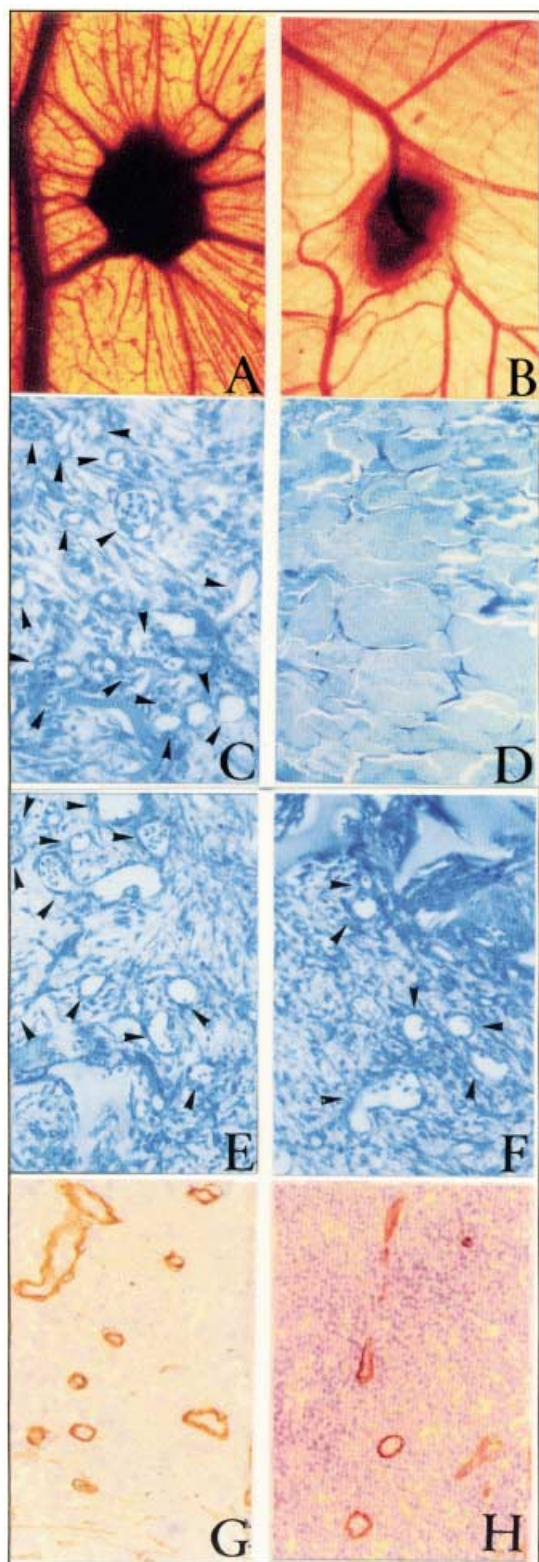
The angiogenic response was evaluated as microvessel density in the CAM area under the implant. The area occupied by microvessels was estimated by using a morphometric method of 'point counting' (Ribatti et al., 1995). Briefly, every third section with 60 serial slides from an individual specimen was analyzed simultaneously by two investigators by a 144-intersection point-square reticulum of 0.125 mm² inserted in the eyepiece of a double-head photomicroscope. Six randomly chosen microscopic fields of each section were evaluated at 250× magnification for the total number of the intersection points that were occupied by vessels transversally cut (diameter ranging from 3 to 10 µm). Mean values ± 1 standard deviation (SD) for vessel counts were determined for each analysis.

The vascular density was indicated by the final mean number of the occupied intersection points, expressed again as a percentage of the total number of intersection points. The statistical significance of differences between the mean values of the intersection points in the experimental CAMs and control CAMs were determined by Student's *t* test for unpaired data.

Results

MTf induces an angiogenic response in the chick embryo CAM

Chick embryo CAMs at day 8 of incubation were implanted with gelatin sponges adsorbed with 1 µg of MTF dissolved in



◀ **Fig. 1.** (A) CAM at day 12 of incubation, 96 h after the implant of a gelatin sponge soaked with 1.0 μg of recombinant human MTF. (B) Sponge treated with PBS only. (C–F) Histological analysis of CAMs grafted with MTF (C), PBS (D), MTF plus anti-FGF-2 (E) and anti-VEGF (F) antibodies. In (C, E, F) arrowheads indicate newly-formed blood vessels. Immunohistochemical staining with antibodies against VEGFR-2 demonstrates an increased staining in the endothelial cells of the newly-formed blood vessels in CAM treated with MTF (G), as compared to a control (H). Original magnification: A, B, 50 \times ; C–F, 250 \times ; G, H, 400 \times .

Tab. II. Macroscopic evaluation of the angiogenic activity of recombinant human MTF in the chick embryo CAM.

Treatment	Number of blood vessels
PBS	7 \pm 3
FGF-2 (1.0 $\mu\text{g}/\text{embryo}$)	35 \pm 5*
VEGF ₁₆₅ (1.0 $\mu\text{g}/\text{embryo}$)	40 \pm 6*
Melanotransferrin (1.0 $\mu\text{g}/\text{embryo}$)	30 \pm 3*

Recombinant human Mtf was adsorbed into gelatin sponges implanted onto the CAM at day 8. PBS-, FGF-2- and VEGF₁₆₅-treated embryos were used as negative and positive controls, respectively. The angiogenic response was assessed macroscopically at day 12 by counting the number of blood vessels entering the sponge as described in Materials and methods.

* Statistically different from PBS ($p < 0.01$).

radially towards the sponge in a spoked wheel pattern (Fig. 1A). A similar macroscopic angiogenic response was observed in the implants treated with 1 μg of FGF-2 or VEGF₁₆₅ (data not shown), whereas no vascular reaction was detectable around the sponges treated with PBS alone (Fig. 1B).

Microscopically, the sponges adsorbed with MTF showed a collagenous matrix containing numerous small blood vessels and fibroblasts localized among the sponge trabeculae (Fig. 1C). Numerous host capillaries piercing the sponge in some points were also recognizable at the boundary between the sponge and the CAM mesenchyme. Similar finding had been reported previously for FGF-2 treated sponges (Ribatti et al., 1997). No collagenous matrix, blood vessels, or fibroblasts were present among the sponge trabeculae in the samples treated with PBS (Fig. 1D).

In agreement with the observations listed above, macroscopic and microscopic evaluation of the vascular density of the CAM at day 12 of incubation demonstrated that MTF exerted an angiogenic response in the chick embryo quantitatively similar to that elicited by FGF-2 or VEGF₁₆₅ (Tables II, III).

Angiogenic activity of MTF is partly dependent on FGF-2 or VEGF and is associated to an overexpression of VEGFR-2 in the newly formed CAM blood vessels

To assess whether the angiogenic response elicited by MTF was due in part to an increased mobilization of endogenous FGF-2 (Ribatti et al., 1995) or VEGF stored in the CAM extracellular matrix, MTF was added to the CAM in the presence of anti-FGF-2 or anti-VEGF antibodies, both of which abolish the angiogenic activity exerted by recombinant FGF-2 or VEGF exogenously added to the CAM vasculature (Ribatti et al., 1995) (Ribatti, unpublished observations). Anti-FGF-2 antibodies reduced the angiogenic response of MTF by 30% and anti-VEGF antibodies by 50%, as shown in Figure 1E–F and in Table III. Finally, MTF-induced angiogenesis in the CAM assay is not completely blocked by a cocktail of anti-VEGF and anti-

PBS. Sponges adsorbed with vehicle alone or with FGF-2 or VEGF₁₆₅ were used as negative and positive controls, respectively. At day 12 of incubation, macroscopic observations of the CAMs showed that MTF induced an angiogenic response characterized by the presence of allantoic vessels spreading

Tab. III. Microscopic evaluation of the angiogenic activity of recombinant human MTf in the chick embryo CAM.

Treatment	Number of intersection points (mean \pm SD)	Microvessel density (%)
PBS	0	0
Melanotransferrin (MTf)	27 \pm 3	18.7
MTf + anti-FGF-2 Ab	20 \pm 2*	13.8
MTf + anti-VEGF Ab	14 \pm 3*	9.7
MTf + antiFGF-2 Ab + anti-VEGF Ab	13 \pm 4*	9.0
FGF-2	30 \pm 5	20.8
VEGF ₁₆₅	32 \pm 6	22.2

Recombinant human MTf (1.0 μ g/embryo) was adsorbed into gelatin sponges in the absence or in the presence of anti-FGF-2 or anti-VEGF antibodies (Ab) (400 ng/embryo) and grafted onto the CAM at day 8. Recombinant FGF-2 and VEGF₁₆₅ (1.0 μ g/embryo) were used as positive control. The angiogenic response was assessed histologically by a planimetric method of 'point counting' on day 12 as described in Materials and methods.

* Statistically different ($p < 0.001$) from MTf.

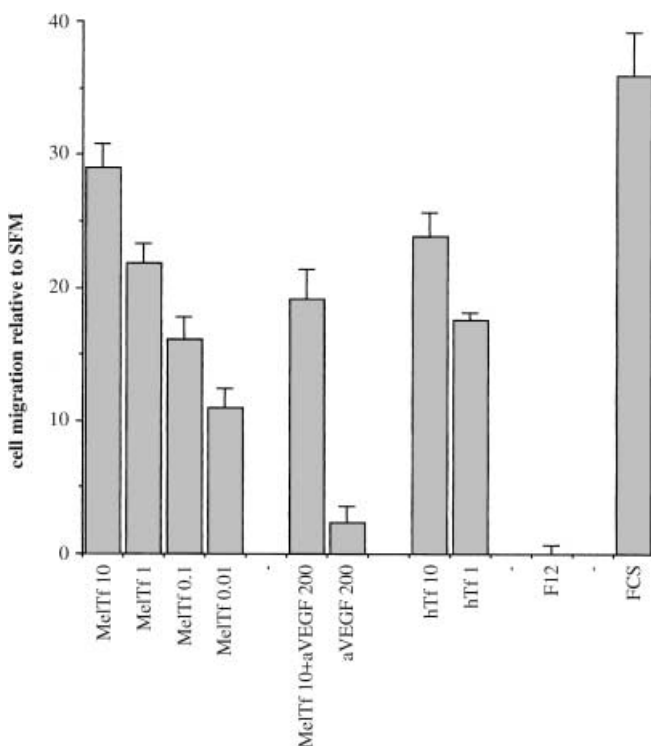


Fig. 2. Chemotactic response of microvascular endothelial cells (HMEC-1) to recombinant human MTf and to human Tf. The factors were added to F12 medium (SFM). F12 medium alone was used as control for background random migration that has been subtracted. Assays were performed in triplicate and repeated at least twice. Five fields were counted on each triplicate filter. Bars indicate SD. Experiments with antibodies against VEGF are indicated by aVEGF.

FGF-2 antibodies, but only slightly decreased as compared to the effect of anti-VEGF antibody alone (Table III).

Immunohistochemical staining with antibodies against VEGFR-2 demonstrated an increased staining in the endothelial cells of the newly-formed blood vessels in CAMs treated with MTf (Fig. 1G), as compared to the controls (Fig. 1H).

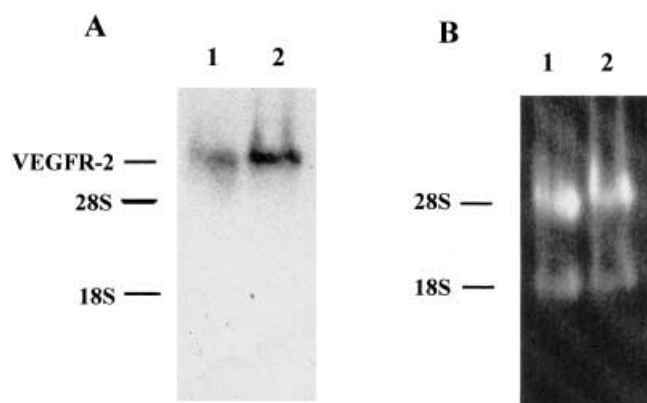


Fig. 3. (A) Northern blot analysis for VEGFR-2 mRNA in HMEC-1 cells. (B) Ethidium bromide staining. Lane 1, untreated cells; lane 2, cells incubated with MTf (5 μ g/ml).

Induction of endothelial cell migration by MTf is partly dependent on VEGF

Purified recombinant MTf induced chemotactic migration by vascular cells in a Boyden chamber assay (Fig. 2) in a dose-dependent manner, with maximal migration at 10 μ g/ml. In agreement with observations in the CAM assay, the presence of anti-VEGF antibodies results in a decrease in the chemotactic response suggesting an involvement of VEGF present in endothelial cells. In fact, endothelial HMEC-1 cells express mRNA for VEGFR-2, which can be overinduced by incubation with MTf (Fig. 3).

MTf does not bind to integrin $\alpha_v\beta_3$ or VEGFR-2

Purified recombinant MTf was tested in a BIAcore assay to determine if it binds to integrin $\alpha_v\beta_3$, due to the presence of a surface available RGD peptide in MTf, or to VEGFR-2. No measurable binding was observed between MTf and integrin $\alpha_v\beta_3$, or between MTf and VEGFR-2. Thus MTf appears not to directly mediate its pro-angiogenic effect by interaction with integrin $\alpha_v\beta_3$, or with VEGFR-2.

Immunohistochemical detection of blood vessels, VEGF and MTf in malignant melanoma

The blood vessels were identified by immunohistochemical reaction with factor VIII-RA. Their number was significantly increased in nevi with ADMA (Fig. 4B), as compared with the nevocytic nevi (Fig. 4A). A further increase was observed in early primary tumours (<0.75 mm Breslow thickness) (Fig. 4C). However, this parameter was substantially unchanged in the evolutionary steps of primary tumours. In addition, a significant increase was observed in the metastases, within the tumour tissue and the surrounding uninvolved tissue (Fig. 4D), as compared with the corresponding areas of advanced (>3.00 mm) primary tumours.

A faint immunoreactivity to MTf and VEGF was detected in the cells of common nevi or cells of the histologically normal dermis. This immunoreactivity significantly increased in nevi with ADMA (Fig. 5A for VEGF and Fig. 5D for MTf), in primary tumours (Fig. 5B for VEGF and Fig. 5E for MTf) and in the metastases (Fig. 5C for VEGF and Fig. 5F for MTf). The

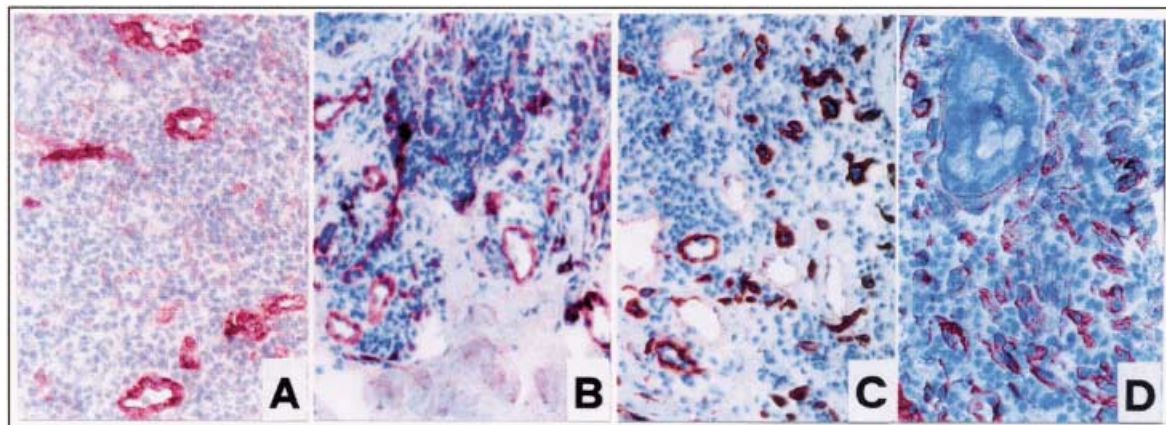


Fig. 4. Immunohistochemical staining of microvessels with anti-factor VIII antibodies in various melanocytic lesions. The number of vessels increases in nevi with ADMA (B), as compared with the nevocytic nevi

(A). A further increase is observed in early primary tumours (C) and in metastasis (D). Original magnification: $250\times$.

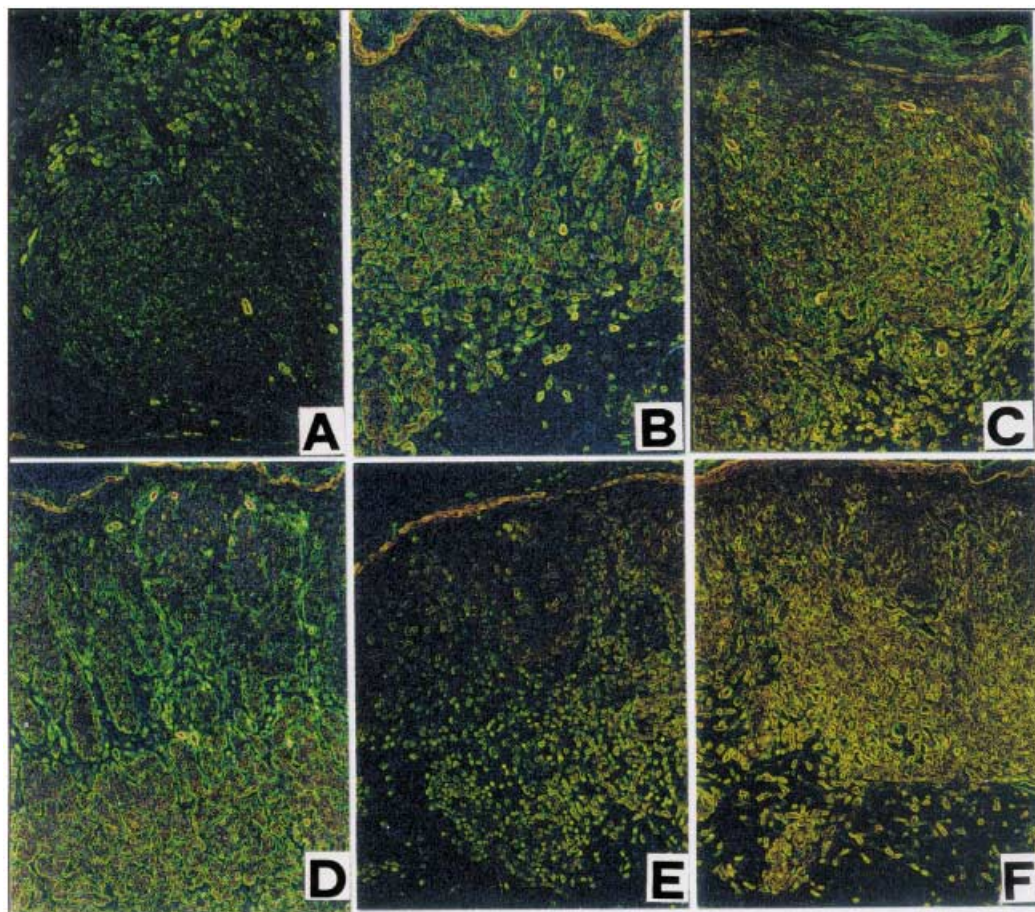


Fig. 5. Immunohistochemical staining of tumour cells with anti-VEGF (A–C) and anti-MTf (D–F) antibodies in various melanocytic lesions. The image was submitted to a binary processing, and the cells marked by the antibodies are coloured in yellow. Tumour cell immunoreactivity is detected according to the cytoplasmic staining to

VEGF and MTf. The immunoreactivity is increased in nevi with ADMA (A, D), in primary tumours (B, E) and in the metastases (C, F). The intensity of staining is scored for both VEGF and MTf as ++ in nevi with ADMA; +++ for primary tumors; ++++ for metastases. Original magnification: $160\times$.

melanomas showed patterns of reaction ranging from homogeneous staining throughout the lesion, to localization of the reaction at the invasive periphery of the lesion, in a part of the

tumour infiltration inflammatory cells. The intensity of staining was scored for both (Table I).

Discussion

Transferrin is expressed in large amounts by hypertrophic chondrocytes in cell culture and in vivo in developing tibia at the level of the diaphysis, at a stage immediately before vascular invasion and bone formation (Gentili et al., 1994). Cell culture medium of hypertrophic chondrocytes promotes endothelial cell migration in vitro. This cell migration is strongly decreased by treatment with polyclonal antibodies against Tf. Cell culture medium and purified Tf promote vascular invasion in vivo in the CAM assay (Carlevaro et al., 1997). Chondrocytes also express VEGF as a major angiogenic molecule in addition to Tf (Carlevaro et al., 2000).

Since melanoma is a highly vascularized tumour (Ribatti et al., 1992) that expresses MTF at high levels, we tested purified recombinant MTF for its capability to induce angiogenesis in an in vivo assay on the CAM. Macroscopic and microscopic evaluation of the vascular density of the CAM demonstrated that MTF exerts an angiogenic response quantitatively similar to that elicited by FGF-2. Overexpression of VEGFR-2 was observed in newly formed vessels, suggesting that MTF angiogenic activity may depend on activation of endogenous VEGF. In addition, when antibodies against VEGF were included in the assay, the angiogenic response was inhibited by 50%. Purified recombinant MTF induced chemotactic migration of vascular cells in a Boyden chamber assay. Migration was decreased in the presence of anti-VEGF antibodies, suggesting endogenous VEGF in endothelial cells to be involved in the migration induced by MTF.

We have also tried to establish a possible correlation between vascularization occurring during melanoma progression (Barnhill et al., 1992; Ribatti et al., 1992) and the expression of MTF and VEGF. MTF and VEGF were expressed at similar levels during melanoma progression. The intensity of staining for both MTF and VEGF increased progressively in nevi with ADMA as compared to nevocytic nevi, and further in primary melanomas and metastatic lesions.

Blood vessels, identified by immunohistochemistry reaction with anti-factor VIII-related antigen, were significantly increased in nevi with ADMA, as compared with nevocytic nevi. A further increase was observed in early primary tumours but the blood vessels remained substantially unchanged in the evolutionary steps of primary tumours. However, a significant increase was observed in the metastases, within the tumour tissue and the surrounding uninvolved tissue, as compared with the corresponding areas of advanced primary tumour.

It was noted that in melanoma several angiogenic cytokines are expressed, including FGF-2, VEGF, IL-8 and GM-CSF (Lazar-Molinar et al., 2000). VEGF is commonly expressed in most solid tumours and the VEGFRs appear predominantly in endothelial cells surrounding or penetrating the malignant tissue (Plate et al., 1993). Otherwise, VEGFRs were absent from vascular cells in the surrounding normal tissue. This finding suggests that VEGFR expression is induced in endothelial cells during tumour angiogenesis by VEGF secreted by tumour cells. VEGFR-1 and VEGFR-2 mRNAs were strongly expressed by vascular endothelium in the numerous vessels elicited by VEGF-overexpressing melanoma cells, whereas neither was expressed in tumours formed from non transfected cells (Claffey et al., 1996).

In conclusion, our in vivo and in vitro data demonstrate a pro-angiogenic activity attributed to human MTF. This is inhibited

by treatment with anti-VEGF antibodies and associated with an overexpression of VEGFR-2 in newly-formed blood vessels.

This supports the hypothesis that MTF may contribute to angiogenesis during melanoma progression, and is likely associated with VEGF overexpression. Limiting the pro-angiogenic activity of MTF may therefore provide a method to decrease the vascularization of tumours and therefore limit tumour progression or growth.

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