Original Basic Research

Alternative angiogenic pathways in melanoma: the role of AQP1

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Abstract

AQP1 belongs to aquaporins family, water-specific, membrane-channel proteins expressed preferentially by microvessels, favoring angiogenesis and increasing permeability. Recently, we have demonstrated that AQP1 has a role as bio-mechanic sensor modulating and stabilizing the organization of cytoskeleton. In the present paper, we have studied the expression of AQP1 and VEGF pathways *in vitro* and in tumors xenografts obtained injecting murine (B16-BL6) or human (WM115) melanoma cells in syngeneic or NOD-SCID mice, respectively. All together, our data show the presence of alternative angiogenic pathways in melanoma tumor xenografts and show, for the first time, a connection between AQP1 and VEGF pathway to facilitate vacuolisation and tube formation.

Key words

Melanoma; Vasculogenic Mimicry; Angiogenesis; AQP1; VEGF; VE-cadherin

INTRODUCTION

Aquaporins are a family of water-specific, membrane-channel proteins expressed in diverse tissues. AQP1 is specifically and strongly expressed in most microvasculature endothelial cells outside the brain. [1] Recently, an impairment of endothelial cell migration without altering their proliferation or adhesion was demonstrated in AQP1 null mice. [2] In this connection, our group demonstrated that silencing AQP1 the cytoskeleton organization is affected through Lin-7/β-catenin pathway. [3, 4] Therefore, AQP1 seems to be a novel promoter of tumor angiogenesis[5] and an intriguing target to modulate angiogenesis/vasculogenic mimicry. [4]

Angiogenesis is a crucial component of tumor growth and metastasis. To target the vascular endothelial growth factor (VEGF) pathway seems to be an interesting therapeutic potential for cancer. [6] However, the recent discovery of alternative vasculogenic pathways like as vasculogenic mimicry (VM), at least in melanoma, opens new perspectives in tumor vascularisation targeting. Interestingly, a recent paper demonstrated that aggressive melanoma tumor cells expressed VE-cadherin, a typical endothelial marker. [7] The functional significance of this remarkable finding is reflected by the role of VE-cadherin in tumor cell formation of embryonic-like vasculogenic networks de novo[7]. In addition, the

microenvironment seems to influence the transendothelial differentiation of aggressive cutaneous melanoma cells, opening new perspectives for therapeutic strategies and novel perspectives on tumor-cell plasticity. [4, 8]

In the present paper, we investigated the possible relationship between AQP1 and VEGF pathways in murine and human melanoma both in vitro and in tumor xenografts.

MATERIALS AND METHODS

Cell Culture and Reagents

The high metastatic murine melanoma B16-BL6 cell line was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 1% non-essential amino acids, 2% MEM vitamin solution, 100 U penicillin, 100 μg streptomycin and 0,25 μg/ml anphotericin B (purchased from Invitrogen, Milan, Italy). Human melanoma WM115 cell line derived from a primary epitheloid tumor was cultured in the basal medium Eagle (BME) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 1% non-essential amino acids, 2% BME vitamin solution, 100 U penicillin and 100 μg streptomycin and 0.25 μg/ml anphotericin B (purchased by Invitrogen, Milan, Italy) as previously described. [9]

Anti-VE-cadherin antibody was a generous gift of Prof. E. Dejana (IFOM, Institute FIRC for Molecular Oncology, Milan).

Cell-tracker Green CMFDA or cell-tracker Red CMFDA were from Molecular Probes.

Spheroids

30 000 cells were plated on 24 non adherent multiwells. 7 days later they growth as spheroids. The same experiments were carried out on plate pre-treated with collagen solution (2mg/ml collagen stock solution extract from tail vein) with or without VEGF (Sigma-Aldrich) for 7/10 days in growth medium without serum. The cells were then photographed every day till 10 days.

Cell Migration

2×10^5 cells were plated on the upper chamber of transwell inserts and maintained in serum-free medium. In the lower chamber was added serum-free medium plus VEGF-A or VEGF-C (as chemoattractant) or no serum (negative control). Cells were incubated for 6 h at 37°C in 5% CO_2. After incubation, the membranes were washed briefly with saline buffer and the non-migrated cells were scraped from the upper surface of the membrane with a cotton swab. Migrated cells were stained for haematoxylin and counted in 20 random high-power microscope fields ($\times$200).

In vivo Tumor Generation

5×10^4 living cells (by tripan blue exclusion) of murine B16-BL6 or human WM115 cells, were injected subcutaneously into the flank of C57BL6 or NOD-SCID mice, respectively. 5-6 animals for each grup were used. Regarding to murine melanoma cells, tumors were detectable 2 weeks later, therefore, animals were sacrificed 10, 14, 17, and 19 days after flank injection. For human cells, a detectable tumor mass appeared 15-19 days.

Light Microscopy

All tissue specimens were divided in two parts. One part, which was immediately fixed in neutral buffered formalin, embedded in paraffin and sectioned was subjected to histopathological characterisation after hematoxillin-eosin staining and appropriate immunohistochemistry. The other part was snap-frozen within 10 min of excision in an isopentane-dry ice mixture and stored at –80°C until processing for immunohistochemistry studies.

For paraffin sections, samples were fixed in buffered formalin for 4h, embed in paraffin and sections of 4-5μm were cut. Slides were deparaffinized using xylene and absolute ethanol, rinsed in distilled water and endogenous peroxidase was blocked washing the sections in 3% H_2O_2 in PBS for 15min, washed and incubated with normal human serum (1:20 Dako). Then the antigen was unmasking exposing the sections to microwave (180 MW, 10 min) in citrate buffer for anti-HMB 45 (anti-human, Dako, 1:50) or anti-aquaporin–1 (anti human and mouse, Sigma, 1:100) and after trypsin digestion for anti- CD31 (monoclonal anti-mouse or anti-human, Dako, 1:20). The sections were incubated with anti-HMB 45 and anti-CD31 for 2h at room temperature or with anti-aquaporin 1 overnight at 4°C.

For frozen sections, 1-2 μm sections were placed on poly-L-lysine coated slides, air-dried overnight at room temperature and then fixed in acetone for 10 minutes. Thus, sections were incubated with primary antibody anti-VE cadherin (1:50) 1h at 37°C.

All the sections were incubated for 30min with biotinylated anti-rabbit or anti-mouse secondary antibody in PBS (Dako, 1:100). This was followed by incubation with streptavidin (Dako, 1:350) coniugated to peroxidase in PBS for 30min, and by a brief rinse in PBS. Color was routinely developed using AEC (3-amino-9-ethylcarbazole, AEC DAKO kit) or VIP (Vector) up to 10 min, rinsed in distilled water, counterstained with Mayer's hematoxillin for 1 min and coverslipped with a permanent mounting medium.

Sections were stained with periodic acid-Schiff (PAS) omitting hematoxylin counterstained to reduce visual noise.

Necrosis was evaluated morphologically and expressed as percentage on the total specimen. Microvessels density was evaluated microscopically. Endothelial vessels were counted at high magnification (×400) with the aid of a grid areas (Leica TMP-TRIBUN ICS version) in 10 different fields for each specimen. The values are expressed as mean ±S.E.

Expression of VE-cadherin and Aquaporin 1 in Melanoma Cells Grown In Vitro

VE-cadherin

Subconfluent cells were grown on 400 mm^2 glass cover and washed three times with Ca^{2+}-Mg^{2+}-PBS according to previously methodology[10] and fixed in acetone– Ca^{2+}-Mg^{2+}-PBS (1:2) for 10-15min. Thus, the cells were incubated with Ca^{2+}-Mg^{2+}-PBS containing 0.5% TRITON X-100 for 5 min and subjected to a second fixation in acetone– Ca^{2+}-Mg^{2+}-PBS (1:2) for 8 min. Cells were incubated in 1% BSA-PBS for 20 min and then incubated with primary antibody anti-VE cadherin (1:50) for 1h at 37°C. After 5 washes in Ca^{2+}-Mg^{2+}-PBS (3 min/each), the cells were incubated with secondary antibody conjugated with TRITC (Alexa 594-conjugated goat anti-mouse, 1:500) for 1h at 37°C and cover slipped with a permanent mounting containing DAPI (Vector) for staining nucleus.

Aquaporin 1

Subconfluent cells grown in 400 mm^2 glass cover were fixed in 4% paraformaldehyde for 20 min and then incubated with anti-AQP-1 (Alpha Diagnostic International, 10 μg/ml) overnight. The cells were then incubated with secondary antibody conjugated with FITC (Alexa 488-conjugated goat anti-rabbit, 1:700) as described above.
The samples were examined with a Leika TCS NT confocal microscope.

Transmission Electron Microscopy

Tumour masses dissected from mice were immediately trimmed in small pieces with a razor blade and immersed overnight at 4°C in the fixative solution (2.5% glutaraldehyde and 0.5% paraformaldehyde in phosphate buffer (PB) 0.1M pH 7.4). After rinsing in PB tissue blocks were post-fixed for 4h at 4°C in 1% osmium tetroxide in PB, gradually dehydrated in ethanol and embedded in Epon-Spurr resin.
Semi thin (1 μm-thick) sections were cut with a Reichert II ultramicrotome, collected on glass slides and stained with toluidine blue, or left unstained, for light microscopic examination. Thin sections collected on copper grids were counterstained with uranyl acetate and lead citrate and examined with a Jeol T8 electron microscope.

Immunoprecipitation

Tumor mass was lysated in buffer A containing: HEPES, pH7.4, 50 mM β-glycerol phosphate, 100 mM NaCl, 2.5 mM EDTA, 1 mM sodiumfloride, 0.5 mM ortovanadate, 0.5 mM PMSF, 1% Triton, 10% glycerol, 10 μg/ml leupeptin and 10 μg/ml aprotinine, and briefly vortexed. DNA and insoluble proteins were removed by centrifugation in a minifuge at 14 000 rpm for 5 min. Proteins (400 μg) were incubated with anti-VEGF antibody (2 μg, monoclonal, Oncogene) overnight at 4°C. The immunocomplex was precipitated with immobilized on Sepharose CL-4B Protein A for 2h at 4°C (Sigma). The bears were washed with the same buffer A four times and proteins were dissolved in SDS sample buffer and loaded on 10% polyacrilamide gels. [11] The specificity of primary antibody was checked silver staining the gels and western blotting the immunoprecipitated samples.

Protein determination

The protein concentration in all the sample was determined by the method of Lowry using serum albumin as the standard. [12]

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Statistics

The ANOVA one-way test was used to determine statistical significance. A P value of less than 0.05 was considered to be statistically significant.

RESULTS

Histology and transmission electron microscopy of murine and human melanoma microcirculation in tumor xenografts

5×10^4 living murine B16-BL6 melanoma cells were injected subcutaneously in C57BL/6 syngeneic mice and tumors were collected after 10, 14, 17 and 19 days (Figure 1). When the tumor mass increased, necrosis increased and microvessels density decreased (Table 1). In particular, there are areas without necrosis and apparently without endothelium but containing blood cells at all the time (Figure1, B). Accordingly, human tumor xenografts gave similar results (Table 1, Figure 2). Mosaic vessels were seldom detected in human tumor xenografts only (Figure 2, panel B).

Table 1 Microvessel density and necrosis in murine and human tumor xenografts

	Necrosis	Microvessel density
BL6 10 days	＜10%	20.3±4.0
BL6 14 days	10-50%	9.3±3.0
BL6 17 days	＞50%	8.0±1.0
BL6 19 days	＞50%	4.6±0.7
WM115 19 days	＜10%	8.1±1.1

5×10^4 living cells (by tripan blue exclusion) murine BL6 or human WM115 cells were injected subcutaneous into syngeneic mice or NOD-SCID mice for murine and human cell line, respectively. 5-6 animals are used for each group. The tumor mass was immediately fixed in neutral buffered formalin, embedded in paraffin, sectioned and stained with hematoxillin-eosin. Necrosis evaluated morphologically has been expressed as percentage on the total specimen. For microvessel desity, endothelial vessels have been counted at high magnification (×400) with the aid of a grid areas (leica TMP-TRIBUN ICS version) in 10 different fields for each specimen. The values are expressed as mean±S.E.

To characterise better pseudovascular spaces, we used a typical endothelium markers,VE-cadherin, and AQP1 (1, 3) while HMB-45 was used as melanoma marker. The latter is an antigen present in the cytoplasm of neoplastic and immature melanocytic cells, including junctional nevi and malignant melanoma but not in adult differentiated nevocytes and melanocytes[13-15]. Moreover, VE-cadherin was also demonstrated to play a fundamental role in both the formation and maintenance of capillary tubes indicating that it participates in formation of specific cell-cell interactions that are involved in the formation of capillary[16]. As shown in Figure 3, melanoma cells in contact with blood vessels show a strong positive signal for HMB-45. In contrast, endothelial cells lining vascular wall are completely negative (Figure 3). In addition, both endothelial and melanoma cells are VE-cadherin positive cells (Figure 3). Finally, we used another endothelial marker, CD31. As shown in Figure 3, vessels in the soft tissue outside the tumor mass are positive for anti-mouse CD31 (Insert A, Figure 3) while spaces surrounded by melanoma cells are CD31 negative (Figure 3 arrows). Furthermore, using monoclonal anti-human CD31 antibody, pseudovascular spaces lined by human melanoma cells are negative (not shown).

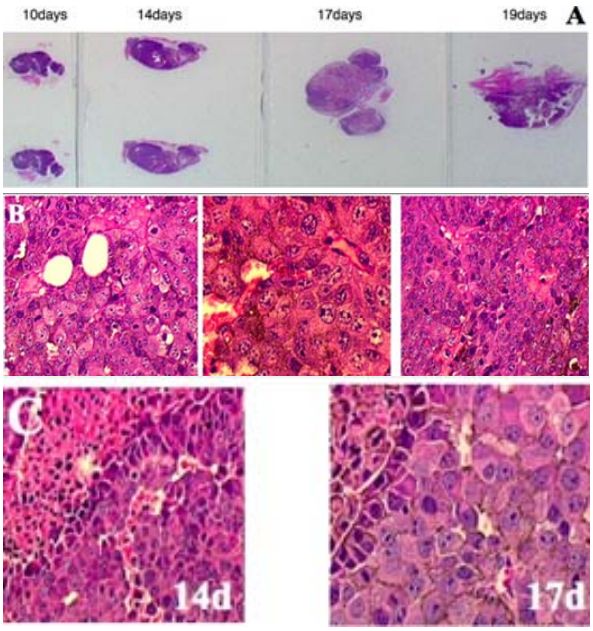

Figure 1 5×10^4 living murine BL6 melanoma cells were injected subcutaneously in C57BL/6 syngeneic mice. Tumors were collected 10, 14, 17 and 19 days after the injection (A). In panel B, three distinct hematoxillin-eosin stained tumors after 10 days are shown (400×). Area without endothelium but with blood vessels are present in all the tumors. Panel C shows the hematoxillin-eosin stained tumor xenografts after 17 and 19 days.

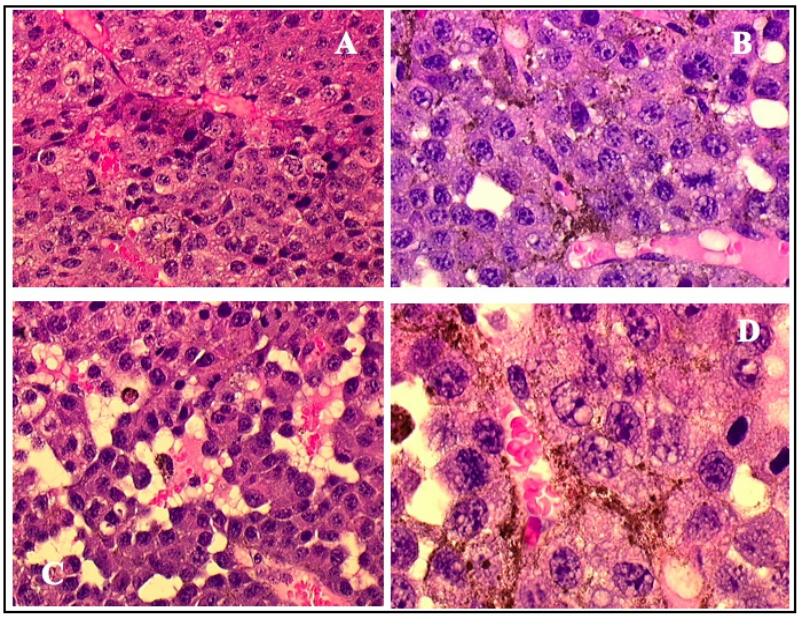

Figure 2 5×10^4 living WM115 cells were injected subcutaneously in immunodeficient mice. Tumors were collected after 19 days and stained with hematoxillin-eosin (four distinct xenograts are shown).Magnification 400×. Panel A-D shows four independent tumors.

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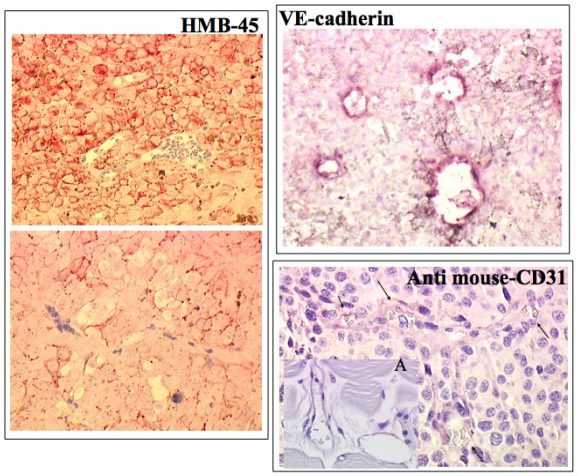

Figure 3 Tumors obtained injecting WM115 living cells (5×10^4) in NOD-SCID mice were collected 19 days after and processed for immunohistochemistry. The slides (4-5μm) were deparaffinised and incubated with anti-human HMB45 (1:50, Dako) or after a tripsin digestion with anti-mouse CD31(1:20, Dako) for 2h at room temperature. Frozen sections (1-2μm) were used immediately and used for VE-cadherin. The latter was incubated with anti-human VE-cadherin (1:50) at 1h at 37°C and then with biotinylated secondary antibody for 30 min (Dako, 1:100) and streptavidin conjugated to peroxidase. The colour was developed using AEC up to 10min for HMB-45 which gives a red colour or VIP (Vector) which gives a purple color for VE-cadherin and CD31. The slides were finally counterstained with Mayer's hematoxillin for 1 min. Magnification, 400×.

Insert A, Positive CD31 endothelium lined vessels in the soft tissue outside the tumor.

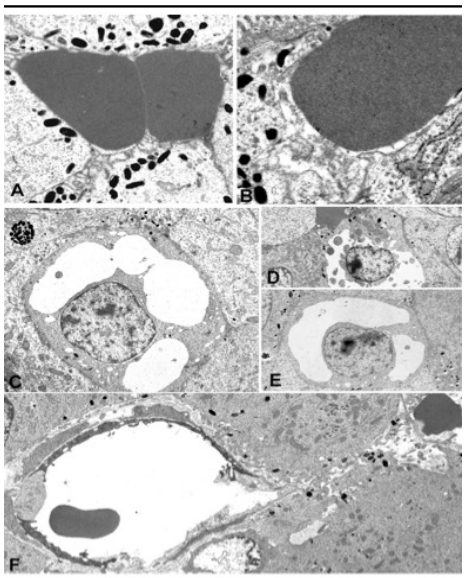

Figure 4 Electron micrographs of vascular channels and blood vessels in murine xenografts. Panel A. A blood vessel (BV) with incomplete and dark endothelium (EN) is close (arrow) to a vascular channel (asterisk, top right) lined by melanoma cells and containing an erythrocyte (E); 13 days after injection; original magnification 3 000 X. Panel B. Two erythrocytes (E) are trapped in a vascular channel lined by melanoma cells; arrows point to electron dense material lining the membranes of melanocytes. 13 days after injection; original magnification 6 500×. Panel C. Higher magnification of an erythrocyte (E) in a vascular channel lined by melanoma cells and containing electron dense material (asterisk). 10 days after injection; original magnification 10 000×. Panels E, F, G. Electron micrographs showing melanoma cells with cytoplasmic vacuoles (asterisks) at 10 (D) and 14 (E, F) days after injection; N, nuclei; original magnification 5 000× (D) and 3 000× (E, F).

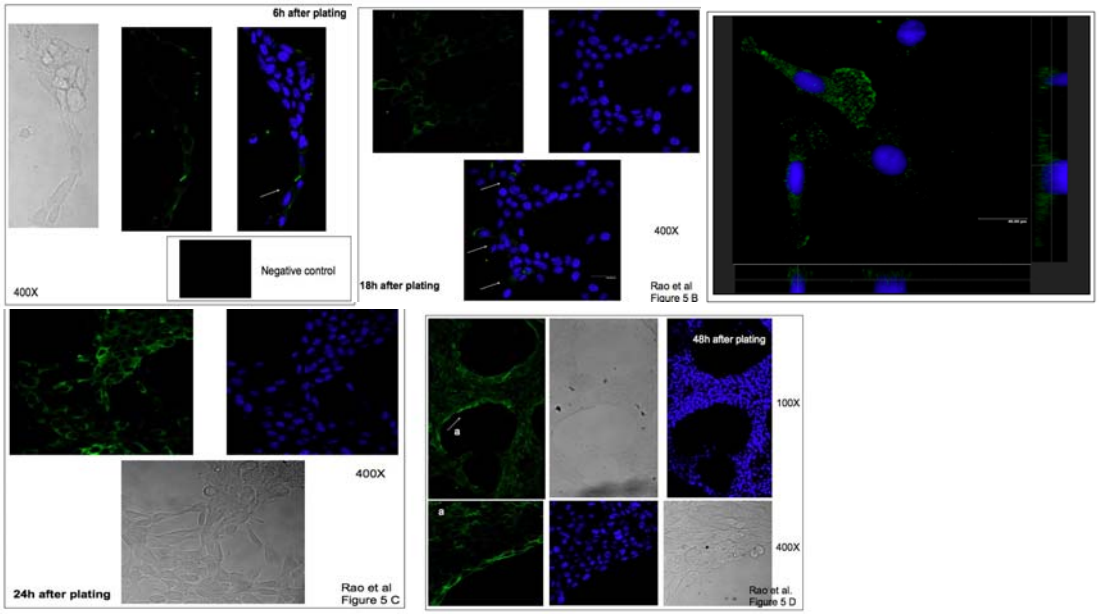

Figure 5 Immunofluorescence of AQP1 in WM115 organising cord like structures (from 6 to 48 hours, panels A-D). 80000/35 cm^2 cells were plated and fixed 48 hours later. The cells were fixed in 4% paraformaldeide for 20min. Incubated with 0.5%Triton X-100 for 5min and with anti-AQP1 overnight (1:100). Nuclei were stained with DAPI. In panel E is shown immunofluorescence of AQP1 in WM115 cells grown in collagen coated plate. The cells were fixed in 4% paraformaldeide for 20min. Incubated with 0.5%Triton X-100 for 5min and with anti-AQP1 overnight (1:100). Nuclei were stained with DAPI.

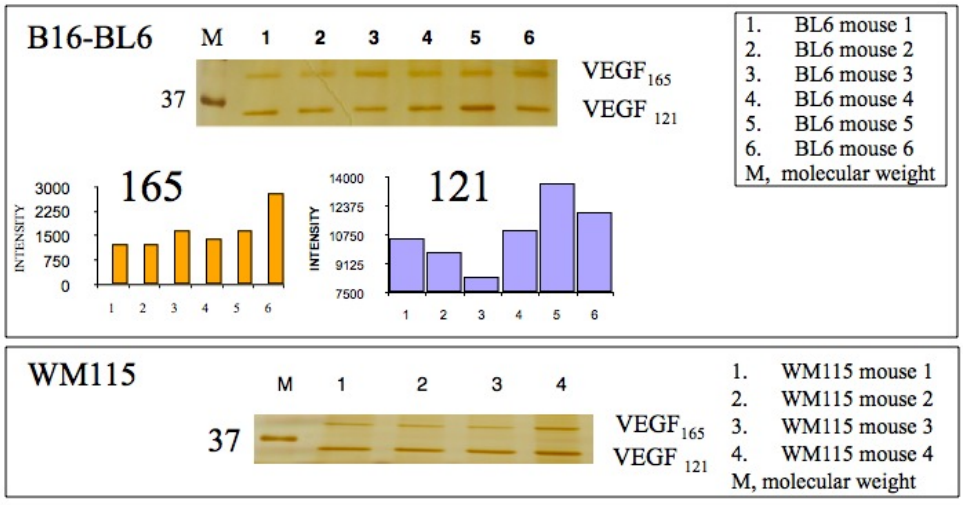

Figure 6 Immunoprecipitation of VEGF in WM115 cells and tumor xenografts. VEGF was immunoprecipitated by 500µg (anti-VEGF, 2µg/ml, Santa Cruz) protein obtained by murine BL6 and human WM115 melanoma cells. Densitometric analyses (OD × mm^2) of both bands are shown.

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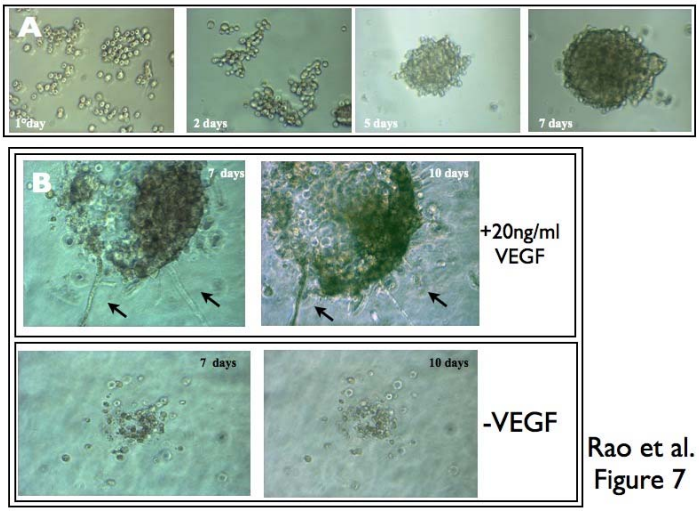

Figure 7 Human melanoma spheroids.Panel A. 30 000 cells were plated on 24 non adherent multiwell for 1-7 days. Panel B. 30 000 cells were plated on pre-treated collagen 24 multiwell in the presence of VEGF (20ng/ml) or without in the basal medium without serum. 7 days later they growth as spheroids. The same experiments were carried out on plate were pre-treated with 40µl of collagen solution (2mg/ml collagen stock solution extract from tail vein) with or without VEGF for 7/10 days. The cells were then photographed every day up to 10 days.

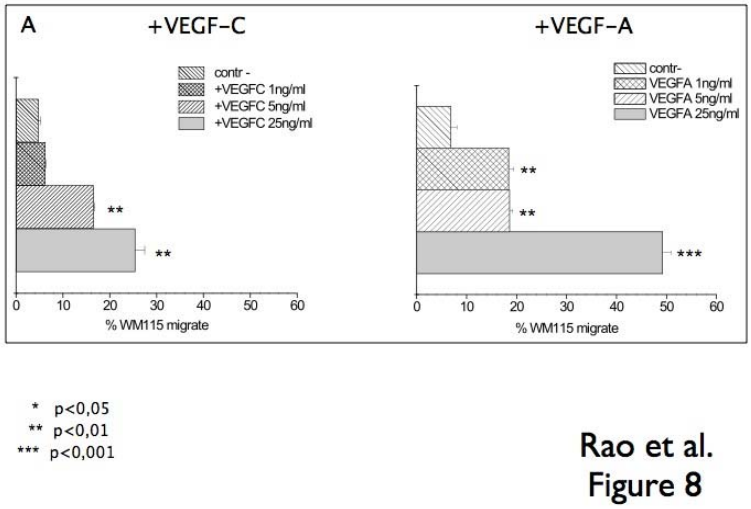

Figure 8 Effect of VEGF-A or VEGF-C on WM115 migration capability.2×10^5 cells were plated on the upper chamber of transwell inserts and maintained in serum-free medium while at the lower chamber serum-free medium plus VEGF-A or VEGF-C was added at the different concentration (chemoattractant) or no serum (negative control). Cells were incubated for 6 h at 37°C in 5% CO_2. Migrated cells remaining on the bottom surface were stained for haematoxylin. The migrated cells were counted in 20 random high-power microscope fields (×200). In the histograms are reported the mean ± SD of the percentage of cells migrate (**P<0,01; ***P<0,001) versus negative control.

Ultrastructural analysis of B16-BL6 syngeneic tumor shows vascular channels not lined by endothelium but directly by tumor cells containing melanosomes and premelanosomes (Figure 4). In particular, melanoma cells are associated with these vascular channels situated on the outer surface of the tubular wall and defined by electrondense material forming a basal lamina (Figure 4, A). In addition, endothelium of blood vessels is often incomplete and very close to small vascular channels containing erythrocytes (Figure 4 B). In 10-14 days injected tumor xenografts several vacuoles of different size was shown in cytoplasm of melanoma cells (Figure 4 D-F). Similar results were obtained for human melanoma xenografts (not shown).

Cord-like Networks and AQP 1 Expression

Since our group demonstrated recently that silencing AQP1, the cytoskeleton organisation was affected through Lin7/β-catenin pathway, changing, in particular, the migration capability of melanoma cells, [3] we focused, herein, on the possible relationship between AQP1 and cord-like network both in vitro and in vivo. Herein, we show that during the organisation of cord like structures in vitro, few cells are AQP1 positive according to previous results. [4] Moreover it appears to be preferentially localised at the plasma membrane of cells exposed versus the hole during the organisation of a cord-like network in vitro (Figure 5, A-D) and in 3D at the tip of lamellipodium[3] (Figure 5E).

VEGF Expression in Murine and Human Tumor Xenografts

VEGF and its receptors Flt-1 and Flk-1/KDR play a major role in angiogenesis as well as in vasculogenesis. [17] Herein, we have investigated the expression of two secreted forms ($VEGF_{121}$ and $VEGF_{165}$.) in murine and human melanoma xenografts. As shown in Figure 7, both isoforms are expressed (Figure 7). Moreover, human melanoma WM115 cells are able to grow as spheroids in non adherent plate but not to sprout (Figure 8, panel A). However, WM115 cells can sprout when they are in collagen coated plate in presence of VEGF only (Figure 8, panel B). Accordingly, as shown in Figure 9, both VEGF-C and VEGF-A are able in vitro to enhance the migration capability of WM115 cells.

DISCUSSION

AQP1 is expressed by human endothelium and melanoma cells and affects the cytoskeleton organisation through Lin7/β-catenin, altering physiological functions such as migration capability. [3] Herein, we used a classical hystological/immunohistochemistry/electron microscopy analysis of mouse and human melanoma grafts in order to investigate if alternative vascular pathways are present independently on the model used (syngeneic or xenotransplantation). In both murine and human melanoma grafts we have shown the presence of alternative vasculogenic pathways AQP1 and VE-cadherin positive. Tumor vasculature in malignant melanoma appears structurally heterogeneous with an irregular diameter, infrequent smooth muscle coat, incomplete basement membrane, and an abnormal perycite coat according to previous papers. [17] Interestingly, VE cadherin and CD31 showed a different expression. VE-cadherin, an adhesive protein promoting homotypic cell to cell interaction[14] which plays a pivotal role in vasculature structure assembly and in the process of vacuolisation or vacuole fusion leading to intercellular lumen formation, [18] expressed by both endothelium and melanoma cells. In contrast, CD31 which is required for cell elongation, migration and/or invasion and for cell-cell association to form the network structures, are expressed by endothelium cells in soft tissue outside the tumor growth. [19] *In vitro*, we followed the organisation of cord-like structures and the localisation of AQP1 positive cells. Our data confirms that only few cells are AQP1 positive[4] and that AQP1 positive cells are preferentially localised to the hole of the cord-like structures. These findings are consistent with the functional role of AQP1 on the cytoskeleton organisation. [3] In fact, the presence of AQP1 at the lamellipodium tip is consistent with its role as bio-mechanic sensor. [3, 4]

It is quite obvious that other factors might be involved in the organisation of pseudovascular spaces. WM115 cells express both receptors of VEGF (Flt1 and Flk1). [9] In tumor xenografts we found that both soluble forms (VEGF165 and VEGF121) are present and both exogenous VEGF-A and VEGF-C are able to enhance the migration capability of these cells. Therefore, VEGF mediated pathway helps the organisation of alternative vasculogenic pathway. This aspect is not surprising since recently Cheng et al demonstrated that under certain conditions, tumors have the potential to feed themselves forming vasculogenic mimicry induced by VEGF-Flt-1[21]. Therefore, the interaction of soluble forms of VEGF with their receptors might trigger intracellular pathways that cooperate with AQP1 to the organisation of a cord like structure. All together our data confirm the interesting possibility to use different drug to target multiple targets like as AQP1 and pro-angiogenic factors like as VEGF.

Conflicting Interest: The authors and peer reviewers report no conflicts of interest.

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