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ORIGINAL PAPER

Anti-angiogenic effects of resveratrol mediated by decreased VEGF and increased TSP1 expression in melanoma-endothelial cell co-culture

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Abstract Resveratrol, a naturally occurring polyphenol, has been reported to be an anti-tumor and chemopreventive agent. Recent data show that it may also exert anti-angiogenic effects. We hypothesized that the anti-angiogenic activity of resveratrol may be caused by modulation of tumor cell release of thrombospondin-1 (TSP1) and vascular endothelial growth factor (VEGF) into the extracellular matrix, leading to vascular endothelial cell (VEC) apoptosis. We therefore evaluated the effects of resveratrol on melanoma cell lines co-cultured with vascular endothelial cells in monolayer and in three dimensional spheroids. We found that resveratrol stimulated isolated VEC proliferation, while it caused growth inhibition of VECs grown with melanoma cells in three-dimensional co-culture. This effect was associated with increased melanoma cell expression of tumor suppressor protein 53 and matrix protein TSP1, as well as decreased hypoxia-driven expression of hypoxia inducible factor-1α and inhibition of VEGF production.

Keywords Angiogenesis · Co-culture · Hypoxia inducible factor-1α (HIF-1α) · Resveratrol · Spheroid · Three-dimensional · Thrombospondin-1 (TSP1) · Tumor supressor protein 53 (p53) · Vascular endothelial growth factor (VEGF)

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Introduction

Angiogenesis, the creation of new blood vessels, is critical for cancer development and progression and is associated with an invasive phenotype and poor prognosis [1, 2]. Angiogenesis is regulated by numerous activators and inhibitors [3, 4]. As malignant cells outgrow their oxygen supply, hypoxia inducible factor-1α (HIF-1α) is stabilized in response to decreased oxygen tension [5, 6]. HIF-1α up-regulates several pro-angiogenic factors [7] such as vascular endothelial growth factor (VEGF) [8, 9]. VEGF promotes angiogenesis by stimulating vascular endothelial cells to migrate towards and into hypoxic zones of the tumor while protecting them from apoptotic death during migration [10, 11]. Increased VEGF expression has also been associated with increased metastases and poorer survival [12–15].

Hypoxia inducible factor-1α has been found to bind to the tumor suppressor protein p53 (p53) [16–18]. Like HIF, p53 is stabilized under hypoxic conditions, although they have somewhat opposing actions with regards to angiogenesis [19, 20]. p53 has been shown to suppress angiogenesis by up-regulating transcription of anti-angiogenic factors, including thrombospondin-1 (TSP1) [21, 22]. TSP1, a natural inhibitor of angiogenesis, counterbalances the effects of VEGF on endothelial cells [23]. TSP1 binding to endothelial cell CD36 receptors, or other receptors such as CD47, leads to downstream apoptosis [24, 25]. We previously established a relationship between p53, TSP1, and angiogenesis in clinical tumor specimens of melanoma, ovary, and prostate [26–28]. Our data demonstrated that the presence of mutant (mt) p53 was significantly associated with loss of wild-type (wt) p53 function manifested by decreased TSP1 expression and increased angiogenesis. Patients with lower TSP1 had

shorter survival. We noted that a large percentage of metastatic melanoma specimens demonstrated p53 accumulation as measured by IHC [26]. This was initially interpreted to be caused by mutations in genomic p53 that lead to a prolonged protein half life [26, 29]. However, it has subsequently come to light that p53 mutations are rare in melanoma, perhaps on the order of 10%, suggesting that protein–protein interactions affected by p53 phosphorylation or acetylation status may be involved in p53 accumulation in melanoma [30–37].

Resveratrol (trans-3,4′,5-trihydroxystilbene) is a polyphenol compound found naturally in grapes, red wine, peanuts, blueberries, cranberries, eucalyptus, and many other plants. It is a phytoalexin and an antioxidant [38, 39]. Resveratrol has been found to inhibit events associated with tumor initiation, promotion, and progression [40, 41]. It has been shown to induce cancer cell apoptosis and/or senescence [42–45]. Recent data suggest that resveratrol exhibits natural anti-angiogenic activity [46–48]. Mechanisms of resveratrol's anti-angiogenic effects include inhibition of hypoxia-induced accumulation of HIF-1α [49, 50] and down-regulation of VEGF [50, 51]. It has also been demonstrated that resveratrol can increase p53 function in a dose-dependent manner in both cancerous and non-cancerous cell lines [52–54].

Although resveratrol has been reported to confer a protective effect against oxidized low-density lipoprotein damage of endothelial cells [55], enhance endothelial cell proliferation at low doses [56], and induce the proliferation and migration of endothelial progenitor cells [57], we hypothesize that resveratrol acts as an antiangiogenic compound indirectly by simultaneously increasing expression of TSP1 and decreasing expression of VEGF in cancer cells, thereby inhibiting angiogenesis through vascular endothelial cell (VEC)-cancer cell and VEC-matrix protein interactions.

In order to interrogate the relevance of such interactions to the effects of antiangiogenic agents, we developed an in vitro model of tumor angiogenesis that incorporates VECs into three-dimensional cancer spheroids [58]. VECs are fluorescently transduced to identify and monitor their migration into tumor spheroids as well as their response to treatment. Whereas numerous models for angiogenesis exist, few allow for the direct analysis of VEC-cancer cell and VEC-stromal interactions. Here we show that resveratrol stimulates VEC growth in monolayer culture but causes a decrease in VEC survival in the three-dimensional tumor co-culture as a result of shifting the ratio between matrix TSP1 and VEGF expression. In this study we report first evidence that resveratrol treatment results in increased levels of TSP1, a downstream target of p53.

Materials and methods

Cell culture

YUZAZ6 (Dr. Halaban, Yale, New Haven CT) and M14 melanoma cell lines were maintained in RPMI 1640 medium (Invitrogen, Carlsbad CA) supplemented with 10% fetal bovine serum (FBS; Gemini Bio-Products West Sacramento CA) and 1% penicillin/streptomycin solution (PS; Invitrogen, Carlsbad CA). A375 melanoma cells (ATCC, Manassas VA) were maintained in Advanced Minimum Essential Medium (AMEM; Invitrogen, Carlsbad CA) supplemented with 2 mM L-glutamine, 10% FBS, and 1% PS. HMVECad (Cascade Biologics, Portland OR), HUVEC, or HAAE1 (both ATCC, Manassas VA) endothelial cells were maintained in F12/K media (Invitrogen, Carlsbad CA) supplemented with 5% FBS, 0.2 μg/ml hydrocortisone (Sigma, St. Louis MO), 5 ng/ml EGF, 0.5 ng/ml VEGF, 10 ng/ml FGF, 20 ng/ml IGF (all from Peprotech, Rocky Hill NJ), 1 μg/ml ascorbic acid (Sigma, St. Louis MO), and 1% PS. Unless otherwise noted, cells were grown under standard normoxic (21% O_2) conditions at 37°C in a 5% CO_2 incubator. Hypoxic conditions were generated in Forma Series II water jacketed incubator (Thermo Fisher Scientific, Marietta, OH) set at 2% O_2, 5% CO_2, and balance N_2.

Fluorescent labeling of endothelial cells

The retroviral vector pBMN-mcherry (Provided as a gift by Dr. Steven George, University of California Irvine, Irvine, CA) was used to transduce HMVECad endothelial cells with RFP for two consecutive days as described previously [59]. Briefly, Phoenix cells (Orbigen, San Diego CA) were transfected for a period of 6 h with pBMN-cherry vector using Lipofectamine 2000 (Invitrogen, Carlsbad CA) per manufacturer's instructions. Viral supernatants were collected at 48 and 72 h, sterile filtered to remove floating cells, mixed with polybrene at a final concentration of 5 μg/ml, and added fresh to HMVECad cells for a period of 6 h. The viral supernatant was replaced with fresh complete EC media overnight and the procedure was repeated on day two. Cells expressing mcherry were sorted by FACSaria (Becton Dickinson, San Jose, CA).

Thrombospondin-1 knockdown

Three Expression Arrest GIPZ lentiviral shRNAmir clones for THBS1 and one non-silencing control (Open Biosystems, Huntsville, AL) were cultured overnight and plasmids were purified using a Plasmid Mini Kit (Qiagen, Valencia, CA). A375 cells were transfected using Arrest-In (Open Biosystems, Huntsville, AL) per manufacturer's

instructions. Initial selection was carried out based on puromycin (InvivoGen, San Diego, CA) resistance and GFP expression. Puromycin resistance was defined as survival after exposure to 0.5 μg/ml puromycin for a period of 5 days. GFP expression was defined as GFP fluorescence intensity 10× greater than auto-fluorescence as determined by FACSaria (BD Biosciences, San Jose, CA). Individual clones were then isolated, grown up, and screened for TSP1 knockdown by ELISA as described below.

Treatment with resveratrol

A 100 mM stock solution of resveratrol (Sigma, St. Louis, MO) was prepared in DMSO and stored as frozen aliquots at −20°C. For treatment of cells, resveratrol was further diluted in appropriate cell culture media.

XTT assay for metabolic activity

Cells were plated into 96 well tissue culture plates at a density of 3,000 cells per well and allowed to attach overnight. Cells were treated with resveratrol for a period of 72 h and metabolic activity was measured using the XTT assay. Briefly, 75 μl of XTT reagent (Sigma, St. Louis, MO) was added to each well already containing 150 μl of media. Plates were incubated for 4 h and optical densities were read at a wavelength of 490 nm with a reference wavelength of 630 nm.

Cell lysates

Cells were lysed using RIPA buffer (Sigma, St. Louis, MO) supplemented with 1% Halt Protease Inhibitor Cocktail with EDTA (Pierce, Rockford, IL) per manufacturer's instructions and 0.2% PMSF (Sigma, St. Louis, MO) and cleared by centrifugation.

Conditioned media experiments

Forty-eight hour conditioned media was collected and syringe filtered through a 0.22 μm PES membrane (Millipore, Billerica, MA) to remove any cells in suspension. Endothelial cells were plated overnight, then two volumes of conditioned media was added to the cells and incubated for an additional 72 h.

Western blotting

Protein bands were resolved on 10–20% Tricine SDS–PAGE gels (Invitrogen, Carlsbad, CA), transferred to PDVF Immobilon-FL membranes (Millipore, Billerica, MA), and blocked for 1 h using a 5% milk solution prepared in phosphate buffered saline (Invitrogen, Carlsbad, CA) supplemented with 0.05% Tween 20 (PBS-T; Sigma, St. Louis, MO), followed by an overnight incubation with primary antibody at 4°C. Antibody dilutions and conditions were as follows: p53 1:500 (Santa Cruz clone DO-1) and TSP1 1:50 (clone 8A6b Novocastra/Leica, Bannockburn, IL). Membranes were incubated with HRP conjugated anti-biotinylated (Cell Signalling, Danvers, MA) and goat anti-mouse IgG:HRP (Stressgen, Ann Arbor, MI) in 5% milk for 1 h at RT and developed using the SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL). Blots were imaged on a Kodak Image Station 2000MM.

ELISAs

VEGF, TSP1, HIF-1α, and p53 levels were measured using DuoSet IC ELISA kits (R&D system, Minneapolis, MN) per manufacturer's instructions.

RT-PCR

Total RNA was extracted from one-third of the pelleted cells using Qiagen RNeasy Mini kit per manufacturer's protocol (Qiagen, Valencia, CA). One microgram of total RNA was reverse transcribed using the Superscript III First Strand kit (Invitrogen, Carlsbad, CA). Reverse transcription was carried out for 3 min at 85°C, 60 min at 42°C, and 10 min at 92°C. Human THBS1 cDNA real-time PCR was performed using an ABI 7000 (Applied Biosystems, Foster City, CA). Approximately 25 ng of reverse-transcribed cDNA per sample was used with a start cycle of 2 min at 50°C and 10 min at 95°C, and 40 cycles of 15 s at 95°C and 1 min at 60°C. THBS1 and GAPDH primers were used with Taqman Universal PCR Mastermix (Applied Biosystems, Foster City, CA) to perform Real-time PCR. Relative quantification (RQ) values were expressed using the formula: ($2^{Ctr - Ctt}$) where $Ctr - Ctt$ represents the difference between delta Ct between the reference sample (untreated control) and tested samples (resveratrol treatment). Delta Ct is the difference between mean Ct values of samples in the target wells and those of endogenous control for the same wells (GAPDH). Values are expressed as fold increases relative to reference sample (untreated control).

Production of spheroids

Serum-free RPMI 1640 medium was prewarmed to 60°C, 125 ml of media was added to 3.0 g of autoclaved methylcellulose (Sigma, St. Louis, MO), and the mixture was stirred continuously for 20 min under aseptic conditions. The methylcellulose was diluted with 125 ml of media at room temperature (RT) and stirred for an additional 2 h at 4°C. The viscous solution was aliquoted into tubes and centrifuged at 2500×*g* at RT for 2 h. The clear, highly

viscous supernatant was removed and stored at 4°C for future use. Methylcellulose was never warmed and re-stored at 4°C. 300,000 cells were re-suspended in 10 ml of media supplemented with 20% methocel stock. A volume of 100 µl of cell suspension was aliquoted into each well of a 96 well round bottom suspension culture plate (Greiner Bio One, Monroe, NC) and incubated at 37°C, 5% CO_2 in a humidified atmosphere. Optimal spheroid formation for some cell lines required modification in methocel concentration or FBS supplementation or a decrease in cell density.

Effect of resveratrol on angiogenesis in vitro

A 1:1 ratio of pBMN-mcherry transduced HMVECad and untransfected or shRNA transfected tumor cells were grown as spheroids for 3 days and subsequently treated with resveratrol for 3 days. At the end of treatment, eight spheroid co-cultures were pooled and disaggregated in 1 ml Accumax (Innovative Cell Technologies, San Deigo, CA). A standard volume of 50 µl of CountBright beads (Invitrogen, Carlsbas, CA) was added to each pooled sample to aid in cell enumeration. After disaggregation, cells were washed once in annexin V binding buffer (140 mM NaCl, 4 mM KCl, 0.75 mM $MgCl_2$, 10 mM HEPES, 1.5 mM $CaCl_2$, pH 7.4). Annexin V binding (BD Biosciences, San Jose, CA) was analyzed per manufacturer's recommendation. Briefly, annexin V-FITC was added to the cell suspension at a 1:20 dilution and incubated for 15 min. Samples were then diluted fivefold with annexin V binding buffer and run by flow cytometry on a FACSaria (BD Biosystems, San Jose, CA). Endothelial cells were distinguished based on their fluorescent label and cells were enumerated based on a ratio of endothelial cells to CountBright beads per sample.

Statistical analysis

GraphPad Instat version 3.0 and Prism version 4.0 (GraphPad software, San Diego, CA) were employed for data analysis. Calcusyn software (Biosoft, Ferguson, MO) was used to determine cell line IC_{50} values. Data are represented as mean values ±SD for three separate experiments. One-tailed paired student's *t*-tests were used to determine statistical significance. *P*-values <0.05 were considered significant.

Results

Effect of resveratrol on cell viability

Melanoma cell lines (A375, M14, and YUZAZ6) were treated with resveratrol for a period of 72 h. All cell lines showed a dose-dependent decrease in metabolic activity as measured by the XTT assay (Fig. 1a). A375 cells were the most sensitive to resveratrol with an IC_{50} dose of 28.79 µM, YUZAZ6 cells had an IC_{50} dose of 36.46 µM, and M14 cells were the most resistant with an IC_{50} dose of 103.44 µM. Resveratrol was not found to cause a dose-dependent dependent decrease in the viability of any of the three endothelial cell lines tested after a 72 h treatment period (Fig. 1b). Resveratrol was found to have a proliferative effect on HAAE1 cells.

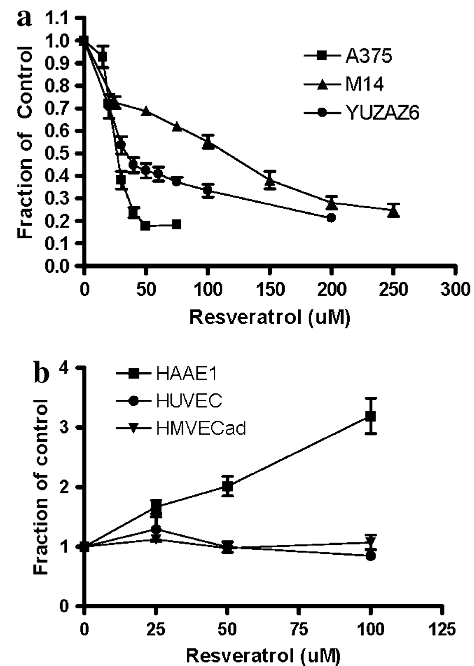

Fig. 1 Effect of resveratrol on cell line viability. Dose response of various melanoma (**a**) or endothelial (**b**) cell lines exposed to resveratrol for 72 h as determined by XTT. Performed in triplicate. *Error bars* represent ±SD

Resveratrol decreased survival of VECs in an in vitro angiogenesis model

Angiogenesis was evaluated as a function of endothelial cell survival in our three-dimensional spheroidal co-culture. M14 cells could not be tested because they did not form uniform spheroids. The WM3211 cell line which formed adequate spheroids was therefore utilized as a third melanoma cell line. Resveratrol treatment (50 µM, 48 h) caused a decrease in endothelial cell viability when grown in co-culture with A375, YUZAZ6, or WM3211 melanoma cells (Fig. 2).

Increased p53 and TSP1 expression after resveratrol treatment

We compared the effects of resveratrol on the YUZAZ6 melanoma line that expresses wt p53 with the M14 line that

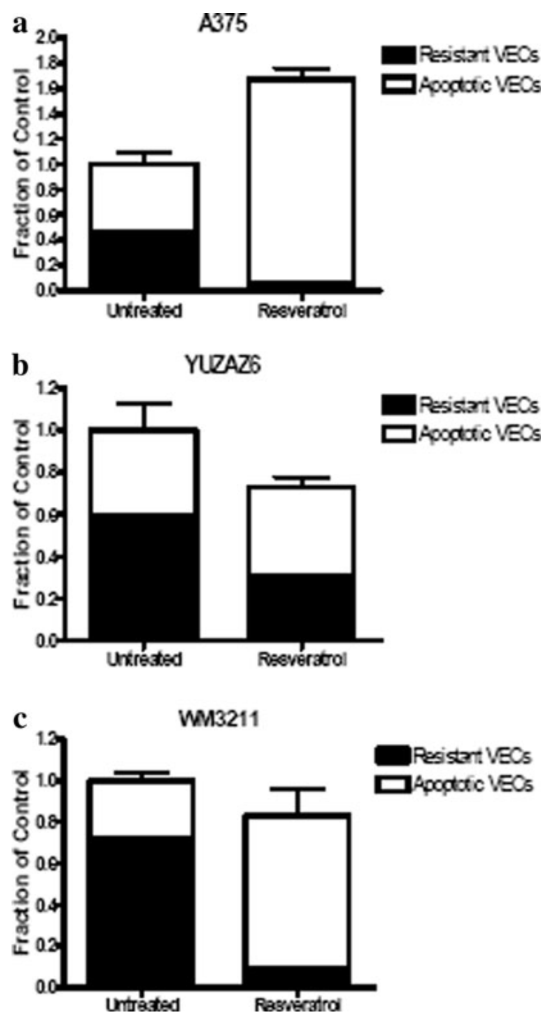

Fig. 2 Effect of resveratrol on HMVEC viability in a three-dimensional co-culture model of tumor angiogenesis, when co-cultured with A375 (**a**) YUZAZ6 (**b**) or WM3211 (**c**) cells, as determined by flow cytometry. Performed in triplicate. *Error bars* represent ±SD

expresses mt p53 [60]. YUZAZ6 and M14 cells were treated with resveratrol and their protein expression assayed by western blot. Both wt and mt p53 were detected by the anti-p53 clone DO-1. Cell lines expressing wt p53 showed a time- and dose- dependent increase in p53 and TSP1 band intensity (Fig. 3). On the other hand, the M14 cell line that expresses mt p53 showed decreased p53 staining intensity but increased TSP1 band intensity. Representative blots of the wild-type p53 melanoma cell line YUZAZ6 (Fig. 3a, b) and mutant p53 melanoma cell line M14 (Fig. 3c, d) are shown. We found this phenomenon to be reproducible by western blot in other melanoma cell lines including SKMEL5 and WM3211 as well as in breast cancer cell lines (data not shown). To show that the increase in TSP1 expression levels occurred at a transcriptional level, we performed RT-PCR on A375 cells treated with resveratrol and compared it to untreated

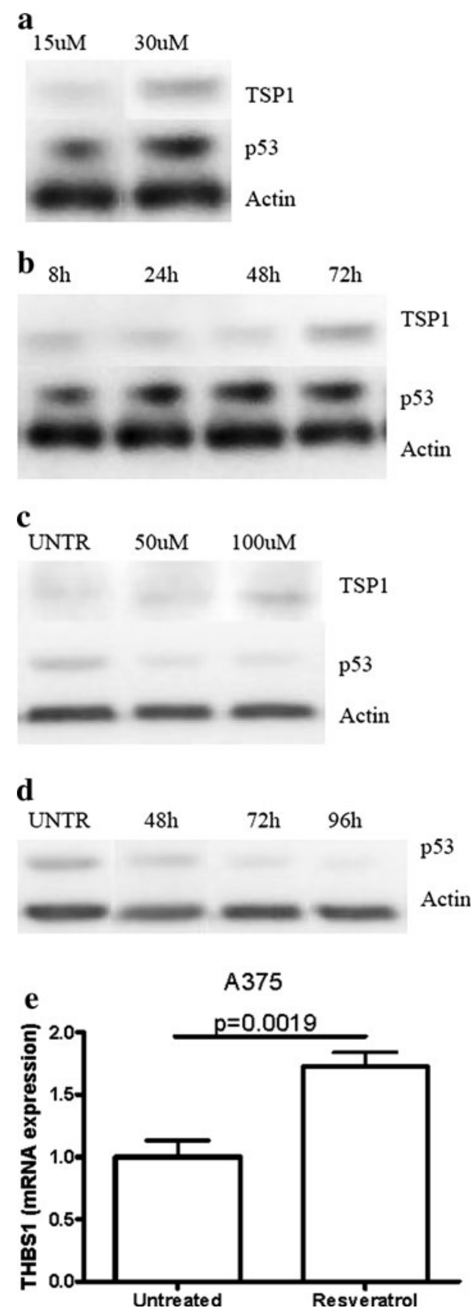

Fig. 3 Effect of resveratrol by western blot. Dose dependent response of YUZAZ6 wild-type p53 line (**a**) or M14 mutant p53 line (**c**) to resveratrol at 72 h. Time dependent response of YUZAZ6 wild-type p53 line to 30 μM resveratrol (**b**) or M14 mutant p53 line to 50 μM resveratrol (**d**). Effect of resveratrol by RT-PCR. Increased thrombospondin expression is caused by a transcriptional increase in THBS1 (**e**). Performed in triplicate. *Error bars* represent ±SD

control cells. We found that treatment with 50 μM resveratrol for 48 h caused a 1.7-fold increase in thrombospondin-1 (gene: THBS1) mRNA expression and was considered significant with a *P*-value of 0.0019 (Fig. 3e).

In order to more accurately assess the changes in p53 levels with resveratrol treatment and the effect of hypoxia

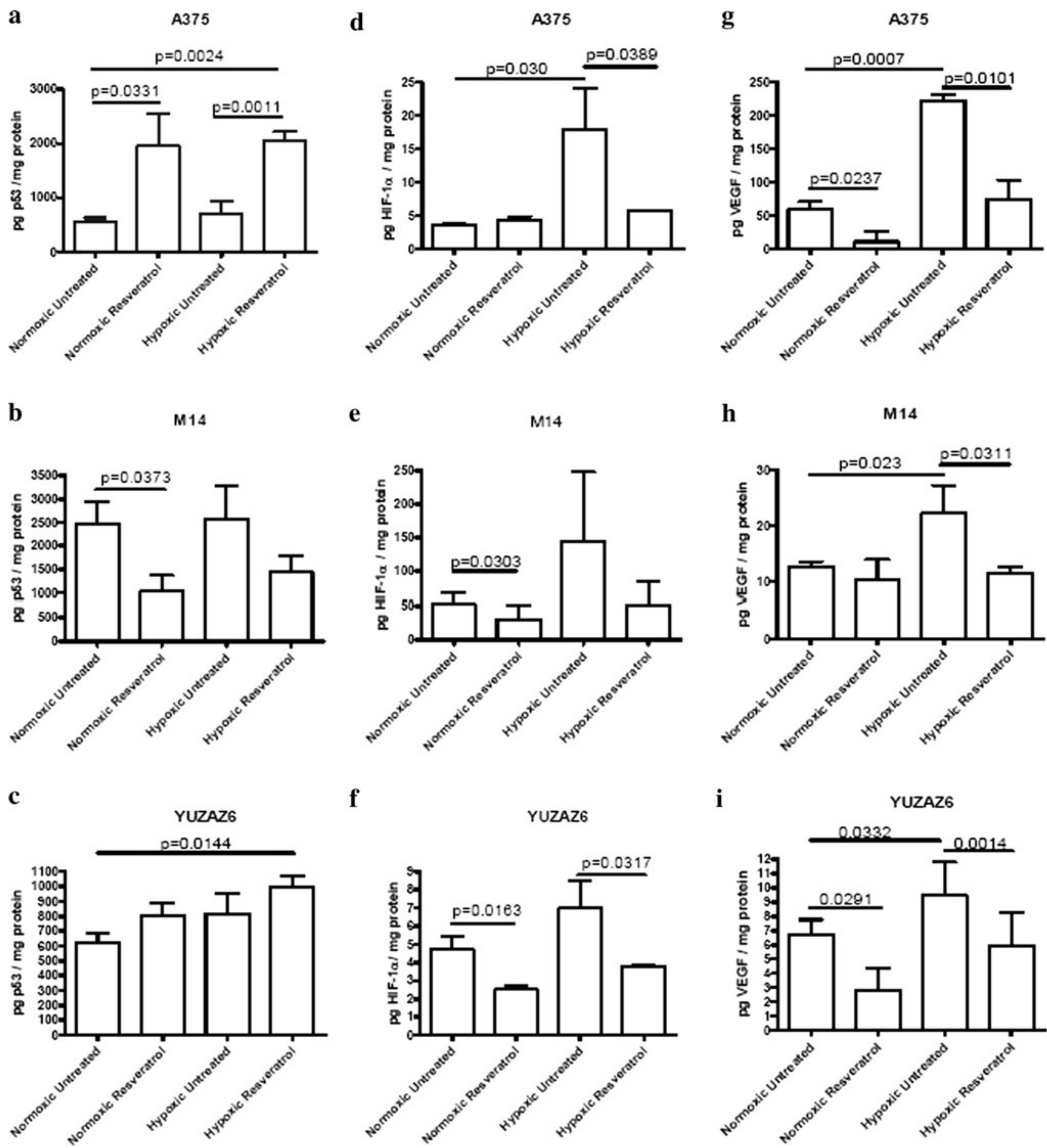

Fig. 4 Effect of resveratrol on expression of p53 (**a–c**), HIF-1α (**d–f**), or VEGF (**g–i**) on normoxic and hypoxic monolayers as determined by ELISA in A375 (**a**, **d**, **g**), M14 (**b**, **e**, **h**), or YUZAZ6 (**c**, **f**, **i**) melanoma cell lines. All cells were lysed after 48 h treatment with resveratrol with the exception of M14 cells that were lysed at 72 h for evaluation of p53 (**b**). Performed in triplicate. *Error bars* represent ±SD

on p53 stabilization, A375, M14, and YUZAZ6 cells grown under both normoxic and hypoxic (2% O_2) conditions were exposed to 50 μM resveratrol for a period of 48 and 72 h and protein expression was determined by ELISA. Both hypoxic conditions and resveratrol resulted in wt p53 accumulation in YUZAZ6 and A375 cell lines, while resveratrol treatment led to decreased mt p53 accumulation in the M14 cell line (Fig. 4a–c). For A375 cells, the degree of resveratrol-induced accumulation of p53 under both normoxic and hypoxic conditions

was considered significant ($P = 0.0331$ and $P = 0.0011$ respectively), while hypoxia-induced accumulation of p53 was not significant ($P > 0.05$). For YUZAZ6 cells, resveratrol-induced or hypoxia-induced accumulation of p53 alone was not significant, but accumulation of p53 induced by resveratrol under hypoxic conditions was considered significant ($P = 0.0144$). For M14 cells, only the resveratrol-induced decrease in p53 accumulation under normoxic conditions was considered significant ($P = 0.0373$).

Inhibition of HIF1 stabilization and VEGF expression by resveratrol

All three cell lines were also assayed for HIF-1α (Fig. 4d–f) and VEGF (Fig. 4g–i) expression after a 48 h period of hypoxia with or without resveratrol. Melanoma cell lines were found to have increased baseline levels of HIF-1α stabilization, likely secondary to ROS production [6]. Hypoxia was found to cause additional stabilization of HIF-1α in all three cell lines, but the degree of HIF-1α accumulation was found to be significant only for the A375 cell line ($P = 0.030$) with a trend toward significance in the other lines (M14, $P = 0.1102$ and YUZAZ6, $P = 0.0948$). Resveratrol significantly inhibited normoxic stabilization of HIF-1α in M14 and YUZAZ6 cell lines ($P = 0.0303$ and $P = 0.0163$ respectively), but did not inhibit the low baseline levels of A375 cell line HIF-1α. Resveratrol also inhibited hypoxia-induced stabilization of HIF-1α in all three cell lines, which was significant for A375 and YUZAZ6 cell lines, with a trend for M14 cells (A375, $P = 0.0389$; YUZAZ6, $P = 0.0317$; M14, $P = 0.0783$).

As expected, hypoxia-induced accumulation of HIF-1α was closely followed by a significant increase in VEGF expression for all cell lines (A375, $P = 0.0007$; M14, $P = 0.023$; YUZAZ6, $P = 0.0332$). Resveratrol down-regulated both hypoxia-induced and normoxic baseline VEGF. This down-regulation was significant under normoxic conditions for A375 ($P = 0.0237$) and YUZAZ6 ($P = 0.0291$) cell lines, and significant under hypoxic conditions for all three cell lines (A375, $P = 0.0101$; M14, $P = 0.0311$; YUZAZ6, $P = 0.0014$). HIF-1α expression was strongly and significantly associated with VEGF levels for all three cell lines (A375: $r = 0.9649$, $P = 0.0351$; M14: $r = 0.9943$, $P = 0.0057$; YUZAZ6: $r = 0.09722$, $P = 0.0228$).

Effect of resveratrol on HIF-1α, VEGF, p53, and TSP1 in melanoma spheroids

A375 and YUZAZ6 cells were grown as spheroids for 72 h and treated with resveratrol for an additional 72 h. M14 cells do not form uniform spheroids using our spheroid-formation method and were not assayed. Spheroids were lysed and assayed for HIF-1α, VEGF, p53, and TSP1 by ELISA (Fig. 5a–h). We found that resveratrol decreased spheroid-related stabilization of HIF-1α in both A375 and YUZAZ6 cell lines. The decrease in HIF-1α stabilization was found to be significant in both cell lines (A375, $P = 0.0004$; YUZAZ6, $P = 0.0112$). Inhibition of HIF-1α stabilization by resveratrol was also associated with decreased VEGF expression; however the 62% decrease in A375 VEGF expression and 14% decrease in YUZAZ6 VEGF expression were of borderline significance (A375, $P = 0.0586$; YUZAZ6, $P = 0.0651$). As in monolayer cultures, resveratrol significantly increased levels of p53 in both of these wt p53 cell lines grown as spheroids (A375, $P = 0.0270$; YUZAZ6, $P = 0.0422$). Resveratrol also

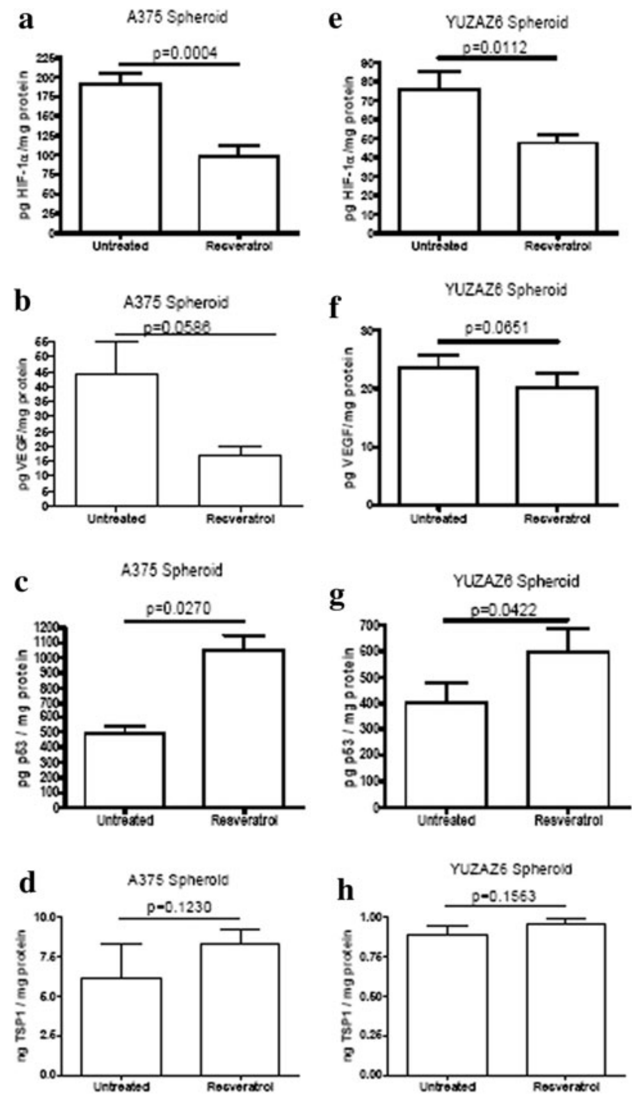

Fig. 5 Effect of resveratrol on expression of HIF-1α (**a**, **e**), VEGF (**b**, **f**), p53 (**c**, **g**), or TSP1 (**d**, **h**) in A375 (**a–d**) or YUZAZ6 (**e–g**) spheroids. Cells were grown as a spheroid for a period of 72 h and treated for an additional 72 h. Protein levels were determined by ELISA. Performed in triplicate. *Error bars* represent ±SD

increased levels of TSP1 in both cell lines, with a trend toward significance (A375, $P = 0.1230$; YUZAZ6, $P = 0.1563$).

The effect of resveratrol on endothelial cells is facilitated by proteins secreted by melanoma cells into the conditioned medium

We set out to demonstrate that the effect of resveratrol on endothelial cells is not direct, but instead facilitated by melanoma cells. Conditioned media from A375 melanoma cells that were untreated or treated with resveratrol for 48 h was collected and added to endothelial cells in monolayer culture. We found that after a period of 72 h, endothelial viability was significantly decreased by 35% ($P = 0.0075$) (Fig. 6a).

Thrombospondin knockdown in melanoma cells decreases the effect of resveratrol on endothelial cells in three-dimensional culture

To show that the effect of resveratrol on endothelial cells in co-culture was caused, in part, by thrombospondin, we eliminated that variable by knocking down THBS1 expression in A375 cells. We determined by ELISA that during these experiments, our non-silenced control cells expressed 9.988 ng TSP1/mg protein whereas our THBS1 silenced cells expressed 2.928 ng TSP1/mg protein. Angiogenesis was evaluated as a function of endothelial cell survival in our three-dimensional spheroidal co-culture. We found that compared to their respective untreated controls, resveratrol-treated endothelial cells co-cultured with cells with knocked-down levels of THBS1 had 3.9-fold better survival than those co-cultured with control transfected cells. This change was considered significant with a P-value of 0.0150 (Fig. 6b).

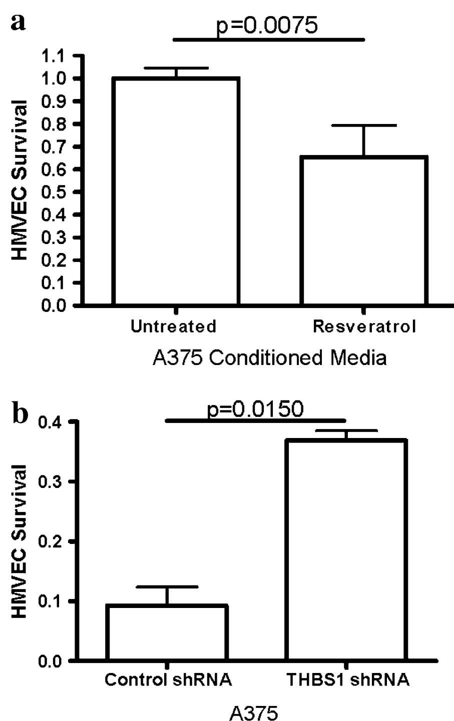

Fig. 6 Effect of media conditioned by A375 melanoma cells treated with resveratrol or untreated on endothelial cell viability in monolayer, as determined by XTT (**a**). Effect of THBS1 knockdown in A375 melanoma cells on HMVEC response to resveratrol in a three-dimensional co-culture model of tumor angiogenesis, as determined by flow cytometry. All data is expressed as a fraction of its respective untreated control. Performed in triplicate. *Error bars* represent ±SD

Discussion

Consistent with the "French Paradox" [38], we found that resveratrol promoted proliferation of endothelial cells in monolayer culture while resveratrol caused apoptosis in HMVECs co-cultured with melanoma cells. Brakenhielm and others have shown that resveratrol inhibits angiogenesis as well as processes dependent on angiogenesis, such as wound healing and tumor growth [46–49, 61]. We hypothesized that this anti-angiogenic activity was caused by resveratrol's modulation of VEC-stroma interactions or more specifically, its induction of secreted matrix protein TSP1 and inhibition of the elaboration of secreted matrix protein VEGF by hypoxic cancer cells. These proteins, when released into tumor stroma affect the delicate balance between endothelial cell growth and death, thereby turning off the angiogenic switch.

It has previously been shown that resveratrol upregulates p53 [52–54] and downregulates both HIF-1α [49, 50] and VEGF [50, 51]. Others have shown that TSP1 is regulated by p53 and that cells with mutant p53 have lower levels of TSP1 expression [26, 61, 62]. Tumor hypoxia is known to stimulate tumor angiogenesis [63, 64]. In addition to studies with normoxic cells, we worked with cells under hypoxic conditions and in spheroids to more closely mimic in vivo conditions. As expected, we found that hypoxia caused a significant increase in HIF-1α levels. We further found that a dose of 50 μM resveratrol for a period of 48 h significantly decreased hypoxia-induced HIF-1α levels in all three cell lines and also significantly decreased baseline HIF-1α levels in the M14 cell line. It has been well established that HIF-1α upregulates VEGF [7–9]. We found that VEGF levels in hypoxic and/or resveratrol treated cells strongly correlated with HIF-1α protein expression levels.

Wt p53 has been shown to be stabilized under hypoxic conditions [19, 20]. It has also been shown to be induced by treatment with resveratrol, most likely as a function of increased phosphorylation at serine 15 [52–54, 65–67]. We

found that wt p53 levels were increased in all cell lines when placed under hypoxic conditions and/or when treated with a dose of 50 μM resveratrol for a period of 48 or 72 h. Resveratrol-induced p53 accumulation under both normoxic and hypoxic conditions was found to be significant in the A375 cell line. The TSP1 promoter has been found to be positively regulated by wild-type p53 [21]. We found that p53 was stabilized by resveratrol (Figs. 3, 4) suggesting the wild-type form may be more active after drug treatment. We also found that bax, another transcriptional target for wild-type p53, was upregulated in melanoma after treatment with resveratrol (data not shown). We therefore fully expected that the effect on TSP1 (Figs. 3a–c, 6d, h) was transcriptional. In fact, we found that treatment with resveratrol caused an increase in THBS1 mRNA expression (Fig. 3e). Resveratrol-mediated changes in p53 and TSP1 expression levels were found to be dose and time dependent.

We believe that resveratrol acts as a multifactorial anti-angiogenic agent by knocking down levels of HIF-1α protein, thereby suppressing HIF related pro-angiogenic factors such as VEGF, and by stabilizing p53 protein levels, leading to increased TSP1. To test this hypothesis, we looked at endothelial cell response to resveratrol in our three-dimensional co-culture model of angiogenesis. We found that resveratrol caused an increase in expression of p53 and TSP1 and a decrease in expression of HIF-1α and VEGF in tumor spheroids. When co-cultured with tumor cells in the spheroid model, resveratrol demonstrated an anti-angiogenic effect on endothelial cells (Fig. 4). Conditioned media from melanoma cells treated with resveratrol also caused a decrease in HMVEC metabolic activity. This further confirms that the anti-angiogenic effect of resveratrol is caused by a resveratrol-induced change in melanoma cell expression of secreted factors. In order to confirm the role of thrombospondin-1, we showed that knock-down of the protein in A375 cells mitigated resveratrol's effect on endothelial cells in co-culture. We expect that the remaining anti-angiogenic effect is caused, at least in part, by a decrease in the secretion of the VEGF survival signal from melanoma cells secondary to decreased HIF-1α.

We believe that it is the resveratrol-induced shift in the TSP1-VEGF balance that is responsible for the change in HMVEC survival that we see in the 3D model after treatment with resveratrol or in monolayer culture after treatment with resveratrol-induced melanoma conditioned media. These data show the importance of matrix interactions and cell–cell interactions in determining VEC response to resveratrol and the value of using angiogenesis models that incorporate a tumor compartment.

Angiogenesis is critical for cancer development and progression. Recent approval of anti-angiogenesis agents for the treatment of colon, breast, lung, and kidney cancer demonstrates the clinical value of this therapeutic modality. In this paper, we showed that resveratrol caused proliferation of endothelial cells in monolayer culture, but acted as an anti-angiogenic agent in a three-dimensional co-culture model of tumor angiogenesis, likely through downregulation of VEGF and upregulation of TSP1. This experimental in vitro system has intrinsic limitations due to the lack of other physiologic process encountered in vivo, potentially limiting its clinical applicability. The limited bioavailability of resveratrol could potentially further limit the clinical applicability of these findings. Bioavailability of unmetabolized resveratrol in humans is relatively low with an average AUC of 1,319 ng h/ml (5.8 h μM) and average maximum concentration of 51.90 ng/ml (227 nM) lasting an average of 1.5 h after a 5 g dose of resveratrol in the form of uncoated immediate-release caplets. On the other hand, a novel resveratrol formulation, SRT501 developed by Sirtris, possesses roughly five times higher bioavailability than native resveratrol [68]. In a phase I trial of oral dosing of 5 g per day for 7 days, SRT501 produced blood levels of approximately 10 μM without toxicity, with AUC values of 44 h μM, comparable to those with demonstrated activity in vitro [69]. In addition, metabolites of resveratrol, such as glucouronide 1 and 2 and 3-sulfate forms are 6.4–23.4× more bioavailable, in terms of AUC, at the same intake dose [70]. It is possible that one or more of these metabolites would have a similar effect as unmetabolized resveratrol. In fact, we have found that trans-resveratrol-3-o-b-d-glucuronide has a similar anti-melanoma effect as unmetabolized resveratrol (data not shown). Patient tolerance for resveratrol is excellent and dose escalation studies of resveratrol that have shown that adverse events caused by resveratrol are nearly non-existent. Further, most pharmacokinetic studies have looked at single doses of resveratrol [70], yet resveratrol can be taken daily and this may increase circulating blood levels. Newer resveratrol dosing formulations appear to reach in vivo exposures comparable to those employed in our study. Cancer trials are currently underway with SRT501. Our findings suggest that measurement of tumor or circulating serum levels of VEGF and TSP1 in cancer patients receiving resveratrol may be worthy of further study as potential biomarkers of resveratrol's anti-angiogenic activity.

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