Effects of Angiogenesis Inhibitors on Vascular Network Formation by Human Endothelial and Melanoma Cells

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Endothelial cells involved in vasculogenesis and angiogenesis are key targets in cancer therapy. Recent evidence suggests that tumor cells can express some genes typically expressed by endothelial cells and form extracellular matrix–rich tubular networks, phenomena known as vasculogenic mimicry. We examined the effects of three angiogenesis inhibitors (i.e., anginex, TNP-470, and endostatin) on vasculogenic mimicry in human melanoma MUM-2B and C8161 cells and compared them with their effects in human endothelial HMEC-1 and HUVEC cells. Anginex, TNP-470, and endostatin markedly inhibited vascular cord and tube formation by HMEC-1 and HUVEC cells in vitro, whereas tubular network formation by MUM-2B and C8161 cells was relatively unaffected. Endothelial cells expressed higher mRNA and protein levels for two putative endostatin receptors, α3 integrin and heparin sulfate proteoglycan 2, than melanoma cells, suggesting a mechanistic basis for the differential response of the two cell types to angiogenesis inhibitors. These findings may contribute to the development of new antivascular therapeutic agents that target both angiogenesis and tumor cell vasculogenic mimicry. [J Natl Cancer Inst 2004;96:1473–77]

Therapeutic strategies that target angiogenesis and vasculogenesis hold great promise in the treatment of cancer (1–7). During the last decade, many new angiogenesis inhibitors have been identified, and several have shown efficacy against tumor growth in vivo (8,9). However, results of early clinical trials with these inhibitors have not yet paralleled the success achieved in animal models (10,11). Although there are several plausible explanations for this incongruity, a key factor may be the heterogeneous composition of growing tumors, which is directly linked to the plasticity of tumor cells. The heterogeneity of the vascular supply to a growing tumor is also a critical factor for tumor survival (12,13). A recent gene expression profiling study revealed that aggressive melanoma cells express genes whose expression is associated with different cellular phenotypes, including genes that are usually expressed by endothelial, epithelial, hematopoietic, or kidney cells; by pericytes; or by several other cell types (14). These results suggest that aggressive melanoma cells might be capable of reverting to an undifferentiated, plastic phenotype.

Additional studies have demonstrated that melanoma cells have a vascular phenotype that is characterized by their ability to form tubular networks when grown in three-dimensional (3-D) culture, concomitant with their expression of genes typically expressed by endothelial cells, phenomena referred to as vasculogenic mimicry (15,16). These observations have prompted us to investigate the potential relevance of a plastic tumor cell phenotype because they challenge our current thinking about how to identify and target tumor cells that might masquerade as endothelial cells or other cell types. Thus, we examined the effects of specific angiogenesis inhibitors on melanoma cells to see if they inhibit vasculogenic mimicry in a manner similar to the way they inhibit endothelial cell–driven angiogenesis.

To test this premise, we treated human endothelial and melanoma cells (seeded at an initial density of 100 000 cells/well on 12-well plates containing 3-D Matrigel or collagen I) with one of three angiogenesis inhibitors of differing specificities—anginex (17), TNP-470 (18), or endostatin (19)—or the aqueous-based vehicle only—for various times and examined the effects of the treatments on the ability of the cells to form vascular cords and tubular networks. We used human metastatic melanoma MUM-2B (uveal) and C8161 (cutaneous) cells, which form tubular structures with lumen(s) and networks when cultured on 3-D collagen matrices (15,16), and human microvascular endothelial cells [HMEC-1; (20)] and human umbilical vein endothelial cells (HUVECs), which form cords and vascular networks when cultured on Matrigel or 3-D collagen matrices (15,16). MUM-2B cells were cloned from a heterogeneous MUM-2 uveal melanoma cell line derived from a liver metastasis (21); C8161 cells were isolated from an abdominal wall metastasis (22). Data were photographically recorded daily; the endothelial cell cultures were morphologically assessed using bright-field microscopy; the melanoma cultures were stained with periodic acid Schiff to visualize tubular networks. Histologic cross-sections of these networks were stained with hematoxylin–eosin to assess luminal integrity. The average total length and mean total number of junctions for treated and control endothelial cords were further analyzed using two-sided Mann–Whitney U tests (17,18).

As shown in Fig. 1 (panels A, D, G, J, and M), HMEC-1 cells treated with each of the three angiogenesis inhibitors had statistically significantly shorter cords and fewer junctions (a measure of the extent of vascular network formation) (23) than HMEC-1 cells treated with the corresponding vehicle. For example, compared with vehicle control, 25 μM anginex and 10 μM anginex decreased mean cord length by 71% (95% confidence interval [CI] = 44% to 98% decrease; P < .001) and 63% (95% CI = 38% to 88% decrease; P = .001), respectively; 25 μM anginex and 10 μM anginex decreased the mean number of
are shown in control was used for all three drugs tested. Representative images from three independent experiments

A vehicle only (D, E, G, and J) Bright-field images of HMEC-1 cultured on 3-D matrices for 3 days in medium containing vehicle only (A, control), anginex (D), TNP-470 (G), or rh endostatin (J). An aqueous-based vehicle-only control was used for all three drugs tested. Representative images from three independent experiments are shown in A, B, and C. B, E, H, and K) Periodic acid Schiff (PAS)-stained images of C8161 cells cultured on 3-D matrices for 6 days in medium containing vehicle only (B, control), anginex (E), TNP-470 (H), or rh endostatin (K). C, F, I, and L) PAS-stained images of MUM-2B cells grown on 3-D matrix for 6 days in medium containing vehicle only (C, control), anginex (F), TNP-470 (I), or rh endostatin (L). (Magnification bar in A is applicable to the images in B–L.) Insets in C, F, I, and L are hematoxylin–eosin-stained cross-sections of the corresponding MUM-2B 3-D cultures. (Magnification bar in panel C inset is applicable to F, I, and L insets.) M) Concentration-dependent effects of the angiogenesis inhibitors on cord formation by HMEC-1 endothelial cells, as determined by measuring the average total cord length (left) as well as the mean number of junctions formed during the assay [right: Rybak et al. (23)] at various drug concentrations. All experiments were performed in triplicate (n = 3 to 8 parameters/test) and analyzed using the two-sided Mann–Whitney U test. Left panel: *, statistically significant differences in the mean total cord length between control and 25 μM anginex (P < .001); 10 μM anginex (P < .001); 100 ng/mL TNP-470 (P = .007); 30 ng/mL TNP-470 (P = .008); 10 ng/mL TNP-470 (P = .038); and 10 μg/mL endostatin (P = .005). Right panel: *, statistically significant differences in the mean total number of junctions between control and 25 μM anginex (P < .001); 10 μM anginex (P < .001); 100 ng/mL TNP-470 (P < .001); 30 ng/mL TNP-470 (P = .011); 10 ng/mL TNP-470 (P = .035); and 10 μg/mL endostatin (P < .001). Error bars correspond to 95% confidence intervals.

By contrast, we observed no statistically significant difference in mean total cord length or in mean total number of junctions in C8161 or MUM-2B melanoma cells treated with these drugs at any concentration and the corresponding cells treated with the vehicle (data not shown). Both anginex and TNP-470 have been described as being cytotoxic for activated endothelial cells in previous studies (17,18); endostatin has been shown to be an inhibitor of endothelial cell migration (19). By comparison, none of the angiogenesis inhibitors, at any concentration, had a statistically significant effect on melanoma cell vasculogenic mimicry, specifically on the ability of melanoma cells to form extracellular matrix–rich vasculogenic-like networks in 3-D matrices, compared with vehicle-treated control cells (Fig. 1, B, C, E, F, H, I, K, and L). Analysis of histologic cross-sections of the networks formed by MUM-2B cells treated with the angiogenesis inhibitors revealed that the morphologic integrity of the tubular structures that contained lumens remained undisrupted compared with that of vehicle-treated control MUM-2B cells (insets of Fig. 1, C, F, I, and L). Thus, these comparative analyses revealed that these angiogenesis inhibitors...
Fig. 2. Effects of anginex (25 μM), TNP-470 (100 ng/mL), and recombinant human (rh) endostatin (10 μg/mL) on cell proliferation, percentage of apoptotic cells, and expression of putative endostatin receptors in human endothelial HUVEC and melanoma C8161 and MUM-2B cells. A) Changes in proliferation of HUVEC, C8161, and MUM-2B cells in response to anginex (TNP-470), and endostatin compared with vehicle-only control cells normalized to 100% as measured by [3H]thymidine incorporation, as previously described (17), and analyzed using two-sided Mann–Whitney U tests. *, statistically significant differences in proliferation between control-treated and anginex-treated HUVEC (P = .002) and C8161 cells (P = .045), and between control-treated and TNP-470–treated HUVEC (P = .003), MUM-2B cells (P = .047), and C8161 cells (P = .016). B) Percentage of apoptotic C8161, MUM-2B, and HUVEC cells in response to anginex, TNP-470, and endostatin compared with vehicle-treated control cells, measured as a subdiploid peak using flow cytometry, was analyzed using the two-sided Mann–Whitney U test. *, statistically significant differences compared with control (anginex: P = .023; endostatin: P = .047). (Error bars correspond to 95% confidence intervals.) C) Semiquantitative reverse transcription–polymerase chain reaction (PCR) demonstrating the relative expression levels of integrin α5-subunit and HSPG2 in untreated HUVEC, C8161, and MUM-2B cells. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were used as controls for PCR amplification, and relative expression levels were quantified using ScionImage Beta 4.0.2 software (Scion Corp., Frederick, MD) normalized against GAPDH expression. Numbers represent relative areas of intensity versus control. D, E) Western blot analysis of α5-integrin subunit (D) and HSPG2 (E) levels in whole-cell lysates of untreated C8161, MUM-2B, HUVEC, and HMEC-1 cells using primary antibodies (Chemicon AB1928 and US Biological H1890–93, respectively) according to the manufacturers’ suggested antibody dilutions, followed by an appropriate horseradish peroxidase–conjugated secondary antibody (Amersham Biosciences, Piscataway, NJ), and enhanced chemiluminescence (SuperSignal West Pico Chemiluminescent Substrate; Pierce, Rockford, IL). A β-actin antibody (MAB1501; Chemicon) was used as a control for equal loading, and relative amounts of protein were determined from digital images obtained using ScionImage normalized against the β-actin controls. Numbers represent relative areas of intensity versus control. F) Mean fluorescence (detected by fluorescence-activated cell sorter analysis of 10^5 events) of HSPG2 (detected by using antibody CBL 497F; Chemicon) in untreated C8161, MUM-2B, HUVEC, and HMEC-1 cells. All experiments (C–F) were performed in duplicate.
crease; \( P = .047 \) (Fig. 2, B). Identical results were obtained with HMEC-1 cells (data not shown). By contrast, neither melanoma cell line showed statistically significant changes in their respective apoptotic indices when treated with any of the three inhibitors compared with vehicle-treated control cells. These data demonstrate that endothelial cells and melanoma cells respond dramatically differently to angiogenesis inhibitors with respect to the induction of apoptosis. Interestingly, previous findings have shown that co-cultures of aggressive vascularogenic melanoma MUM-2B cells and endothelial HUVEC cells result in lysis of endothelia within 48 hours (24), suggesting a complex interaction between these two cell populations. The differential response of endothelial cells and melanoma cells to angiogenesis inhibitors that we report here may provide additional insight into the mechanistic interactions between endothelia and proliferating tumors (and demonstrate dual targets for antivascular drug therapy).

Finally, to address a possible mechanistic basis for the different effects of the inhibitors on endothelial and melanoma cells, we used semiquantitative reverse transcription–polymerase chain reaction, Western blot, and fluorescence-activated cell sorter analyses to examine whether endothelial cells and melanoma cells expressed different levels of the recently reported receptors for endostatin, \( \alpha_\beta_1 \) integrin (25) and heparin sulfate proteoglycan 2 (HSPG2; also known as perlecan, 26,27). We found that endothelial HUVEC cells displayed robust expression of HSPG2 and the integrin \( \alpha_\beta_3 \)-subunit at the mRNA and protein levels (Fig. 2, C–F). By contrast, the melanoma cells expressed only modest levels of integrin \( \alpha_\beta_3 \)-subunit mRNA and protein and barely detectable levels of HSPG2 mRNA and protein. Thus, these data reveal that the receptors for one of the angiogenesis inhibitors tested, endostatin, are differentially expressed in the endothelial and melanoma cells. This observation of vastly different levels of the integrin \( \alpha_\beta_3 \)-subunit and HSPG2 may provide important clues as to the disparate responses of these cell types to antivas-

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**References**


NOTES

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