In Vivo Intracellular Signaling as a Marker of Antiangiogenic Activity

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Abstract

Alterations in endothelial cell (EC) signaling could serve as a marker of effective antiangiogenic therapy. We determined the effect of an antiangiogenic tyrosine kinase inhibitor, SU6668, on tumor EC signaling in liver metastases in mice. In vitro immunofluorescence verified that pretreatment of ECs with SU6668 before exposure to VEGF decreased in vitro phosphorylation of Erk and Akt. Using double-fluorescence immunohistochemistry, phosphorylated Erk and Akt were constitutively expressed in ECs in liver metastases in untreated mice, but SU6668 blocked activation of these signaling intermediates. Determining the activation status of the Erk and Akt signaling pathways in tumor ECs may serve as a surrogate marker for the effectiveness of antiangiogenic regimens.

Introduction

VEGF is the angiogenic factor most closely associated with aggressive disease in numerous solid malignancies (1–5). VEGF is known to induce intracellular signaling in ECs, activating the MAP kinase/Erk and PI-3 kinase/Akt pathways (6–8). These two pathways are prototypical survival pathways in ECs (8–12). It is now well documented that VEGF is not only an angiogenic factor, but is also a survival factor for normal and tumor endothelium (13–16). An important antiangiogenic strategy is to neutralize the activity of VEGF. It may be possible to determine the extent of such therapies by examining EC signaling in biopsy specimens. Tumor angiogenesis is a dynamic process where ECs in different areas of a tumor may undergo different processes (i.e., proliferation and apoptosis) concurrently. Changes in EC intracellular signaling presumably occur before the alterations in the architecture in the tumor microvasculature (15, 17). In addition, alterations in EC signaling may indicate an abrogated mitogenic response to angiogenic factors. In the current study, we sought to determine the feasibility of examining EC signaling as a surrogate marker for antiangiogenic therapy designed to target VEGF. Our goal was to demonstrate that in the presence of angiogenic tyrosine kinase inhibitor, VEGF would be unable to activate the EC signaling pathways that mediate angiogenesis (EC proliferation and/or survival).

Materials and Methods

ECs and Culture Conditions. Neonatal HDMECs (Cascade Biologicals, Inc., Portland, OR) were cultured and maintained in growth-supplemented medium 131 as recommended by the supplier. For immunohistochemical analyses, HDMECs were plated on two chamber slides (Becton Dickinson Labware, Franklin Lakes, NJ) at a concentration of 3 × 10⁵ cells/chamber. Adherent cells were then serum-starved 12 h before treatment in MEM supplemented with 1% fetal bovine serum. For Western blotting, cells were starved overnight in MEM supplemented with 5% fetal bovine serum.

Reagents and Antibodies. SU6668 and SU5416 were provided by Sugen, Inc. (South San Francisco, CA), PD98059 (2-(2′-aminomethyl-3′-methoxyphenyl)-oxanaphthalene-4-one) and wortmannin were purchased from New England Biolabs (Beverly, MA). SU6668 was chosen for use in in vitro studies because it is more soluble than SU5416. hrVEGF was obtained from R&D Systems (Minneapolis, MN). Hoechst dye 3342 MW 615.9 (Hoechst, Warrington, PA) was used to counterstain nuclei, and Prolong (Molecular Probes, Eugene, OR) was used as mounting medium. Antibodies were purchased as follows: polyclonal rabbit antihuman phospho-p44/42 MAP kinase (Erk1/2), polyclonal rabbit anti-p44/42 MAP kinase (Erk1/2), polyclonal rabbit antihuman phospho-Akt (Thr308), and polyclonal rabbit anti-Akt from New England Biolabs and Alexa Fluor 488 goat antirabbit IgG (H+L) and Alexa Fluor 594 goat antimouse IgG (H+L) from Molecular Probes. Antibodies for CD31/PECAM-1 staining were purchased as described previously (15).

Western Blot Hybridization. Cells were pretreated for 30 min with either medium alone (control), PD98059 (50 µM), wortmannin (200 nM), or SU6668 (1 µM) before hrVEGF (20 ng/ml) stimulation for 15 min. Blots were done as described previously (18) using a primary antibody dilution of 1:1000. Relative expression of the phosphorylated forms of the proteins was determined using NIH Image 1.61 software.

Immunofluorescence Double Staining in Colon Cancer Liver Metastases. We used harvested liver metastases from a previous study (15) to develop an in vivo immunohistochemical assay to evaluate Erk and Akt phosphorylation.
tion in the tumor endothelium. The assay uses a double-labeling technique in which ECs in liver metastases are labeled red (anti-CD31 antibody), and cells with activated signaling intermediates are labeled green (anti-pErk or anti-pAkt antibody). Colocalization of both colors leads to yellow, which represents ECs in which the Erk or Akt pathway has been activated. As described in a previous report, mice that had been implanted with human colon cancer cells were given daily 200-μl i.p. injections of either control vehicle [30% PEG-300 (w/v) in 0.1 M sodium phosphate buffer (pH 8.2), SU6668 (60 mg/kg in control vehicle), or SU5416 (12mg/kg in control vehicle)]. Specimens from seven mice/treatment group were analyzed. We examined viable ECs that did not have the morphology of apoptotic ECs. Tissue preparation and CD31/PECAM stains were done as described previously (15). The second primary antibody (anti-phospho-Erk, 1:100; or anti-phospho-Akt 1:400) was added and incubated overnight at 4°C. The second primary antibody was removed by rinsing with TBS, the second secondary antibody (fluorescent green-conjugated; 1:400) was added, and the sections were incubated for 1 h at room temperature. Finally, the sections were washed with TBS for 30 min, the nuclei were counterstained, and the sections were mounted for microscopic analysis. The double-staining procedure was repeated on four separate occasions with reproducible results.

Results

In Vitro Activation of Erk and Akt in HDMECs as Determined by Western Blotting. We pretreated cells with rhVEGF in the presence or absence of the MAP kinase inhibitor PD98059, the PI-3

Fig. 1. Effect of VEGF on signaling pathways in ECs. Blockade of MAP kinase, PI-3 kinase, or VEGFR2 blocks the activation of Erk and Akt by VEGF. HDMECs grown in vitro were serum-starved overnight and then stimulated with VEGF. Erk and Akt phosphorylation increased after treatment with VEGF. However, blockade of MAP kinase [with PD98059 (PD; 50 μM)] or PI-3 kinase [with wortmannin (WT; 200 nM)] blocked the activity of Erk and Akt, respectively, after VEGF stimulation. Treating the HDMECs with SU6668 (a tyrosine-kinase inhibitor of the VEGFR2) likewise prevented the induction of Erk and Akt phosphorylation by VEGF.

Fig. 2. Immunofluorescent evaluation of signaling pathways in HDMECs. A. column 1, HDMECs stained for phosphorylated Erk, which appears green; column 2, HDMECs stained for CD31 (red) and pErk (green) that, when superimposed, appear yellow. Treatment conditions are shown at left. SU6668 effectively decreased the phosphorylation of Erk in the absence (Control) or presence of VEGF stimulation. B. column 1, cells stained for phosphorylated Akt (green); column 2, staining for CD31 (red) and pAkt (green), which, superimposed, appear yellow. Treatment conditions are shown at right. SU6668 significantly inhibited phosphorylated Akt in the absence (Control) or presence of VEGF stimulation.
Panel A

Fig. 3. Immunofluorescent evaluation of EC signaling pathways in human colon cancer liver metastases in nude mice. A, row 1, CD31-positive ECs (red); row 2, pErk-positive cells (green); row 3, an overlay of rows 1 and 2, where yellow fluorescence indicates pErk-positive ECs. Representative sections demonstrate a significant decrease in Erk phosphorylation in SU6668-treated animals relative to those in the control group. B, row 1, CD31-positive ECs (red); row 2, pAkt-positive cells (green); row 3, an overlay of rows 1 and 2, where yellow fluorescence indicates pAkt-positive ECs. Representative sections demonstrate a significant decrease in pAkt in SU6668-treated animals relative to those in the control group.

Discussion

In this report, we assessed whether markers of intracellular endothelial signaling could be used to measure the effectiveness of antiangiogenic therapy targeting the VEGF system receptor. Results from our in vitro model verified that VEGF activated the MAP kinase/Erk and PI-3 kinase/Akt pathways in HDMECs. We also showed that SU5416 and SU6668 inhibited EC signaling in liver metastases. The tumor tissue for our in vivo experiment came from a previous study of ours in which mice were implanted with human colon cancer cells in the spleen, leading to liver metastases (15). In that study, two tyrosine-kinase inhibitors were examined; one targeting the receptor for VEGF (SU5416) and the other targeting the receptors for VEGF, platelet-derived growth factor, and fibroblast growth factor (SU6668). Both were found to inhibit tumor angiogenesis and growth and to induce apoptosis of ECs and tumor cells. These results, in combination with those of the present study, demonstrate that antiangiogenic
therapies that target VEGF in this murine model significantly increase EC apoptosis and decrease EC signaling. VEGF protects ECs from apoptosis in vivo by activating appropriate intracellular survival pathways. The activation of these pathways can be documented by immunohistochemistry and could potentially serve as a surrogate marker of the effectiveness of antiangiogenic therapies.

We theorized that VEGF-directed antiangiogenic therapies would block important EC signaling cascades, and that this would serve as early surrogate markers of their effectiveness. To prove this hypothesis, we sequentially devised in vitro and in vivo models. Using Western blotting, pretreating the HDMEC with PD98059 effectively inhibited the activation of Erk after VEGF stimulation. Wortmannin prevented activation of Akt and, to some extent, activation of Erk. This is a finding consistent with previous studies demonstrating that wortmannin inhibits VEGF-induced Erk activation (in HUVECs) in a dose-dependent manner (8) and implicating PI-3 kinase as a modulator of MAP kinase activation by VEGF. Once we confirmed that VEGF induced activation of the signaling pathways expected, we then proceeded with the development of an in vitro immunohistochemical technique to determine the feasibility of examining signaling activation ECs, in situ. In this in vitro assay, we used immunohistochemical techniques to assess VEGF-stimulated HDMECs pretreated with specific inhibitors of signaling molecules. In the absence of VEGF stimulation, SU6668 treatment led to a decrease in the basal level of activated Erk; in the presence of VEGF, SU6668 produced an almost complete abrogation of Erk activation. Pretreatment of HDMECs with SU6668 also led to complete abrogation of any basal or stimulated levels of activated Akt in EC in vitro. In previous studies (11), VEGF was proven to ameliorate the time-dependent increase in apoptosis of serum-starved HDMECs through activation of the MAP kinase/Erk pathway together with inhibition of stress-activated protein kinase/c-jun-NH2-kinase. Other reports found that VEGF acts as a survival factor for HUVECs through its effects on the VEGFR2 (Flk-1/KDR) leading to the activation of the PI-3 kinase/Akt signaling pathway, which in turn plays a critical role in preventing HUVEC apoptosis (8–10, 19).

For our in vivo model, we used harvested liver metastases from a previous study of colon cancer metastasis in a murine model and examined viable ECs that did not have the morphology of apoptotic ECs. The antiangiogenic agents SU6668 and SU5416 block important signaling cascades in tumor ECs. The altered signaling events observed after treatments are likely to be upstream and earlier mediators of alterations in either EC proliferation (Erk), survival (Akt), or both. SU6668 antiangiogenic effects could arise from any or all of the potential receptor systems targeted by the compound. SU6668 treatment effects on ECs and tumor cells were similar to the effects seen with SU5416 treatments, pointing to VEGF as the main angiogenic and EC survival factor in this murine model.

In summary, our results demonstrate that antiangiogenic therapies that target VEGF, in this murine model, significantly increase EC apoptosis and decrease EC signaling. VEGF protects ECs from apoptosis by activating appropriate intracellular survival pathways acting in vivo as a tumor EC survival factor and an angiogenic factor. The inhibition of activation of these pathways after antiangiogenic therapies can be documented by immunohistochemistry and could potentially serve as a surrogate marker of the effectiveness of these therapies. Additional studies are necessary to confirm these results in human tissues.

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References

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