Hyperthermia Inhibits Angiogenesis by a Plasminogen Activator Inhibitor 1-dependent Mechanism

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ABSTRACT

Hyperthermia (HT) associated with radiotherapy or chemotherapy is a promising method for cancer treatment, although the molecular mechanisms of this process are not well understood. HT exhibits various anti-tumor effects, including damage of tumor vasculature. Here, we investigate the effect of HT on in vitro and in vivo angiogenesis. We show that heat treatment of endothelial cells (ECs) affect their differentiation into capillary-like structures in two models of in vitro angiogenesis. Furthermore, the formation of new vessels promoted by angiogenic inducers in the chick embryo chorioallantoic membrane assay is impaired after heat treatment. These effects cannot be explained by direct cytotoxicity but are dependent on modulation of angiogenesis-involved genes. Gene expression profile of ECs subjected to heat shock demonstrates that plasminogen activator inhibitor 1 (PAI-1), a protein involved in the control of extracellular matrix degradation, is specifically up-regulated. The use of anti-PAI-1-neutralizing antibodies reverses the effect of HT on the in vitro EC morphogenesis and in vivo vessel formation. Moreover, microvesSEL outgrowth from PAI-1−/− aortic rings was not affected by HT compared with aortic rings from PAI-1+/+ mice. Heat treatment of murine mammary adenocarcinoma results in inhibition of tumor growth, associated with a reduction of microvesSEL number and an increase of PAI-1 expression. These results indicate that heat-mediated PAI-1 induction is an important pathway by which HT exerts its antitumor activity and may represent a rationale for a combined cancer therapy based on HT associated with antiangiogenic molecules.

INTRODUCTION

Radiotherapy is an important standard cancer treatment for local and regional disease. The importance of improving radiotherapy is additionally highlighted by the fact that ~20–30% of cancer deaths are attributable to local/regional failures, and 50–60% of cancer patients die with local tumors as a component of ineffective treatment (1). Recent advances in radiotherapy are attributed to combination of radiotherapy with antineoplastic drugs and other physical treatments such as HT (2, 3). HT acquires a clinical therapeutic effect when such as HT3 (2, 3). HT acquires a clinical therapeutic effect when heat-mediated PAI-1 induction is an important pathway by which HT exerts its antitumor activity and may represent a rationale for a combined cancer therapy based on HT associated with antiangiogenic molecules.

Cell Cultures and Heat Treatment. Human ECs were prepared from umbilical cord veins, characterized and grown as described previously (26).

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Rossi, Istituto Mario Negri Sud, Lanciano, Italy) were cultured in DMEM HEPES modified (BioWhittaker, Europe, Verviers, Belgium), supplemented with 10% FCS.

For heat treatment, cultured ECs were added with the appropriate pre-warmed medium and then incubated at 39°C, 41°C, 43°C, and 45°C for 1 h in a CO2 incubator calibrated to ±0.1°C. The temperature at the cell surface within the culture plates was measured with a microthermocouple (Model HH21 microprocessor thermometer, type T, Omega). Equilibration from 37°C to 43°C was attained within 14 ± 3 min (n = 20). To test if heat treatment could denature VEGF-A165 protein (R&D Systems, Wiesbaden-Nordenstadt, Germany), we performed some preliminary experiments with a prewarmed gelatin (Sigma-Aldrich, Milano, Italy), a water-soluble tetrazolium salt, into a water-insoluble formazan product (28). After addition of MTT (0.2 mg/ml), incubation at 37°C for 2 h, and solubilization of the formazan product with DMSO (Sigma-Aldrich), absorbance was measured by spectrophotometry at 595 nm, using a microplate reader (Perkin-Elmer, Norwalk, CT).

Cell proliferation was measured by using the Biotrak cell proliferation ELISA system (Amersham Life Science, Little Chalfont Buckinghamshire, United Kingdom) according to the manufacturer’s instruction. Briefly, ECs were seeded at 1.0 × 105 cells/well in a 48-well plate in Medium199 10% FCS and 20 ng/ml VEGF-A165. After 60 min at 37°C, ECs were incubated at different temperatures (39°C, 41°C, 43°C, and 45°C) for 1 h, and cell viability was determined immediately and 16 h after heating by measuring the ability of cells to metabolize MTT (Sigma-Aldrich, Milano, Italy), a water-soluble tetrazolium salt, into a water-insoluble formazan product (28). After addition of MTT (0.2 mg/ml), incubation at 37°C for 2 h, and solubilization of the formazan product with DMSO (Sigma-Aldrich), absorbance was measured by spectrophotometry at 595 nm, using a microplate reader (Perkin-Elmer, Norwalk, CT).

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In Vitro Matrigel Assay. Matrigel (150 μl; BD Biosciences, Bedford, MA) was added to each well of a 48-well plate and incubated at 37°C to allow gel formation (29). ECs were plated on Matrigel at 2.5 × 104 cell/well in Medium199 10% FCS. Cells were allowed to adhere to the gel coating for 60 min at 37°C and then were incubated for 1 h at different temperatures (39°C, 41°C, 43°C, and 45°C). Incubation was then continued for 16 h at 37°C and cells photographed. In some experiments, 2, 5, 10 μg/ml of the neutralizing anti-PAI-1 antibody MA-33H1F7 or of the MA-31C9 antibody (30) were added to the medium.

Capillary-like structures were quantitated (31) by automatic counting in triplicate of low power fields (∼40) using the ImageProPlus 4.0 imaging software (Media Cybernetics, Silver Spring, MD), and percentage inhibition was expressed as untreated differentiated ECs at 100%.

Endothelial Spheroid Assay. EC spheroids were generated as described previously (32). The spheroid-containing gels were heated at 43°C for 1 h and then incubated at 37°C for 16 h. In some experiments, 2, 5, 10 μg/ml of neutralizing anti-PAI-1 antibody MA-33H1F7 or of the MA-31C9 antibody were added to the medium. Capillary-like sprouts were quantitated by automatic measurement of the cumulative length of all sprouts originating from the central plain of an individual spheroid using the ImageProPlus 4.0 imaging software (33).

Western Blot Analysis. Total cellular proteins were extracted from ECs maintained at 37°C and from heat-treated ECs (1 h 43°C) by adding Laemmi Buffer [62.5 mm Tris-HCl (pH 6.8), 2% w/v SDS, 10% glycerol, 50 mM DTT, and 0.1% w/v bromphenol blue], separated by SDS-PAGE (10%), transferred to nitrocellulose membranes, blocked with 3% BSA in Tris-buffered saline, and incubated with primary antibodies as described previously (34). Horseradish peroxidase-conjugated secondary antibodies and chemiluminescence with Western Lighting system (Perkin-Elmer Life Sciences, Inc., Boston, MA).

CAM Assay. Chick embryo CAM assay was performed as described previously (35). Gelatin gels (Gelfoam; Upjohn Co., Kalamazoo, MI), adsorbed with 40 ng of VEGF-A165 or PBS as control, were implanted on the top of the growing CAMs. In selected experiments, eggs were treated by injecting CAM with 40 μg of MA-33H1F7 or MA-33B8 or MA-31C9 anti-PAI-1 antibodies. Eggs were subsequently incubated in a 37°C or in a 43°C incubator for 2 h. The temperature inside the eggs were measured with a microthermocouple, and equilibrium from 37°C to 43°C was attained within 52 ± 4 min. CAMs were examined daily until day 12 and photographed in ovo under an Olympus stereomicroscope SZX9. The number of vessels was quantified with ImageProPlus 4.0 imaging software in three randomly selected areas (1 mm2) as described previously (36).

Aortic Rings Assay. Angiogenesis was studied by culturing rings of mouse aorta in Matrigel gel (37). Thoracic aortas were removed from PAI1+/− and PAI1−/− C57B1/6 mice and immediately transferred to a dish containing ice-cold serum-free DMEM. One mm-long aortic rings were sectioned and rinsed extensively in five consecutive washes with DMEM. Plates were coated with 150 μl of Matrigel; after gelling, the rings were placed in the wells, sealed in place with an overlay of 100 μl of Matrigel and covered with 250 μl of Medium199 containing 20% FCS. Embded aortic rings were cultured for 2 days, treated for 1 h at 43°C, and then replaced at 37°C. After an additional 4 days of culture, phase contrast images were captured by using a cooled digital charged couple device Hamamatsu ORCA camera (Hamamatsu Photonics Italia, Aree, Italy).

cDNA Microarray Hybridization and Northern Blot Analysis. Total cellular RNA was extracted from control and heat-treated ECs by the RNAzol (Tel-Test, Inc., Friendswood, TX) method, according to the manufacturer’s instruction. cDNA probe synthesis was performed following procedures in Atlas cDNA Expression Array User manual. The resulting 32P-cDNA probes were hybridized to the Atlas Human Cancer 1.2 Array (Clontech, Palo Alto, CA). For Northern blot analysis, RNA hybridization was carried out overnight with 2 μg of [α-32P]dCTP-labeled (3000 Ci/mmol; Amersham Life Science) human PAI-1 probe (a 1.0-Kb BglI-BglI fragment of PAI-1). Both Northern blot membranes and arrays were exposed to phosphor image screens for 16 h. Images were acquired by using a Cyclone Phosphor System (Packard, Meriden, CT). Arrays were analyzed and normalized according to the manufacturer’s instructions.

In Vivo Tumor Model and Immunohistochemistry. Subconfluent cultures of TSA mammary adenocarcinoma or 3LL Lewis lung carcinoma cell lines were harvested, resuspended in a sterile solution of PBS, and cells (5 × 105) were injected respectively s.c. into the left hind foot of BALB/c (Charles River Laboratory) or PAI-1+/− and PAI-1−/− C57B1/6 mice. Tumors were measured with a caliper, and the volumes were calculated according to the formula: width2 × length × 0.52. When tumors reached a volume of 90–100 mm3, mice legs were heated for 1 h at 43°C in a modified water bath. In our previous experiments, it had been established that intratumor temperature stabilized at 0.2°C below the water temperature within a maximum of 2 min (38). The fractional tumor volume indicates the ratio between the volume recorded after 7 days from the treatment (V) and that at the day of HT (V0) (39). For histological analysis, tumors were resected, fixed with 4% buffered formalin, and embedded in paraffin. Tissue sections (5-μm thick) were stained with H&E. The immunohistochemical studies used the monoclonal antibody anti-vonWillebrand Factor (1:100; Dako, Glostrup, Denmark) and the monoclonal antibody anti-PAI-1 (1:200; American Diagnostica, Inc., Greenwich, CT), following the procedures described previously (29).

RESULTS

Hyperthermia Inhibits in Vitro Angiogenesis but Does Not Affect EC Viability and Proliferation. ECs plated on three-dimensional ECM preparation such as Matrigel spontaneously differentiate into multicellular capillary-like structures in a process named in vitro angiogenesis (40, 41). We explored the ability of ECs subjected to hyperthermic conditions to form capillary-like structures in this in vitro morphogenesis assay. ECs were seeded on Matrigel, incubated for 60 min at 37°C to allow their adhesion, incubated for 1 h at 39°C, 41°C, 43°C, or 45°C, and replaced in a 37°C incubator for 16 h. Heat treatment at 43°C and 45°C strongly affected EC assembly into capillary-like structures in a significant manner (P < 0.0001), with cells showing only a minimal ability to differentiate as opposite to cells maintained at 37°C (Fig. 1, A, C, and D). ECs treated at 41°C and 39°C were still able to make capillary-like structures, although with a decreased efficiency as shown by the lesser tube number. The inhibition was significant for the treatment at 41°C (P < 0.0001) but not at 39°C (Fig. 1A). The addition to the culture medium of an angio-
genic factor such as VEGF-A165 did not counteract the inhibitory effects of HT on ECs in vitro angiogenic activity (data not shown).

To evaluate whether HT inhibits in vitro angiogenesis through its cytotoxic effect, we evaluated ECs viability after heat shock. It was estimated using a colorimetric assay based on the reduction of MTT by viable cells, which has been shown to produce results comparable with the clonogenic assay (42). ECs were incubated for 1 h at the above mentioned temperatures in 5% CO₂ incubator and immediately processed for MTT assay. ECs treated for 1 h at 39°C, 41°C, and 43°C showed a slight decrease in viability, whereas a significant effect (P < 0.001) was only observed at 45°C (Fig. 1B).

Because the formation of capillary-like structures occurred within 16 h, we considered the possibility that HT could induce a delayed decrease in cell viability. The response of ECs assayed after 16 h of heating was similar to that observed immediately after treatment, except for cells treated at 45°C, which showed a reduced viability (Fig. 1B).

These data suggest that HT blocks in vitro angiogenesis in a range of temperatures lower than those needed to kill ECs. Particularly, heating at 43°C does not induce a significant cell death, although it inhibits almost completely capillary-like structures formation.

To additionally investigate this phenomenon, we studied the effects of 43°C HT in the collagen spheroid model. Spheroid aggregates of ECs sprout and invade the surrounding collagen gel forming a ring of capillary-like structures in the presence of an angiogenic inducer (Fig. 1F) (32). Heat treatment at 43°C completely inhibited EC sprouting induced by VEGF-A165 (Fig. 1G). Notably, the same treatment did not affect the pre-established capillary-like structures both in Matrigel and in collagen, suggesting that HT selectively impair ECs differentiation into capillaries (Fig. 1, E and H). Furthermore, we examined whether HT causes a cell cycle delay when ECs were induced to proliferate by VEGF-A165. The BrdUrd incorporation analysis indicated that there was no significant difference between ECs maintained at 37°C and heat treated for 1 h at 43°C, suggesting that the cell cycle is not perturbed by HT (Fig. 1I). Moreover, apoptotic cells, evaluated by the presence of histone-associated DNA fragment, were never detected in 43°C heat-incubated ECs, confirming the very low cytotoxic effect exerted by this experimental condition (data not shown).

Heat Treatment Blocks Angiogenesis in the Chick CAM. Because HT blocks EC morphogenesis in vitro, we extended our observations to a well-established in vivo model of angiogenesis, the chick CAM assay (35). Gelatin sponges, adsorbed with VEGF-A165 or vehicle alone, were implanted on the top of growing CAMs. Two days after stimulation, VEGF-A165 induced a strong angiogenic response in the CAM tissue, as shown by the increased number of branching vessels converging toward the center of the sponge (Fig. 2A and Table 1). To evaluate the effect of heat shock, eggs were incubated for 2 h...
at 43°C after sponge implantation and then replaced at 37°C. After 2 days, we observed the complete inhibition of VEGF-A165-induced angiogenesis in CAM (Fig. 2B and Table 1). In addition, we did not observe any macroscopic sign of tissue damage in preexisting CAM vessels (Fig. 2B).

**Heat Shock Increases PAI-1 Expression.** To elucidate the molecular mechanisms by which heat inhibits angiogenesis, we investigated the gene expression profile of ECs after heat shock. By using the Atlas cDNA expression array, spotted with 1150 human cancer-related genes, we identified genes modulated by HT in ECs. Cells were heated for 1 h at 43°C, and the gene expression profile was evaluated both immediately and after 6 h. Exposure of ECs to heat treatment resulted in up-regulation of mRNA levels of PAI-1. This protein is involved in the homeostasis of ECM degradation occurring in angiogenesis, tumor invasion, and metastasis spreading (43). Validation of microarray data by Northern blot analysis, performed on RNA isolated from heat-treated EC, confirmed that PAI-1 expression was up-regulated 6 h after heat treatment at 43°C and persisted through 12 h (Fig. 2D). Western blot analysis of cell lysates derived from ECs maintained at 37°C or exposed to 43°C for 1 h was performed using anti-PAI-1 antibody. PAI-1 expression was evaluated immediately, 6, 12, and 24 h after heat treatment. An increased expression rate of PAI-1, which correlates with the mRNA levels, was observed beginning from 6 h up to 24 h (Fig. 2E).

**Anti-PAI-1 Antibody Antagonizes the Effects of HT on in Vitro and in Vivo Angiogenesis.** To demonstrate that in vitro and in vivo angiogenesis inhibition by HT could depend on elevated levels of PAI-1, we analyzed the effect of a neutralizing antibody anti-PAI-1 (MA-33H1F7) in angiogenesis assays. This antibody converts active PAI-1 into the inactive form but does not interfere with vitronectin/PAI-1 interaction (30).

The addition of MA-33H1F7 antibody in Matrigel and in collagen-sprouting assays inhibited, in a dose-dependent manner, the impairment caused by HT on the assembly of vascular structures (Fig. 3, A and E). In Matrigel and in collagen-sprouting assays, 5 and 10 µg/ml of MA-33H1F7 were respectively sufficient to recover by ∼75% the heat-mediated angiogenesis inhibition (Fig. 3, I and L). The heat-induced inhibition of capillary-like structures was not affected by the addition of an anti-PAI-1 antibody (MA-31C9; Ref. 44) that binds PAI-1 without affecting its catalytic activity (Fig. 3, B and F).

We evaluated the effects in CAM assay of MA-33H1F7 and MA-33B8 antibodies able to block PAI-1 activity. Quantitative analysis of these results demonstrated that MA-33H1F7 but not MA-31C9, injected into the CAM cavity, abrogated the inhibitory effect of heat shock on VEGF-A165-induced angiogenesis (Table 1). MA-33B8, which has been shown to convert active PAI-1 into a latent form (45) and to decrease the interaction between PAI-1 and vitronectin, rescued the CAM vascularization with quantitative results comparable to those obtained with MA-33H1F7.

**Impaired Heat Effect on Microvessel Outgrowth in PAI-1−/− Mice.** We additionally investigated the involvement of PAI-1 in the inhibition of new vessel formation induced by HT, using aortic

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**Table 1.** Effect of anti-PAI-1 antibodies on angiogenic response to VEGF-A165 of heat-treated CAM.

<table>
<thead>
<tr>
<th>Stimulation</th>
<th>No. of CAMs examined</th>
<th>Vessels/mm²</th>
<th>Statistical significance</th>
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<tbody>
<tr>
<td>Vehicle</td>
<td>5</td>
<td>3.88 ± 1.75</td>
<td>§</td>
</tr>
<tr>
<td>Vehicle + HT</td>
<td>5</td>
<td>4.10 ± 1.25</td>
<td>§</td>
</tr>
<tr>
<td>VEGF-A165</td>
<td>8</td>
<td>14.83 ± 4.08</td>
<td>§</td>
</tr>
<tr>
<td>VEGF-A165 + HT</td>
<td>8</td>
<td>5.67 ± 2.32</td>
<td>§</td>
</tr>
<tr>
<td>VEGF-A165 + MA33B8</td>
<td>4</td>
<td>12.75 ± 1.55</td>
<td>*</td>
</tr>
<tr>
<td>VEGF-A165 + HT + MA33B8</td>
<td>7</td>
<td>13.17 ± 2.88</td>
<td>*</td>
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<tr>
<td>VEGF-A165 + MA33H1F7</td>
<td>4</td>
<td>13.61 ± 1.03</td>
<td>*</td>
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<td>5</td>
<td>14.00 ± 1.78</td>
<td>*</td>
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<td>VEGF-A165 + MA31C9</td>
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<tr>
<td>VEGF-A165 + HT + MA31C9</td>
<td>7</td>
<td>4.50 ± 1.61</td>
<td>§</td>
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Statistical analysis was performed by one-way analysis of variance (F = 22.37) and Student-Newman-Keuls test (*, P < 0.05 versus vehicle-stimulated CAM; §, P < 0.05 versus VEGF-A165-stimulated CAM; ‡, P < 0.05 versus VEGF-A165 + HT + MA31C9).

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4 L. Primo, C. Roca, and F. Bussolino, unpublished results.
explants embedded in Matrigel isolated from PAI-1+/+ and PAI-1−/− mice (37). Both aortic rings showed a vigorous angiogenic response after 6 days of culture in presence of 20% FCS (Fig. 4, A and C). One h of heat treatment at 43°C, performed after 2 days of culture, inhibited microvessel formation in aortic explants arising from PAI-1+/+ mice but did not impair that from PAI-1−/− aortic rings (Fig. 4, B and D).

**Effects of HT on Murine Tumor Growth and Vascularization.** To test the effects of HT on an in vivo murine tumor model, TS/adenocarcinoma cells were s.c. injected in hind legs of BALB/c mice and allowed to grow till they reached a volume of 90–100 mm³ (day 0) before receiving 1 h of 43°C HT in a modified water bath. A relevant reduction of tumor size in heat-treated mice as compared with control animals was observed after 7 days of tumor growth (P = 0.006; Fig. 5A). Histological analysis of all heat-treated tumors revealed a large area of necrosis (Fig. 5, B and C) that was not present in control animals. This necrotic pattern could be induced by ischemia, resulting from an alteration in microcirculation. To confirm a vascular damage of heat-treated tumors, we performed an immunohistochemical analysis using an antibody against von Willebrand Factor to specifically stain ECs. In contrast to control tumors (Fig. 5D), heat-treated adenocarcinomas showed a reduction of vascular staining, suggesting that HT reduced tumor vascularization (Fig. 5E).

We also examined these tumors for the expression of PAI-1 by immunohistochemistry analysis. PAI-1 was detected at high level in heat-treated TSA adenocarcinomas (Fig. 5G), predominantly in cells that lined the inner necrotic area of the tumor. This pattern of expression was almost undetectable in control tumors (Fig. 5F). Because TSA cells did not express PAI-1 mRNA and did not show an increase of PAI-1 after heat treatment (data not shown), it is likely that the heat-induced PAI-1 modulation is mostly a feature of host cells, including ECs.

To assess the contribution of PAI-1 on heat treatment effectiveness, we examined the effects of HT on the growth of Lewis lung carcinomas, s.c. injected in C57B1/6 PAI-1+/+ and PAI-1−/− mice. One h of heat treatment at 43°C produced significant growth inhibition in PAI-1+/+ mice at day 7 (P < 0.05). In contrast, the effect of HT was dramatically impaired in PAI-1−/− mice (Table 2).

**DISCUSSION**

Inhibition of angiogenesis has been suggested to play a role in tumor regression activity exerted by HT (21). In our study, we performed in vitro and in vivo experiments to better address molecular aspects of this biological effect. The selected temperature routinely used in our in vitro and in vivo assay was 43°C based on preliminary
experiments done in a range from 39°C to 45°C. In our conditions, treatment at 43°C showed a minimal cytotoxic effect, although it completely blocked the in vitro EC differentiation into capillary-like structure.

Heat treatment of ECs strongly inhibits their differentiation into vascular structures both in Matrigel assay, which recapitulates numerous aspects of angiogenesis, including cell migration, differentiation, and metalloproteinase activation (46), and in spheroid assay, which mimics vessel sprouting from a preexisting one (32). We also show that HT inhibits angiogenesis in the aortic ring and in CAM assays. These results paralleled those obtained in mice transplanted with TSA cells where HT reduced tumor vascularization. Therefore, it is intriguing to speculate that the putative in vivo antiangiogenic activity is part of the more general antivascular effect of HT.

Our results suggest that the mechanism of angiogenesis inhibition is not a direct injury to ECs as already reported (21) because they are viable after heat shock, and the preformed capillary structures are not damaged by heat treatment. Moreover we showed a reduced CAM vascularization after the heat shock without any sign of tissue necrosis or vascular damage in chick CAM. On the other hand, we cannot exclude that slow heating rate in CAM experiments could result in development of thermotolerance, reducing the direct cytotoxic effect of HT (47).

The gene profile analysis performed on heated ECs clearly shows that HT activates a specific gene response, involving the transcription of PAI-1, the key regulator of the plasminogen activation pathway. This is a proteolytic cascade implicated in many physiological and pathological processes, including vascular thrombosis, metastasis diffusion, inflammation, and angiogenesis. During neo-vascularization, this system is pivotal in remodeling ECM and allows ECs to find a more favorable microenvironment for their proliferation and differentiation (48). PAI-1 is the primary inhibitor of uPA and tissue-type plasminogen activator, which both activate plasminogen to the broad spectrum enzyme plasmin, which is thought to directly degrade ECM proteins and activate latent metalloproteinases (43). Proteolytic break-
However, the exact role of PAI-1 in regulating tumor growth and angiogenesis has not been clearly defined. Mice with targeted disruption of PAI-1 grow to adulthood and are fertile, but the lack of PAI-1 is associated with impaired tumor angiogenesis (51). Indeed, increased expression of PAI-1 is associated with poor prognosis in many cancers (52, 53). In contrast, tumor cells transduced with PAI-1 cDNA showed a reduction in primary tumor growth, tumor-associated angiogenesis, and metastasis (54). This is consistent with recent results showing that high levels of recombinant PAI-1 inhibited angiogenesis in CAM through two distinct mechanisms that depend either on its ability to inhibit proteinase activity or to block αvβ3 integrin binding to vitronectin (55). Our results demonstrate that elevated levels of endogenous PAI-1 in ECs are sufficient to inhibit angiogenesis in vitro and in vivo, and this antiangiogenic activity could be neutralized by anti-PAI-1 antibody. Our in vitro experiments on heat-treated ECs suggest a physiological role of PAI-1 in regulating ECM degradation and vascular assembly, indicating that after HT, an excess of PAI-1 is released by ECs and contribute to inhibit angiogenesis, probably through a paracrine mechanism. The important role of PAI-1 in angiogenesis is reinforced by experiments in CAM, where we almost completely restored the ability of VEGF-A165 to induce vascularization by blocking PAI-1 activity with different neutralizing antibodies, and by the aortic ring assay, which demonstrates that in absence of PAI-1 gene HT is ineffective in inhibiting microvessel outgrowth.

The contribution of PAI-1 to the inhibitory effect of HT on tumor growth was evaluated in Lewis lung carcinoma implanted in PAI-1−/− mice. The absence of PAI-1 significantly affects the response of heat-treated tumors, indicating that PAI-1 regulation is an important mechanism by which HT exerts its antitumor activity. However, the antitumor effect of HT is not exclusively mediated by PAI-1 because the treatment efficacy in PAI-1−/− mice was not completely abolished, suggesting that different molecular mechanisms such as the increase of vascular permeability consequent to EC cytoskeleton collapse could be involved (12, 56).

Besides the previously described reasons why HT and radiation work synergistically (5), we suggest that antiangiogenic effect of heat treatment could play a role in the synergism between radiation therapy and HT because angiogenesis inhibitors enhance the tumor response induced by radiation (39, 57–59). Recent studies have demonstrated that combination of radiation with anti-VEGF-A165 antibody or angiostatin increases the antitumor effects of radiation (39, 59). Moreover, VEGF-A expression is induced in tumor cells in vitro and in vivo after exposure to ionizing radiation (58, 60, 61), and it has been suggested that radiation-induced VEGF-A specifically protects tumor capillaries from the toxic effects of radiation. Here, we show that mitogenic concentrations of VEGF-A165 do not revert the effects of HT on capillary-like structure formation in vitro. Furthermore, the CAM vascularization induced by VEGF-A165 is completely abolished by heat treatment suggesting that heat-treated tissue is not responsive to VEGF-A165. Thus, it is tempting to speculate that HT improves the efficacy of radiotherapy in tumor killing because it blocks the proangiogenic activity of VEGF-A165 elicited by radiation itself. Additional investigations are required to evaluate how the heat-mediated inhibition of angiogenesis could contribute to tumor regression when combined with radiation therapy and what temperatures and treatment times could improve the clinical applications of HT.

In conclusion, we report a prominent example of physical treatment able to modulate a complex biological response such as angiogenesis by a specific and unique molecular mechanism. Our observations demonstrate for the first time that up-regulation of endogenous PAI-1 in ECs is sufficient to inhibit angiogenesis both in vitro and in vivo, contributing to clarify the contradictory role of PAI-1 in tumor growth and angiogenesis. Finally these findings could open new perspectives in therapeutic applications of HT, improving its effectiveness by combining with antiangiogenic drugs.

**ACKNOWLEDGMENTS**

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**REFERENCES**

Announcements

(Requests for announcements must be received at least three months before publication.)

FUTURE ANNUAL MEETINGS OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH

2004 March 27–31, Orlando, FL
2005 April 16–20, Anaheim, CA

AACR SPECIAL CONFERENCES IN CANCER RESEARCH

A number of meetings are now being organized in the AACR’s series of smaller scientific meetings. Following are the topics, dates, locations, and program committees for these meetings. When full details of each meeting are available, AACR members will be the first to receive complete brochures and application forms for participation in these important conferences. Nonmembers may receive this information by sending their names and addresses to Meetings Mailing List, American Association for Cancer Research, Public Ledger Building, 150 South Independence Mall West, Suite 826, Philadelphia, PA 19106-3483. Up-to-date program information is also available via the Internet at the AACR’s website (http://www.aacr.org).

SNPs AND CANCER: MOLECULAR EPIDEMIOLOGY IN HUMAN POPULATIONS
September 13–17, 2003
Sonesta Beach Resort, Key Biscayne, FL

Chairpersons
Timothy R. Rebbeck, Philadelphia, PA
Fred F. Kadlubar, Jefferson, AR

ADVANCES IN BREAST CANCER RESEARCH: GENETICS, BIOLOGY, AND CLINICAL IMPLICATIONS
October 8–12, 2003
Hyatt Regency Huntington Beach Resort & Spa, Huntington Beach, CA

Chairpersons
Carlos L. Arteaga, Nashville, TN
Lewis A. Chodosh, Philadelphia, PA

NEW DIRECTIONS IN TUMOR ANGIogenesis
October 15–19, 2003
Sheraton Chicago, Chicago, IL

Chairpersons
Judah Folkman, Boston, MA
Zena Werb, San Francisco, CA
Peter Carmeliet, Leuven, Belgium

SECOND ANNUAL INTERNATIONAL CONFERENCE ON FRONTIERS IN CANCER PREVENTION RESEARCH
October 26–30, 2003
JW Marriott Desert Ridge Resort, Phoenix, AZ

Chairperson
Raymond N. DuBois, Nashville, TN

AACR-NCI-EORTC INTERNATIONAL CONFERENCE ON MOLECULAR TARGETS AND CANCER THERAPEUTICS
November 17–21, 2003
Hynes Center, Boston, MA

Chairpersons
Charles L. Sawyer, Los Angeles, CA
Edward A. Sausviile, Bethesda, MD
Jaap Verweij, Rotterdam, The Netherlands

CALENDAR OF EVENTS

16th Annual Meeting of the American Society of Pediatric Hematology & Oncology, May 1–4, 2003, Sheraton Seattle Hotel and Tours, Seattle, WA. Contact: Marilyn Rutkowski. Phone: 847.375.4830; E-mail: mrutkowski@amctec.com; Website: www.aspho.org.

Thomas L. Petty Aspen Lung Conference 46th Annual Meeting: Lung Cancer: Early Events, Early Interventions, June 4–7, 2003, Given Institute, Aspen Colorado. Contact: Jeanne Cleary, University of Colorado Health Sciences Center, Box C272, 4200 East 9th Avenue, Denver, CO 80262. Phone: 303.752.2681; Fax: 303.752.2678; E-mail: jeanne.cleary@uchsc.edu; Website: www.aspenlungconference.org.

16th International Symposium of the Journal of Steroid Biochemistry & Molecular Biology: Recent Advances in Steroid Biochemistry and Molecular Biology, June 5–8, 2004, Seefeld, Tyrol, Austria. Contact: Prof. J. R. Pasqualini, Steroid Hormone Research Unit, Institut de Puericulture, 26 Boulevard Brune, 75014 Paris, France. Phone: 33.1.4539.9109; Fax: 33.1.4542.6121; E-mail: jorge.pasqualini@wanadoo.fr.

MASCC/ISOO 15th International Symposium of Supportive Care in Cancer, June 18–21, 2003, Berlin, Germany. Contact: Kinga M. Tahy/Julia Boettger, Dachauer Str. 44a, D-80335 Munich, Germany. Phone: 49.89.5490.96.70; Fax: 49.89.5490.96.75; E-mail: mascc@emc-event.com; Website: www.symposium-online.de/mascc.

Advances in Breast Cancer: From Molecular Pathology and Imaging to Therapeutics, June 20–21, 2003, Sutton Place Hotel, Toronto, Ontario, Canada. Contact: Continuing Education, Faculty of Medicine, 500 University Avenue, Suite 650, Toronto, Ontario, MSG 1V7, Canada. Phone: 416.978.2719; Fax: 416.971.2200; E-mail: ce.med@utoronto.ca; Website: www.cme.utoronto.ca.

First Annual Melanoma Research Congress, June 21–24, 2003, Wyndham Franklin Plaza Hotel Philadelphia, PA. Contact: Sandy Parsons, The Wistar Institute, 3601 Spruce Street, Philadelphia, PA 19104. Phone: 215.898.3959; Fax: 215.898.0980; E-mail: parsons@wistar.upenn.edu.

Fourth International Symposium on Hormonal Carcinogenesis, June 21–25, 2003, Palau de la Musica, Valencia, Spain. Contact: Tandria Price/Dr. Jonathan J. Li, Dept. of Pharmacology, Toxicology and Therapeutics, Mail Stop 1018, University of Kansas Medical Center, 3901 Rainbow Blvd., Kansas City, KS, 66160-7417. Phone: 913.588.4744; Fax: 913.588.4740; E-mail: tprice@kumc.edu; Website: http://www.kumc.edu/hormonecancers.

6th Cancer Research UK Beatson International Cancer Conference: Cell Signaling and Cancer, July 6–9, 2003, Glasgow, Scotland. Contact: Tricia Wheeler, Beatson Institute for Cancer Research, Garscube Estate, Switchback Road, Bearsden G61 1BD, Scotland, United Kingdom. Phone: 44(0).141.942.0855; Fax: 44(0).141.330.6426; E-mail: t.wheeler@beatson.gla.ac.uk; Website: http://www.beatson.gla.ac.uk/conf.
ANNOUNCEMENTS

First Annual Opinion Leader Summit: Targeted Therapies in the Treatment of Hematological Malignancies, July 9–12, 2003, Kona, HI. Contact: Ora Guy, S. G. Madison/CBCE, 8445 Freeport Parkway, Suite 680, Irving, TX 75063. Phone: 972.929.1900; Fax: 972.929.1901; E-mail: oguy@sgmadison.com; Website: http://www.sgmadison.com.

Third International Symposium on Translational Research in Oncology, July 23–27, 2003, Dublin, Ireland. Contact: Carl Wilson, S. G. Madison/CBCE, 8445 Freeport Parkway, Suite 680, Irving, TX 75063. Phone: 972.929.1900; Fax: 972.929.1901; E-mail: cwilson@sgmadison.com; Website: www. sgmadison.com.

12th World Conference on Tobacco or Health: Global Action for a Tobacco Free Future, August 3–8, 2003, Helsinki, Finland. Contact: Conference Secretariat. E-mail: wctoh2003@congcreator.com; Website: www.wctoh2003.org.

Second Annual Symposium on Anti-Receptor Signaling in Human Neoplasia, August 14–16, 2003, Barcelona, Spain. Contact: Carl Wilson, S. G. Madison/ CBCE, 8445 Freeport Parkway, Suite 680, Irving, TX 75063. Phone: 972.929.1900; Fax: 972.929.1901; E-mail: cwilson@sgmadison.com; Website: www. sgmadison.com.

Breast Cancer Symposium, September 12, 2003, University of Michigan, Ann Arbor, MI. Contact: Registrar, Department of Medical Education, PO Box 1157, Ann Arbor, MI 48106–1157. Phone: 734.763.1400 or 800.800.0666; Fax: 734.936.1641.


10th Hong Kong International Cancer Congress, November 19–21, 2003, Faculty of Medicine Building, The University of Hong Kong, Hong Kong. Contact: 10th HKICC Congress Secretariat, Department of Surgery, University of Hong Kong Medical Centre, Queen Mary Hospital, Hong Kong. Phone: 852.2818.0232 or 852.2855.4235; Fax: 852.2818.1186; E-mail: mededcon@ hku.hk; Website: www.hkicc.org.

Correction

In the article by C. Roca et al., entitled “Hyperthermia Inhibits Angiogenesis by a Plasminogen Activator Inhibitor 1-dependent Mechanism,” which appeared in the April 1, 2003 issue of Cancer Research (pp. 1500–1507), footnote 1 appeared incorrectly. A grant from CNR-MIUR (Progetto “Terapia preclinica molecolare in oncologia” Legge 449-97) was omitted.

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Luca Primo
Donatella Valdembri
Anna Cividalli
Paul Declerck
Peter Carmellet
Pietro Gabriele
Federico Bassolino
Hyperthermia Inhibits Angiogenesis by a Plasminogen Activator Inhibitor 1-dependent Mechanism

Cristina Roca, Luca Primo, Donatella Valdembri, et al.


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