Amifostine Inhibits Angiogenesis in Vivo

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ABSTRACT
Amifostine (WR-2721) is an inorganic thiophosphate-cytoprotective agent developed to selectively protect normal tissues against the toxicity of chemotherapy and radiation. We have previously shown that amifostine protects both chicken embryo chorioallantoic membrane (CAM) vessels and cells from the effects of X-rays. In the present work, we studied the effect of amifostine on angiogenesis in vivo, using the CAM model. Amifostine decreased the number of CAM vessels in a dose-dependent manner, without being toxic for the tissue. It also decreased the mRNA levels of both vascular endothelial growth factor (VEGF) isoforms VEGF165 and VEGF165, 6 and up to 48 h after its application onto the CAM. Similarly, it decreased the mRNA levels of inducible nitric-oxide synthase, 24 and 48 h after drug application. Furthermore, amifostine decreased the deposited amounts of laminin and collagen I 24 h after its application, without affecting the expression of the corresponding genes. The protein amounts and activity of matrix metallopeptinase-2 were not affected, whereas the expression of the corresponding gene was decreased up to 48 h after drug application. Finally, the activity of plasmin was increased 6 h after amifostine application and remained increased at later time points. These findings suggest that amifostine alters the expression of several molecules implicated in the angiogenesis process and affects the composition of the extracellular matrix in a way that leads to inhibition of angiogenesis. Such an antiangiogenic action of amifostine, together with its radioprotective effects, further supports its use in combination with radiotherapy for increased therapeutic efficacy.

Amifostine (WR-2721) exerts a broad-spectrum cytoprotection against side effects of chemotherapy and radiotherapy (Capizzi and Oster, 2000). WR-2721 is the inactive form of the drug which is rapidly metabolized to the active form WR-1065 by the membrane-bound alkaline phosphatase. WR-1065 is further metabolized through intracellular oxidation to the symmetric disulfide WR-33278, cysteamine, and mixed disulfides (Capizzi, 1996). The active metabolite WR-1065 and the symmetric disulfide WR-33278 resemble endogenous polyamines, like spermine and spermidine, and may bind to and stabilize any part of the DNA helix that is not protected by histones (Grdina et al., 2000).

A major pharmacological benefit of amifostine ensues from the fact that it protects normal tissues from the toxic effects of chemotherapeutic agents and ionizing radiation, leaving the antitumor effects of these agents either unchanged or enhanced (Santini, 2001). The selective protection of normal tissues by amifostine is based on the higher amounts of alkaline phosphatase in normal compared with tumor tissues. In addition, the activity of the enzyme is relatively lower in tumor tissues, since the suitable pH for alkaline phosphatase is 6.6 to 8.2 and the environment of the tumors is more acidic (Dorr, 1998). The fact that amifostine enhances the antitumor effects of some chemotherapeutic agents and ionizing radiation can be explained by the fact that proliferating tumor cells maintain a high cellular polyamine content via ornithine decarboxylase-controlled biosynthesis, thereby blocking WR-33278 cell import by the induction of antizyme. However, the passive diffusion of WR-1065 accelerates polyamine degradation, resulting in lowered apoptotic threshold. In normal cells, polyamine generation is not up-regulated and the disulfide is actively imported into the cell, affording resistance to apoptosis induction (List and Gerner, 2000; Quinones et al., 2002). Preclinical data indicate that amifostine can reduce the risk of secondary cancers caused by radiation and certain forms of chemotherapy (Dorr, 1998) and inhibits the formation of spontaneous metastases (Grdina et al., 2002).

Angiogenesis, the formation of new blood vessels from preexisting ones, is an active process that is dependent upon the
balance of positive and negative regulators. Vascular endothelial growth factor (VEGF) is one of the most potent angiogenic growth factors and plays a significant role in both development and homeostasis (Carmeliet and Jain, 2000). Besides its role in activation of endothelial cells during the initial steps of angiogenesis, VEGF is also very important for the maintenance of the differentiated state of blood vessels (Ferrara and Davis-Smyth, 1997). VEGF-induced proliferation, migration, differentiation of endothelial cells, and angiogenesis are believed to be at least partly mediated by nitric oxide (NO) (Papanastasiou et al., 1997; Fukumura et al., 2001). NO production is catalyzed by a family of enzymes, the NO synthases (NOS), which exist in three isoforms, neuronal (nNOS), inducible (iNOS), and endothelial (Fukumura and Jain, 1998). VEGF induces the expression of both endothelial NOS and iNOS in human umbilical vein endothelial cells (Kroll and Welter, 1998).

The remodeling of the extracellular matrix (ECM) is pivotal for the functioning of the endothelium during the different steps of angiogenesis. The initial steps of angiogenesis involve degradation of the basement membrane and the surrounding extracellular matrix, and proliferation and migration of endothelial cells. During the final steps, endothelial cells differentiate and secrete new basement membrane (Carmeliet and Jain, 2000). Laminin is located mainly in the basement membrane and plays a critical role in tubule formation. Vessel formation also involves the deposition of various collagens, including collagen types I, III, IV, and V (Stromblad and Cheresh, 1996). The proteolytic enzymes that have mainly been implicated in ECM remodeling and angiogenesis are the family of metalloproteinases (MMPs) and the plasminogen/plasmin system (Lijnens, 2002).

In the present study, we examined the action of amifostine on angiogenesis by using the in vivo model of chicken embryo chorioallantoic membrane (CAM). Our results indicate that amifostine inhibited angiogenesis through modulation of both the expression of important angiogenic genes and the composition of the ECM.

### Materials and Methods

**CAM Assay.** The in vivo chicken embryo CAM angiogenesis model was used, as previously described (Papadimitriou et al., 2001). Leghorn fertilized eggs (Pindos, Ioannina, Greece) were incubated for 4 days at 37°C, when a window was opened on the egg shell, exposing the CAM. The window was covered with tape and the eggs were returned to the incubator. Amifostine was diluted in a final volume of 50 μl of H2O and applied at the 9th day of embryo development on an area of 1 cm2 of the CAM, restricted by a plastic ring. To evaluate the effect of amifostine, 48 h after treatment and subsequent incubation at 37°C, CAMs were fixed in situ, excised from the eggs, placed on slides, and left to air-dry. Pictures were taken through a stereoscope equipped with a digital camera, and the total length of the vessel network was measured, as previously described (Papadimitriou et al., 2001). The assay was carried out three times and each experiment contained 10 to 20 eggs per data point.

For the biochemical studies, amifostine was applied on the CAM as described above, and after different time periods of incubation at 37°C, the CAMs were excised, cut in pieces, washed three times in phosphate-buffered saline (PBS), pH 7.4, and stored at −20°C until used (Giannopoulou et al., 2001).

**Reverse Transcriptase-Polymerase Chain Reactions (RT-PCRs).** Total RNA isolation from CAMs was performed as previously described (Giannopoulou et al., 2001). Primers used for fibronectin, A1 chain of laminin (LM), collagen type IV (C-IV), collagen type I (C-I), β3 chain of integrin αβ3 (β3), and proMMP-2 were designed using the corresponding chicken sequences (Giannopoulou et al., 2001). The primers used for iNOS and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were also designed using the corresponding chicken sequences (Pipili-Synetos et al., 2000). The RT-PCRs for all the above mRNAs were performed in a single step with 200 to 250 ng of total RNA, using the Access RT-PCR system (Promega, Madison, WI), as previously described (Pipili-Synetos et al., 2000; Giannopoulou et al., 2001). The primers for VEGF were designed according to the chicken sequence available (GenBank Accession Number AB011078). The sequences of all the used primers and the anticipated product sizes are shown in Table 1.

The PCR primers for VEGF corresponded to sequences of exon 3 (sense) and exon 7 (antisense) (Kim et al., 2000) and amplified two splicing variants of avian VEGF, VEGF190 (456 bp) and VEGF165 (381 bp) (Sugishita et al., 2000). The RT-PCRs for VEGF mRNA were performed in a single step using 200 to 250 ng of total RNA and the One Step RT-PCR kit (Qiagen, Hilden, Germany), as follows. The reverse transcriptase reaction was performed by a mix of Omniscript and Sensiscript Reverse Transcriptases for 30 min at 50°C, followed by an incubation at 95°C for 15 min to activate the HotStar Taq DNA polymerase and inactivate the reverse transcriptases. After the denaturation step, 40 cycles of amplification (94°C for 1 min, 59°C for 1 min, and 72°C for 1 min) were performed and ended with a final DNA synthesis step at 72°C for 10 min.

In all cases, PCRs were not in the saturating phase (data not shown). DNA contamination was excluded by performing PCRs in the absence of the reverse transcription step.

To further establish that the PCR products, although they had the expected size, represented the corresponding cDNAs for VEGF, the PCR products were purified from agarose gels and subjected to restriction enzyme analysis using PstI, which should cleave both products only at one site. The products of the enzyme cleavage were analyzed on 2% agarose gels containing 0.5 μg/ml ethidium bro-

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**Table 1**

Sequences of all the primers used and the anticipated product sizes

<table>
<thead>
<tr>
<th>Protein</th>
<th>Sense Primer</th>
<th>Antisense Primer</th>
<th>Product Size (bp)</th>
</tr>
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<tbody>
<tr>
<td>FN</td>
<td>CACTGAGTGATACATCCTGATGC</td>
<td>GAAATTCTCTTTTCCATTCC</td>
<td>472</td>
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<tr>
<td>LM</td>
<td>TGCTAATGTCCTCAGGACC</td>
<td>GCCACCAACAGAGATCTCC</td>
<td>427</td>
</tr>
<tr>
<td>C-IV</td>
<td>CAAAATCACCAGATACC</td>
<td>TGCATACTCTTAGACC</td>
<td>653</td>
</tr>
<tr>
<td>C-I</td>
<td>TGCAGTCAACAGAAGAG</td>
<td>AGCATCTCTGGAAG</td>
<td>141</td>
</tr>
<tr>
<td>β3</td>
<td>CCTTCACCAACATCAGTCC</td>
<td>ACTCCATCTGAAGAGTCC</td>
<td>580</td>
</tr>
<tr>
<td>proMMP-2</td>
<td>CCTCACCACACATCACCTTCC</td>
<td>AGTCCTAGCAGCACAGTCC</td>
<td>530</td>
</tr>
<tr>
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<td>CACGAGATACATCTGACC</td>
<td>GCAGGATGCAAACATCGGC</td>
<td>1223</td>
</tr>
<tr>
<td>GAPDH</td>
<td>AGGGATGCGGCCATTGCGGC</td>
<td>TGCAGCTCTTTTACTCAAAGC</td>
<td>456 and 381</td>
</tr>
</tbody>
</table>

FN, fibronectin; LM, laminin; C-IV, collagen type IV; C-I, collagen type I; β3, β3 chain of integrin αβ3.
mide and photographed using a digital camera. The PCR product/GAPDH ratio of electrophoretic band values represents the expression of each gene at different time points after amifostine application.

**Western Blot Analysis of ECM Proteins.** CAM tissue was homogenized using a glass-glass homogenizer in 50 mM Tris-HCl, pH 7.4, containing 3.4 M NaCl, 1 mM phenylmethylsulfonyl fluoride, 4 mM EDTA, 2 mM N-ethylmaleimide, and 1 µg/ml aprotinin, as previously described (Giannopoulou et al., 2001). The homogenates were centrifuged at 12,000 g for 20 min at 4°C. Pellets were once more homogenized and centrifuged as described, to ensure complete removal of plasma proteins. The final pellet was resuspended in 50 mM Tris, pH 7.4, containing 4 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 2 mM N-ethylmaleimide, and the total protein concentration was determined using the Bradford (1976) method. Equal amounts of total protein were loaded on 5% SDS-polyacrylamide gel electrophoresis mini-gels, analyzed, and transferred to Immobilon-P membranes. Blocking was performed by incubating the polyvinylidene difluoride membranes with 5% (w/v) nonfat dry milk in Tris-buffered saline (TBS), pH 7.4, for 1 h at room temperature and under continuous agitation. The membranes were then incubated with anti-fibronectin (1:2500; Chemicon International, Temecula, CA), or anti-laminin (1:2500; Chemicon International), or anti-collagen IV (1:500; Chemicon International), or anti-collagen I (1:2,000; Chemicon International), or anti-actin (1:500; Chemicon International), in 3% (w/v) nonfat dry milk in TBS, 0.05% Tween 20 for 3 h at room temperature under continuous agitation, and then with horseradish peroxidase-conjugated goat anti-rabbit IgG or rabbit anti-goat IgG (when the anti-collagen IV antibody was used) at a dilution of 1:2500 in 3% (w/v) nonfat dry milk in TBS/0.05% Tween 20 or for 1.5 h at room temperature under continuous agitation. Detection of immunoreactive bands was performed by diaminobenzidine or, in the case of laminin, by enhanced chemiluminescence (Amersham Biosciences UK, Ltd., Little Chalfont, Buckinghamshire, UK), according to the manufacturer’s instructions. The antibodies used were monospecific and did not show any cross-reactivity (data not shown). The pictures of the gels were digitized and the protein levels that corresponded to each immunoreactive band were quantified using the ImagePC image analysis software (Scion Corporation, Frederick, MD).

**Detection of Apoptosis and Staining of CAM Paraffin Sections.** CAM tissues were excised from the eggs 24 h after amifostine application, washed in PBS, fixed in saline-buffered formalin, dehydrated, and embedded in paraffin. Sections were cut at 4 µm thickness and placed on positively charged glass slides. After rehydration of the tissue sections, the slides were stained with standard hematoxylin-eosin staining. Apoptosis was studied on CAM paraffin sections using a commercially available apoptosis kit (APOPTOS-I.S.; Ylem Srl, Roma, Italy), according to the manufacturer’s instructions. Briefly, the slides were

Fig. 1. Inhibition of angiogenesis by amifostine. A, different amounts of amifostine (0.01, 0.1, 0.15, 0.2, 0.25, 0.5, 1, and 10 µg) in the same final volume of 50 µl were applied on an area of 1 cm² restricted by a plastic ring, at CAMs from day 9, as described under Materials and Methods. After 48 h of incubation at 37°C, the CAMs were fixed, excised from the eggs, and photographed, and the total length of the vessel network was measured using image analysis software. Results are expressed as mean ± S.E.M. of the percentage of change in the number of vessels treated with amifostine compared with the untreated tissue. Asterisks denote a statistically significant difference (unpaired t test) from the untreated tissue (*, P < 0.05; **, P < 0.01; ***, P < 0.001). B, CAM paraffin sections 24 h after amifostine application. a and b: hematoxylin-eosin staining of CAM paraffin sections. a, control; b, tissue treated with amifostine (1 µg/cm²). Magnification ×20. c and d: CAM paraffin sections treated with an in situ apoptosis detection kit. Sections were counterstained with hematoxylin. c, control; d, tissue treated with amifostine (1 µg/cm²). Arrows indicate apoptotic cells (magnification ×40).
incubated with proteinase K for 15 min at room temperature, washed in double-distilled water twice for 5 min each, and covered with peroxidase blocking solution. After 20 min, the slides were washed in double-distilled water twice, and sections were preincubated for equilibration in TdT buffer (30 mM Tris-HCl, 140 mM sodium cacodylate, 1 mM cobalt chloride, pH 7.2) for 10 min at room temperature. For elongation and labeling of 3'-OH DNA termini, each section of the slide was incubated with 50 μl of reaction mixture, containing TdT enzyme and biotinylated dUTP in TdT buffer for 60 min at 37°C. The reaction was stopped by transferring the slides in PBS for 5 min at room temperature, and the slides were covered with blocking solution for 10 min at room temperature. For detection of poly(dUTP)-biotin complexes, the sections were incubated with diluted streptavidin-conjugated peroxidase for 30 min at room temperature. Then, the slides were washed twice for 5 min each in PBS, pH 7.4, and covered with freshly prepared diaminobenzidine solution. After 20 min, the slides were washed with distilled water, counterstained with hematoxylin, mounted in mounting fluid, viewed through a Zeiss microscope, and photographed using a digital camera.

Colorimetric Assay for the Determination of Active Plasmin. Amifostine was applied on the CAM, in an area of 1 cm², restricted by a plastic ring, and after different time periods of incubation at 37°C, the CAMs were excised, cut in pieces, washed three times in PBS, pH 7.4, and homogenized in lysis buffer (0.1 M Tris-HCl, pH 7.4). Homogenates were centrifuged at 20,000 g for 20 min at 4°C. Total protein concentration was determined in the supernatants, using the Bradford assay. Total protein (100 μg) from each sample was diluted in 200 μl of lysis buffer, placed in the wells of a 96-well microplate, and incubated at 37°C for 1 h with the plasmin substrate Val-Leu-Lys-p-nitroanilide (0.6 mM/well) (Sigma, Athens, Greece). Substrate cleavage was determined by monitoring the absorbance at 405 nm, using an enzyme-linked immunosorbent assay microplate reader (Bio-Rad, Hercules, CA). The amounts of active plasmin in each sample (milliunits/milligram of total protein) were determined using a standard curve from assays with purified plasmin (Sigma) (Baldi et al., 1996).

Statistical Analysis. The significance of variability between the results from each group and the corresponding control was determined by unpaired t test. Each experiment included triplicate measurements for each condition tested, unless otherwise indicated. All results are expressed as mean ± S.E.M. from at least three independent experiments.

Results

Amifostine Inhibited Angiogenesis in the Chicken Embryo CAM. As mentioned above, amifostine is a prodrug that can be metabolized by the membrane-bound alkaline phosphatase into the active free thiol WR-1065 (Capizzi, 1996). Previous studies in our laboratory have shown that there is active alkaline phosphatase in the CAM, and the applied drug is metabolized in its active form within 15 min (Giannopoulou et al., 2002a). To study the effect of amifostine on CAM angiogenesis, different concentrations of the drug were applied on the CAM, as described under Materials and Methods, and after 48 h, the total length of the vessel network was measured. As shown in Fig. 1A, amifostine decreased angiogenesis in a dose-dependent and statistically significant manner. The decrease was maximal at the dose of 0.25 μg/cm² and was not due to toxicity, as verified on CAM paraffin sections stained with eosin-hematoxylin (Fig. 1B, a and b) or treated with a kit for in situ detection of apoptosis (Fig. 1B, c and d).

Amifostine Decreased the mRNA Levels of VEGF₁₉₀ and VEGF₁₆₅. VEGF is a multifunctional glycoprotein involved in endothelial proliferation and migration, vascular permeability and angiogenesis. Differential exon splicing of the VEGF gene results in five main mRNA species which code for five secreted isoforms: VEGF₂₀₀, VEGF₁₈₀, VEGF₁₆₅, VEGF₁₄₅, and VEGF₁₂₁ (Ferrara and Davis-Smyth, 1997). Avian VEGF isoforms exhibit more than 70% homology with the corresponding human sequences and are also generated by alternative splicing (Sugishita et al., 2000). As mentioned under Materials and Methods, the PCR primers used in the present study amplified two variants of avian VEGF, VEGF₁₉₀ and VEGF₁₆₅ (Fig. 2). We investigated whether amifostine affects the expression of these VEGF isoforms by using a sensitive, semiquantitative RT-PCR, at several time points after drug application. As shown in Fig. 3, amifostine decreased the mRNA levels of both VEGF₁₉₀ and VEGF₁₆₅ 6 h after its application. The expression of both isoforms remained decreased up to 48 h after amifostine was applied on the CAM.

Amifostine Decreased the mRNA Levels of iNOS. Our previous studies have shown that iNOS is the only isoform of NOS detected in the chicken embryo CAM (Pipili-Synetos et al., 2000; Giannopoulou et al., 2002b). To study the effect of amifostine on the iNOS mRNA, we used a semiquantitative RT-PCR for the corresponding gene, as previously described (Pipili-Synetos et al., 2000). As shown in Fig. 4, the expres-

![Fig. 2. The products of RT-PCRs for CAM VEGF₁₉₀ and VEGF₁₆₅ (456 and 381 bp, respectively) were eluted from agarose gels and subjected to restriction enzyme cleavage using PstI, which cleaves both products only at one site. Lane 1, VEGF₁₉₀ (456 bp). Lane 2, products of VEGF₁₉₀ treatment with PstI. Lane 3, PCR markers: 150, 300, 500, and 750 bp. Lane 4, products of VEGF₁₆₅ treatment with PstI. Lane 5, VEGF₁₆₅ (381 bp).](image-url)
Amifostine Decreased the Deposition of ECM Proteins. In the present work, we studied the effect of amifostine on ECM proteins that are implicated in several steps of the angiogenic cascade (Papadimitriou et al., 1993). The ECM proteins were analyzed by Western blotting techniques. The protein levels that corresponded to each immunoreactive band of fibronectin, B1 chain of laminin, /H9251 chain of collagen I, and collagen IV were quantified using image analysis software and were normalized to the corresponding levels of actin. As shown in Fig. 5, the protein levels of collagen I and laminin were decreased 6 h after amifostine application and remained decreased up to 24 h. Fibronectin and collagen IV were not affected by amifostine at any of the time points examined in our study. To determine whether the above-described changes in the amounts of laminin and collagen I were due to changes in the expression of the corresponding genes, we tested the effect of amifostine on the levels of their mRNAs by semiquantitative RT-PCRs, at several time points after drug application. Amifostine did not affect the expression of any of the tested ECM genes, including integrin /H9251/H9263/H9252 up to 48 h after drug application (data not shown).

Amifostine Decreased the mRNA Levels of proMMP-2. MMP-2 has been implicated in invasive processes, such as angiogenesis and tumor metastasis. In the present work, we studied the effect of amifostine on both MMP-2 expression and activity. By zymography, we found that amifostine did not affect either the protein amounts or the activity of MMP-2 in the chicken embryo CAM (data not shown). However, the expression of the proMMP-2 gene was decreased 6 h after amifostine application and remained decreased at later time points (Fig. 6).

Amifostine Increased the Activity of Plasmin. Plasmin is a protease that hydrolyzes many extracellular proteins (Pepper, 2001). In the present work, we examined the effect of amifostine on plasmin activity at several time points after drug application on the chicken embryo CAM, using a colorimetric assay as described under Materials and Methods. We found that amifostine increased the activity of plasmin within the first 6 h after its application (Fig. 7).

Discussion

Amifostine has been characterized for its cytoprotective activity against the toxic effects of radiation or chemotherapy. Cytoprotection has been shown to rely on at least three different activities of amifostine: binding to and protection of DNA, neutralization of antineoplastic drugs, and antioxidant properties (Dorr, 1998; Grdina et al., 2000). Amifostine can affect several cellular processes, such as the activation of certain transcription factors, the expression levels of genes, and the activities of certain proteins (Murley et al., 1997, 2001; Romano et al., 1999; Shen et al., 2001). In the present...
Fig. 5. Amifostine decreased the deposition of ECM proteins. A, Western analysis for fibronectin (FN), laminin (LM), collagen I (C-I), collagen IV (C-IV), and actin (Ac) at several time points after application of amifostine (1 μg/cm²) on the chicken embryo CAM. C, control; Amf, Tissue treated with amifostine. Representative picture of 4 to 12 independent experiments. B, the protein amounts were quantified by densitometric analysis of the corresponding bands and the ratio ECM protein/actin was calculated for each lane. Results are expressed as mean ± S.E.M. of the percentage of change of the amounts of proteins in the treated compared with the untreated tissue (control). Asterisks denote a statistically significant difference (unpaired t test) from the control. *, P < 0.05.
study, we showed that amifostine inhibited angiogenesis in the chicken embryo CAM by modulating both the expression of important angiogenic genes and the composition of the ECM.

The effect of amifostine on angiogenesis has not been widely studied. To our knowledge, there is only one short study suggesting increased angiogenesis and endothelial cell proliferation after amifostine application on the yolk sac membrane of the chicken embryo (Plaswilm et al., 1999). However, only one very high dose of amifostine (26 μg/cm²) was applied in that study. In the present work, we showed a dose-dependent inhibition of angiogenesis in the chicken embryo CAM, which is supported by the effects of amifostine on several molecules implicated in angiogenesis. In agreement with an antiangiogenic effect of amifostine are studies that show that it down-regulates cell cycle progression (North et al., 2000), represses c-myc gene expression (Liu et al., 1997), inhibits topoisomerase II activity (Snyder and Grdina, 2000), increases the plasma levels of angiostatin (Grdina et al., 2002), and decreases MMP-2 activity (Grdina et al., 2002).

VEGF is considered the key angiogenic factor, expressed under both physiological and pathological conditions of angiogenesis, and many promoters or inhibitors of angiogenesis modulate VEGF and/or its receptors (Ferrara and Davis-Smyth, 1997). This is the first study that shows that CAM cells express at least two VEGF isoforms, VEGF₁₆₅ and VEGF₁₉₀. The former is found both secreted and bound to the ECM, whereas the latter is mainly sequestered on cell surfaces and the ECM. Although VEGF₁₆₅ is considered the most potent mitogen for endothelial cells, VEGF₁₉₀ can also be mitogenic (Ferrara and Davis-Smyth, 1997). What is the exact role and distribution of the two VEGF isoforms in the chicken embryo CAM or the identity of the cells that produce them need further investigation. Amifostine decreased the mRNA expression of both VEGF isoforms, and this may at least partly contribute to its antiangiogenic effect on the CAM.

Fig. 6. Amifostine decreased the mRNA levels of proMMP-2. A, products of RT-PCRs for the mRNAs of proMMP-2 and GAPDH at several time points after application of amifostine (1 μg/cm²) on the chicken embryo CAM. C, control; Amf, tissue treated with amifostine. Representative picture of four independent experiments. B, the mRNA amounts were quantified by densitometric analysis of the corresponding bands and the ratio proMMP-2/GAPDH mRNAs was calculated for each lane. Results are expressed as mean ± S.E.M. of the percentage change of the mRNA amounts in the tissue treated with amifostine compared with the nontreated tissue (control). Asterisks denote a statistically significant difference (unpaired t test) from the control (*, P < 0.05).

Fig. 7. Amifostine increased the activity of plasmin. The activity of plasmin was measured using a colorimetric assay, and results are expressed as mean ± S.E.M. of the percentage of change of the plasmin activity in the tissue treated with amifostine (1 μg/cm²) compared with the nontreated tissue (control) from three independent experiments. Asterisks denote a statistically significant difference from the control (*, P < 0.05).

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time points than that of iNOS, is not known. Moreover, the reduction of iNOS mRNA by amifostine might be an indirect effect due to an increase in the expression of superoxide dismutase (Murley et al., 2001). Changing the redox status of CAM cells may lead to inhibition of iNOS expression (C. Polytarchou and E. Papadimitriou, unpublished data; Saito et al., 2001).

MMPs degrade the ECM and facilitate angiogenesis. In the present study we showed that amifostine decreased proMMP-2 mRNA levels, although it did not affect the total amounts or the activity of MMP-2 in CAM protein extracts. A decrease of MMP-2 protein amounts and activity has been observed in tumor cells (Grdina et al., 2002), as well as in human endothelial cells (E. Giannopoulou and E. Papadimitriou, unpublished observations). The discrepancy between the effect of amifostine on the mRNA and the protein levels of MMP-2 could be due to a concomitant effect of amifostine on the amounts of membrane type 1 MMP or the inhibitor of MMP-2, tissue inhibitor of metalloproteinase 2. Alternatively, platelets also release MMP-2, which may contribute a large amount of the total MMP-2 of the tissue, masking the effect of amifostine on the endothelial MMP-2. In this case, there may be a locally decreased amount or activity of MMP-2 on the surface of migrating endothelial cells, in agreement with decreased angiogenesis (Strombland and Chereshe, 1996).

ECM protein remodeling is very important for the control of angiogenesis and maturation of vessels (Polytarchou et al., 1993; Pepper, 2001). In the present study it was shown that amifostine decreased the deposited amounts of laminin and collagen type I and, to a lesser extent, collagen type IV, without affecting the expression of the corresponding genes. These data suggest that it affects ECM deposition through an effect on mechanisms that control the formation of the matrix.

In the chicken embryo CAM, laminin seems to play a significant role in both the early stages of angiogenesis, when proliferation and migration of endothelial cells takes place, and during differentiation and maturation of the new vessels (Auszprunk et al., 1991; Papadimitriou et al., 1993). Since laminin is detected only in the basement membrane of CAM vessels (Auszprunk et al., 1991), a decrease in the amounts of deposited laminin corresponds well with a decreased number of blood vessels. However, it cannot be established from the present study whether this decrease in the deposition of laminin is the cause or the effect of the reduced number of vessels after amifostine application. The decrease in laminin deposition could be due to activation of proteolytic enzymes, such as plasmin (Pepper, 2001). Amifostine increased the activity of plasmin in the CAM and this may, at least partly, be the mechanism through which it affects the deposited amounts of laminin.

Collagens play an important role in angiogenesis. Inhibition of collagen synthesis, or deposition or cross-linking of collagen chains inhibits angiogenesis (Ingber and Folkman, 1988). Amifostine decreased the deposition of collagen, without affecting its biosynthesis. These data suggest that amifostine may affect collagen cross-linking. Lysyl oxidase is the enzyme that initiates the covalent cross-linking of extracellular collagen molecules, converting these to insoluble fibers (Kagan, 2000). Lysyl oxidase is a copper-dependent amine oxidase and is inhibited by a variety of compounds bearing primary amine functions, such as β-aminoproprionitrile, β-bromoethylamine, ethylenediamine, and others (Kagan, 2000). Amifostine and its metabolites resemble polyamines and, having amine functions, they may inhibit lysyl oxidase and, thus, collagen cross-linking. In the same line, since amifostine is metabolized by copper-dependent amine oxidases to acrolein and cysteamine (Meier and Issels, 1995), it may act as an antagonistic substrate for lysyl oxidase. The product of this reaction, cysteamine, may also act as an inhibitor of lysyl oxidase.

In summary, amifostine affects ECM remodeling and the expression of several angiogenic molecules in a way that inhibits angiogenesis. Taking into account that embryonic angiogenesis may differ in several aspects from human tumor angiogenesis in the adult, further studies are in progress using other angiogenesis models. An antimetastatic (Grdina et al., 2002) and an antiangiogenic (this study) action of amifostine, in addition to its radioprotective effects, further support the use of amifostine in combination with radiotherapy for increased therapeutic efficacy.

References


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