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Amifostine has Antiangiogenic Properties *In Vitro* by Changing the Redox Status of Human Endothelial Cells

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Amifostine is a broad-spectrum cytoprotective agent, selective for normal tissues. It is a pro-drug metabolised to the free thiol WR-1065 that may act as a scavenger of free radicals, generated in tissues exposed to chemotherapeutic agents or radiation. WR-1065 can be further oxidized to its symmetric disulfide WR-33278 or degraded to hydrogen peroxide (H_2O_2). Both WR-1065 and WR-33278 resemble endogenous polyamines. Although amifostine is used in some cases in the clinic, there are only few studies concerning its actions at the cellular level. We have previously shown that amifostine inhibits angiogenesis *in vivo*, affecting the expression of several angiogenic genes. In the present work, we studied the effect of amifostine on human umbilical vein endothelial cell (HUVEC) functions *in vitro*, in order to further clarify its mechanism(s) of action. Amifostine increased HUVEC proliferation, an effect that was reversed by the intracellular H_2O_2 scavenger sodium pyruvate, agents that increase intracellular cAMP levels and L-valine. On the other hand, amifostine decreased HUVEC migration, an effect that was reversed by L-valine or L-arginine but not sodium pyrouvate. The decrease in migration was in line with decreased tube formation on matrigel and decreased amounts of metalloproteinase-2 released into the culture medium of HUVEC. Finally, amifostine reduced tyrosine nitration of the cytoskeletal proteins actin and α-tubulin in a time dependent manner. This last action could be due to the reduced production of nitric oxide (NO) or to other not yet identified mechanisms. Collectively, our results suggest that amifostine acts on endothelial cells through pathways that affect the redox status of the cells, either by producing H_2O_2 or by modulating NO production.

Keywords: Amifostine; Endothelial cells; Angiogenesis; Hydrogen peroxide; Nitric oxide; Tyrosine nitration

INTRODUCTION

Amifostine (WR-2721) protects normal tissues from the damaging effects of radiation and chemotherapeutic agents.[1,2] It is a pro-drug, rapidly converted to the active form WR-1065 by the membrane-bound alkaline phosphatase. WR-1065 is a free thiol that is further oxidised to the symmetric disulfide WR-33278 or mixed disulfides with proteins, glutathione, L-cysteine and cysteamine. Alternatively, WR-1065 is degraded to acrolein, cysteamine, hydrogen peroxide (H_2O_2) and ammonia by Cu-dependent amine oxidases.[3,4] The protective ability of amifostine is based on the fact that WR-1065 and 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. Thus, the DNA molecules become more compact and less vulnerable to attack by radiation- and chemotherapy-induced free radicals. WR-1065 may also directly scavenge free radicals, like hydroxyl radicals that are produced during radiation.[5] The selective protection of normal tissues by amifostine is based on the higher amounts of alkaline phosphatase in normal compared with tumour tissues. In addition, the activity of the enzyme is relatively lower in tumour tissues, since the suitable pH for alkaline phosphatase is 6.6 to 8.2 and the environment of the tumours is more acidic.[6]

Besides its chemo- and radioprotective effects, amifostine enhances the antitumour effects of some

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chemotherapeutic agents and ionising radiation[7,8] and reduces the risk of secondary cancers caused by radiation and certain forms of chemotherapy.[6] In preclinical studies, it inhibits the formation of spontaneous metastasis,[9] as well as tumour-induced angiogenesis observed after irradiation.[10]

Angiogenesis is the formation of new blood vessels from pre-existing ones. It is an active process in which endothelial cells are stimulated to degrade the pre-existing basement membrane and to migrate into the perivascular stroma, where they proliferate and form new blood vessels. Migrating endothelial cells produce several types of matrix metalloproteinases (MMPs) and serine proteinases to remodel the extracellular matrix in front of the sprouting vessel. Inhibition of endothelial cell activation inhibits angiogenesis.[11]

Amifostine inhibits angiogenesis *in vivo*.[12] In agreement with an anti-angiogenic effect of amifostine are also *in vitro* studies that show that it down-regulates cell cycle progression,[13] represses c-myc gene expression,[14] inhibits topoisomerase II activity,[15] increases the plasma levels of angiostatin[9] and decreases MMP-2 activity.[9] However, the effect of amifostine on endothelial cell functions has not been widely studied. Treatment of endothelial cells with amifostine increased[16] or decreased[17] proliferation and inhibited migration,[18] without any information on the mechanisms involved.

Based on our previous results on the antiangiogenic effects of amifostine *in vivo*,[12] in the present work we studied the action of amifostine on several endothelial cell functions that are related to angiogenesis, *in vitro*. Our aim was to clarify molecular pathways that amifostine affects in endothelial cells.

MATERIALS AND METHODS

Cell Culture

Human umbilical vein endothelial cells (HUVEC) were isolated from human umbilical cords and cultured as previously described.[19] The cells were grown as monolayers in medium M199 supplemented with 15% foetal calf serum (FCS), 150 μg/ml endothelial cell growth supplement, 5 U/ml heparin sodium, 100 U/ml penicillin-streptomycin and 50 μg/ml gentamycin (complete growth medium). Cultures were maintained at 37°C 5% CO_2 and 100% humidity and used at passages 1–3.

Cell Proliferation Assay

In order to determine if amifostine affects the proliferation of HUVEC, the 3-[4, 5-dimethylthiazol-2-yl]-2,5-dimethyltetrazolium bromide (MTT) assay was used, as previously described.[19] Briefly, cells were seeded at a density of 2.5×10^4 cells/well in 24-well tissue culture plates. The medium was aspirated later at 24 h, cells were washed twice with phosphate buffered saline (PBS) and 0.5 ml of either complete growth medium or M199 containing 5% FCS was added in each well. The tested agents were added to the medium at the indicated concentrations and the number of cells was measured after 48 h. MTT stock (5 mg/ml in PBS) at a volume equal to 1/10 of the medium was added and plates were incubated at 37°C for 2 h. The medium was removed, the cells were washed with PBS pH 7.4 and 100 μl acidified isopropanol (0.33 ml HCl in 100 ml isopropanol) were added to each well and agitated thoroughly, in order to solubilise the dark blue formazan crystals. The solution was transferred to 96-well plates and immediately read on a microplate reader (Biorad, Hercules, CA, USA), at a wavelength of 490 nm. Results were always confirmed by direct measurements of the cells using a standard Neubauer haemocytometer.

Boyden Chamber Assay

Migration assays were performed as previously described,[19] in 24-well microchemotaxis chambers (Costar, Avon, France), using uncoated polycarbonate membranes with 8 μm pores. Briefly, HUVEC were harvested and resuspended at 10^5 cells/0.1 ml in M199 containing 0.25% bovine serum albumin or in complete growth medium. The bottom chamber was filled with 0.6 ml of the corresponding medium and the tested agents. The upper chamber was loaded with 10^5 cells and incubated for 4 h at 37°C. After completion of the incubation, the filters were fixed with saline-buffered formalin and stained with 0.33% toluidine blue solution. The cells that migrated through the filter were quantified by counting the entire area of each filter, using a grid and an Optech microscope at a 20 × magnification.

Matrigel Tube Formation Assay

The tube formation assay was performed as previously described.[19] Briefly, matrigel was used to coat the wells of 96-well plates (0.04 ml/well) and was left to polymerize for 1 h at 37°C. After polymerization, 15,000 cells suspended in 0.15 ml of medium were added to each well. Amifostine was added to the corresponding wells simultaneously with the cells. After 6 h of incubation, the medium was removed, the cells were fixed and the total length of the tube network was measured in the entire area of the wells.

cGMP Assay

NO production from HUVEC in response to amifostine was assessed by the intracellular

accumulation of cGMP. HUVEC were cultured in 60 mm dishes and used at confluence. The medium was aspirated, cells were washed twice with PBS and 0.5 ml of M199 containing 5% FCS was added in each well. Amifostine at a concentration of 2 mM was added to the medium and the amounts of intracellular cGMP were measured after 4 h. The incubation with amifostine was stopped by removal of the culture supernatant and the cells were immediately covered with 0.1 N HCl and scraped off. The HCl extract was collected, centrifuged for 5 min at 5,000*g* and stored at −20°C until analysed. To normalise cGMP values, protein content in each dish was measured by the Bradford assay and the results were in the range of fmol cGMP/mg protein. Direct measurement of the intracellular cGMP levels was performed using the cGMP direct EIA kit (R & D Systems), according to the manufacturer's instructions.

Zymography

The release of MMP-2 into the culture medium of HUVEC was measured by zymography, following standard procedures.[20] In brief, HUVEC were plated in 24-well plates at a density of 2.4×10^4 cells per well, in M199 containing 5% FCS. The cells were treated with the tested agents, as described above for the cell proliferation studies. At 48 h later, 50 μl of the medium from each group were diluted 1:1 with 2× Laemmli sample buffer without β-mercaptoethanol. The samples were analyzed in 10% SDS-PAGE gels containing 0.1% gelatin. Following electrophoresis, gels were washed twice in 2.5% Triton-X 100 for 20 min at room temperature and incubated for 72 h at 37°C in developing buffer (50 mM Tris–HCl pH 7.5, 200 mM NaCl and 10 mM $CaCl_2$). The gels were stained with Coomassie Brilliant Blue R for 4 h at room temperature, de-stained in methanol-acetic acid-water (4.5:1:4.5 v/v) for 2 h and photographed using a digital camera.

Western Blot Analysis for 3-Nitrotyrosine, Actin and α-tubulin

HUVEC were seeded at a density of 5×10^5 cells per plate in 60 mm tissue culture plates. At 24 h later, the medium was aspirated, cells were washed twice with PBS and 5 ml of M199 containing 5% FCS were added in each plate. Amifostine (2 mM) was added in the cell medium and after different time periods of incubation, the medium was aspirated, cells were washed twice with PBS and lysed in 200 μl of Laemmli sample buffer. The lysates (80 μl from each plate) were analysed by Western blot for 3-nitrotyrosine, actin and α-tubulin, as previously described.[21] Briefly, the samples were loaded on 10% SDS-PAGE gels, analysed and transferred to Immobilon P membranes. For the detection of actin and α-tubulin, blocking was performed by incubation of the PVDF membranes in 5% (w/v) non-fat 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 a monoclonal anti-actin antibody (Chemicon, dilution 1:500), or a monoclonal anti-α-tubulin antibody (Sigma, dilution 1:5,000), in 3% (w/v) non-fat dry milk in TBS containing 0.05% Tween-20 (TBS-T), for 3 h at room temperature under continuous agitation. After three washes in TBS-T, membranes were further incubated with horseradish peroxidase conjugated goat anti-mouse IgG (Sigma, dilution 1:5,000), in 3% (w/v) non-fat dry milk in TBS-T, for 1.5 h at room temperature under continuous agitation. For the detection of nitrotyrosine, blocking was performed by drying the PVDF membrane according to the manufacturer's (Millipore) instructions. The membranes were then incubated with a polyclonal anti-nitrotyrosine antibody (Upstate Biotechnology, 2 μg/ml) in TBS-T for 3 h at room temperature under continuous agitation and then, after washing with TBS-T, with horseradish peroxidase conjugated goat anti-rabbit IgG (Sigma, dilution 1:2,500) in TBS-T, for 1.5 h at room temperature under continuous agitation. Detection of the immunoreactive proteins was performed by diaminobenzidine (DAB) or the chemiluminescence horseradish peroxidase substrate SuperSignal (Pierce), according to the manufacturer's instructions. The protein levels that corresponded to each immunoreactive band were quantified by image analysis of digital photos of the immunoblots (using the ImagePC image analysis software, Scion Corporation).

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 at least triplicate measurements for each condition tested, unless otherwise indicated. All results are expressed as mean ± SEM from at least three independent experiments.

RESULTS

We have previously shown that amifostine inhibits angiogenesis *in vivo* affecting extracellular matrix remodelling, as well as the expression of several angiogenic molecules, like vascular endothelial growth factor.[12] However, the cells that were affected by amifostine and were responsible for its effect on angiogenesis have not been determined. Since endothelial cells are the main cellular component of the vessel wall and have important roles in

the process of angiogenesis, in the present work, we studied the effect of amifostine on HUVEC proliferation, migration and tube formation on matrigel.

Effect of Amifostine on HUVEC Proliferation

Initially, we studied the effect of amifostine on HUVEC proliferation. HUVEC were treated with several concentrations of amifostine (0.125, 0.25, 0.5, 1 and 2 mM) and the number of cells was measured using the MTT assay, as described in "Materials and Methods" section. As shown in Fig. 1A, amifostine had a biphasic effect on HUVEC proliferation: When the concentration of the drug was less than 1 mM it decreased, while at concentrations higher than 1 mM it increased HUVEC proliferation. At amifostine concentrations higher than 4 mM, no further increase in proliferation was observed. Treatment of HUVEC with amifostine 2, 4 and 6 mM increased cell proliferation by $109 \pm 9\%$ ($n = 16$), $154 \pm 18\%$ ($n = 9$) and $156 \pm 16\%$ ($n = 9$), respectively, compared to untreated cells. The effect of amifostine on HUVEC proliferation was similar whether the experiments were performed in M199 containing 5% FCS (Fig. 1) or in complete growth medium (data not shown). Treatment of cells with 600 U/ml catalase (a scavenger of extracellular H_2O_2) 30 min before the addition of amifostine, reversed the decrease in cell proliferation observed with the lower concentrations of amifostine (Fig. 1A). On the other hand, treatment of cells with sodium pyruvate (a scavenger of intracellular H_2O_2) 30 min before the addition of amifostine, reversed the increase in cell proliferation observed with the higher concentrations of amifostine (Fig. 1B).

In order to further define intracellular pathways that amifostine may affect in HUVEC, cells were treated with agents that increase intracellular cAMP, like forskolin and isoproterenol, 20 min before the addition of amifostine. Both agents reversed the increase in HUVEC proliferation caused by high concentrations of amifostine in a concentration dependent manner (Fig. 2). However, amifostine had no direct effect on cAMP levels in the presence or absence of the cAMP modulating agents (data not shown). The increase in HUVEC proliferation by amifostine was not affected by inhibition or down-regulation of protein kinase C by staurosporine, chelerythrin and phorbol-12 myristate-13 acetate or by the intracellular calcium chelator 1,2,-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid-acetoxymethyl ester (data not shown).

L-Valine, an inhibitor of arginase that is implicated in polyamine synthesis, partially inhibited the proliferation of HUVEC induced by amifostine (Fig. 3), even at the higher used concentration (50 mM). Higher concentrations of L-valine could not be used due to solubility problems.

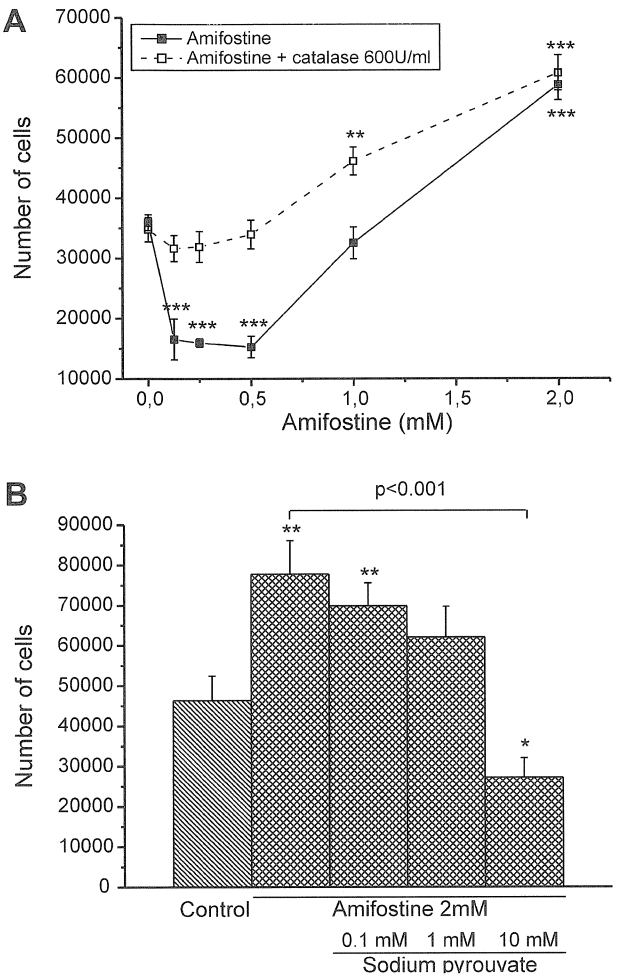

FIGURE 1 Effect of amifostine on HUVEC proliferation. (A) Different doses of amifostine were applied on HUVEC in the presence or absence of catalase and 48 h later, the number of the cells was estimated with the colorimetric MTT assay, as described in "Materials and Methods" section. (B) HUVEC were treated with different concentrations of sodium pyruvate, 30 min before the addition of 2 mM amifostine. Results are expressed as mean ± SEM of the number of HUVEC from at least three independent experiments performed in triplicates. Asterisks denote a statistically significant difference (unpaired t-test) from untreated HUVEC. $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

Effect of Amifostine on HUVEC Migration and Tube Formation on Matrigel

Amifostine inhibited HUVEC migration in a concentration dependent manner. At the concentration of 0.25 mM it caused a 17% decrease compared with the control, non treated cells, while at the concentration of 2 mM the corresponding decrease was 30%. The decrease in migration was reversed by addition of exogenous L-valine or L-arginine but not sodium pyrouvate (Fig. 4). Similar results were obtained when the experiments were performed in complete growth medium (data not shown). Sodium pyrouvate alone at a concentration of 10 mM resulted in a 21 ± 4 decrease in migration of HUVEC, which was statistically significant ($P < 0.05$) and is in line

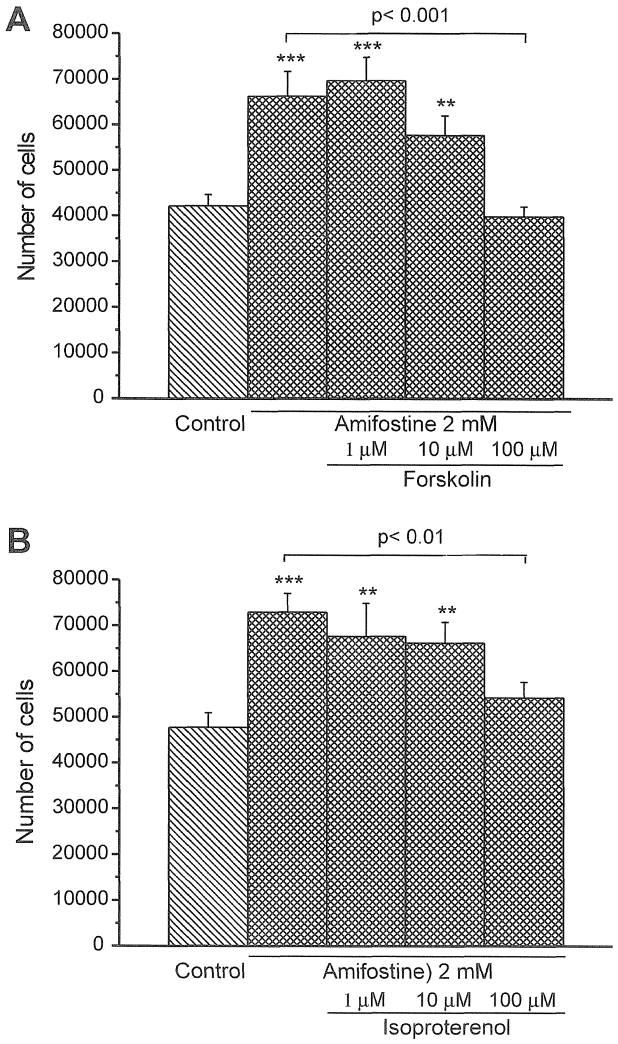

FIGURE 2 Effect of cAMP modulating agents on the increased proliferation of HUVEC caused by amifostine. HUVEC were treated with different concentrations of forskolin (A) or isoproterenol (B), 20 min before the addition of 2 mM amifostine. 48 h after the addition of amifostine, the number of the cells was estimated with the colorimetric MTT assay, as described in "Materials and Methods" section. Results are expressed as mean ± SEM of the number of HUVEC from at least three independent experiments performed in triplicates. Asterisks denote a statistically significant difference (unpaired *t*-test) from untreated HUVEC. $^{**}P < 0.01$ and $^{***}P < 0.001$.

with a stimulating effect of H_2O_2 on endothelial cell migration.[22]

In line with the decrease in migration, amifostine also inhibited tube formation on matrigel, in a modest but statistically significant manner (Fig. 5B).

Amifostine Decreased the Amounts of Intracellular cGMP Produced by HUVEC

Small quantities of NO produced by eNOS activate soluble guanylate cyclase through interaction with the catalytic heme groups of the enzyme and produce cGMP from GTP. Therefore, cGMP production was measured in order to access the effect of amifostine on NO production by HUVEC.

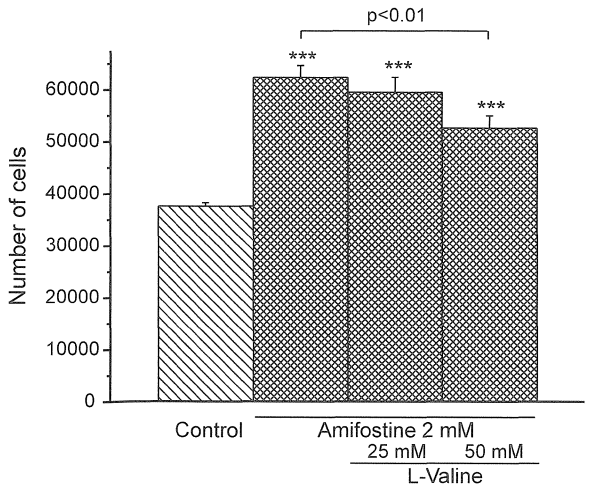

FIGURE 3 Effect of L-valine on the increased proliferation of HUVEC caused by amifostine. HUVEC were treated with 2 mM amifostine in the absence or presence of L-valine (25 and 50 mM). 48 h, after the addition of amifostine, the number of the cells was estimated with the colorimetric MTT assay, as described in "Materials and Methods" section. Results are expressed as mean ± SEM of the number of HUVEC from at least three independent experiments performed in triplicates. Asterisks denote a statistically significant difference (unpaired *t*-test) from untreated HUVEC. $^{***}P < 0.001$.

The amounts of cGMP produced by unstimulated HUVEC were 572 ± 44 fmol/mg protein and were decreased by 15.3 ± 2% 4 h after treatment with 2 mM amifostine. This decrease was statistically highly significant ($P < 0.01$).

Amifostine Decreased the Amounts of MMP-2 Released into the Culture Medium of HUVEC

HUVEC were treated with several concentrations of amifostine and after 48 h, the medium of the cells

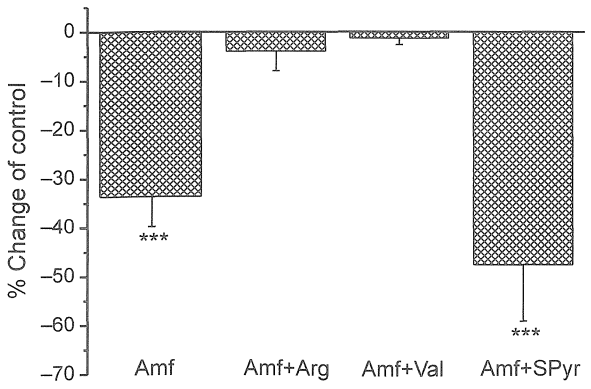

FIGURE 4 Effect of amifostine on HUVEC migration. Migration was measured by using the Boyden chamber assay, as described in "Materials and Methods" section. Results are expressed as mean ± SEM of the % change compared to the untreated cells, from at least three independent experiments. Asterisks denote a statistically significant difference (unpaired *t*-test) from untreated HUVEC. $^{***}P < 0.001$. Amf: Amifostine 2 mM, Val: L-Valine 50 mM, Arg: L-arginine 1 mM and Spyr: Sodium pyrouvate 10 mM. The number of untreated cells that migrated through the membranes were 390 ± 50 cells.

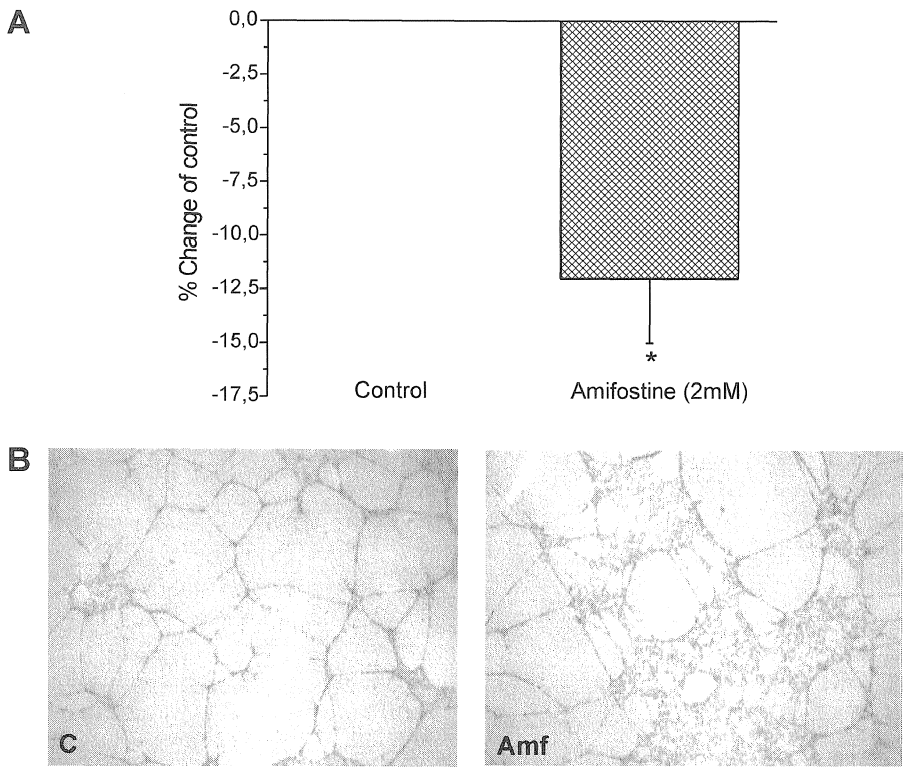

FIGURE 5 Effect of amifostine on tube formation on matrigel. (A) The assays were performed in M199 containing 5% FCS. The cells were fixed and the length of the tube network was quantified using image analysis software. Results are expressed as mean ± SEM of the % change compared to the untreated cells, from three independent experiments performed in triplicates. (B) Representative pictures showing the formation of tubes on matrigel, when the assays were performed in complete growth medium. C: Control; Amf: Amifostine 2 mM.

was collected and analysed, as described in "Materials and Methods" section. Amifostine decreased the amounts of MMP-2 released into the HUVEC medium in a concentration-dependent manner. Catalase (600 U/ml) did not affect this action of amifostine (Fig. 6). As can be seen in Fig. 6, amifostine also had an inhibitory effect on other metalloproteinases, among which MMP-9, that are released in HUVEC medium.

Amifostine Decreased Tyrosine Nitration of Actin and α-tubulin

Previous studies in our laboratory have shown that actin and α-tubulin are tyrosine nitrated in HUVEC under routine culture conditions.[21] Moreover, amifostine reversed the tyrosine nitration of both cytoskeletal proteins induced by irradiation *in vivo*.[10] In the present work, we studied by Western blot analysis whether amifostine affected tyrosine nitration of actin and α-tubulin in HUVEC. Amifostine decreased nitration of actin up to 24 h after its addition in HUVEC medium (Fig. 7A and B). Tyrosine nitration of α-tubulin was decreased up to 1 h after treatment of cells with the drug, was reversed to control levels at 3 h and significantly decreased again 24 h after amifostine addition in HUVEC medium (Fig. 7A and C).

DISCUSSION

Previous studies in our laboratory have shown that amifostine inhibits angiogenesis *in vivo*, by modulating the expression of several genes important for angiogenesis.[12] Since endothelial cells have a pivotal role in angiogenesis,[11] in the present work, we studied the effect of amifostine on endothelial cell functions related to the angiogenic process.

Amifostine exerted a biphasic effect on HUVEC proliferation, being inhibitory at lower and stimulatory at higher than 1 mM concentrations. The decrease in HUVEC proliferation caused by the lower used concentrations of amifostine is probably due to free thiol oxidation taking place in the cell culture medium containing FCS, as suggested by its reversal by catalase. FCS contains transition metals and it has been demonstrated that if transition metals are available to catalyse the oxidation of the thiols to their corresponding disulfides and if the thiols are present at concentrations lower than 1 mM, thiols can extracellularly generate H_2O_2 and subsequently hydroxyl radicals, which are toxic to cells.[23] A biphasic toxicity of WR-1065 was also observed in Chinese hamster fibroblasts, where cell killing occurred at about 0.2 to 1.0 mM thiol, but not at higher or lower drug concentrations. The toxicity was prevented by the addition of catalase, while

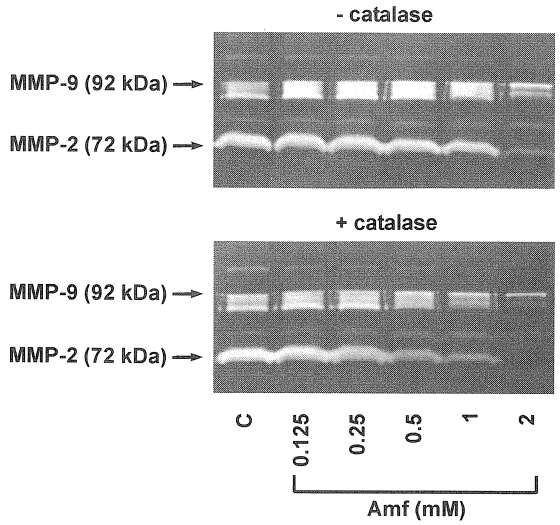

FIGURE 6 Effect of amifostine on MMPs secretion from HUVEC. Fifty il of the culture media of cells treated with different concentrations of amifostine, in the absence or presence of 600 U/ml catalase, were analysed by zymography, as described in "Materials and Methods" section. Representative gels of four independent experiments. C: Control, untreated HUVEC; Amf: HUVEC treated with different concentrations of amifostine, as indicated.

superoxide dismutase had no effect.[24] In contrast to the effect of amifostine on normal cells, the drug had no stimulatory effect on C6 rat glioma cell proliferation, at concentrations up to 8 mM (data not shown).

On the other hand, the increase in HUVEC proliferation caused by the higher used concentrations of amifostine seems to be due to H_2O_2 produced by intracellular degradation of amifostine by Cu-dependent amine-oxidases,[3,25] since it is reversed by sodium pyruvate, an intracellular scavenger of H_2O_2. H_2O_2 increases the activity of Raf-1, which in turn activates MEK1 and the extracellular signal-regulated kinases (ERK)[26] and through this pathway, it leads to an increase in cell proliferation.[27] It is well known that low concentrations of intracellular H_2O_2 stimulate endothelial cell proliferation,[22] something that is also supported by the fact that sodium pyrouvate by itself caused a decrease in HUVEC proliferation, which was approximately 25% at the concentration of 10 mM sodium pyrouvate (data not shown).

Agents that increase intracellular cAMP, like forskolin and isoproterenol, reversed HUVEC proliferation induced by amifostine in a concentration-dependent manner. Since amifostine had no effect on cAMP levels in HUVEC, these data lead to the hypothesis that the effect of the cAMP elevating agents is due to interference with the H_2O_2-induced signalling pathways that increase cell proliferation. It has been shown that treatment of cells with forskolin or isoproterenol inhibits Raf-1 and reduces H_2O_2-induced ERK activity.[28,29]

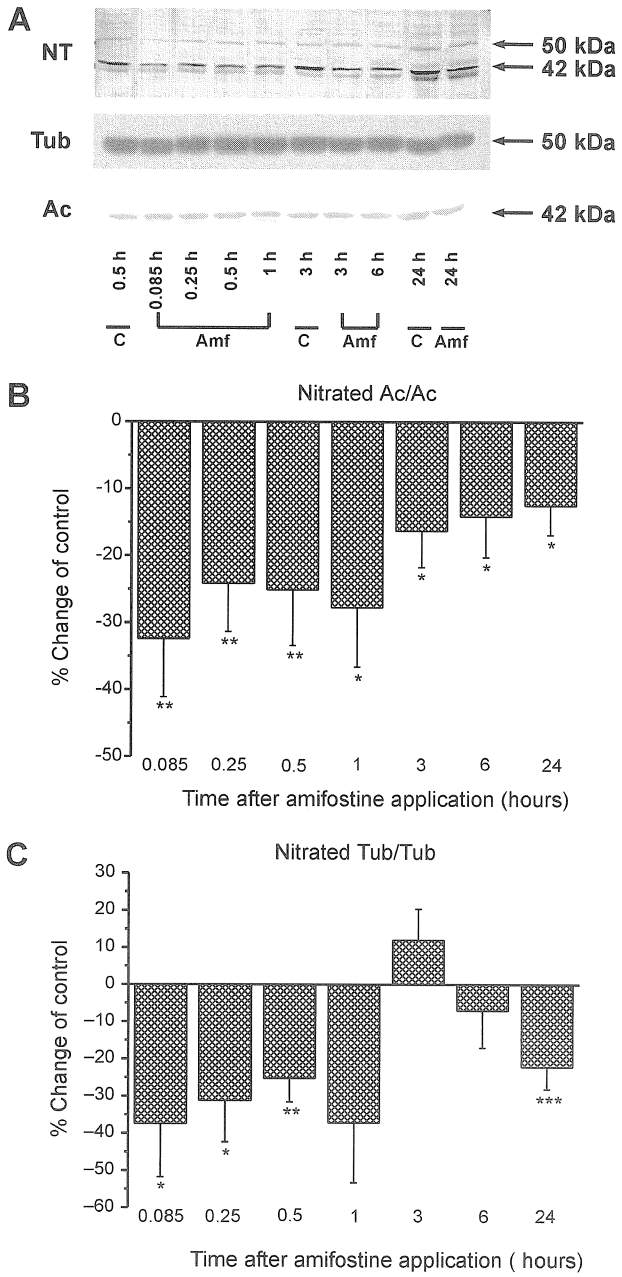

FIGURE 7 Effect of amifostine (2 mM) on the tyrosine nitration of actin and α-tubulin in HUVEC. (A) Western blot analysis of 3-nitrotyrosine (NT), α-tubulin (Tub) and actin (Ac) at several time periods after amifostine application. C: control, untreated HUVEC; Amf: HUVEC treated with 2 mM amifostine. Representative picture of six independent experiments. The protein amounts were quantified by densitometric analysis of the corresponding bands and the ratio of nitrated actin/actin (B) and nitrated α-tubulin/α-tubulin (C) was calculated for each lane. Results are expressed as mean ± SEM of the % change of the fraction of nitrated actin and α-tubulin to total actin and α-tubulin respectively, compared to untreated HUVEC. $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

Treatment of mammalian cells with WR-1065 results in an increase in spermidine synthesis.[30] In order to test if this is also happening in HUVEC, we studied the effect of L-valine, an inhibitor of polyamine synthesis,[31] on the amifostine-induced HUVEC proliferation. L-Valine partially inhibited

amifostine-induced HUVEC proliferation, an indication that this effect is partially due to induction of polyamine synthesis by WR-1065, besides the role of its own conversion to polyamine-resembling molecules. Polyamines are essential for endothelial cell proliferation[32] and can be considered a source of H_2O_2 through their cellular metabolism.[33] The latter may be the mechanism through which they affect HUVEC proliferation in the present study, as supported by the complete inhibition of amifostine-induced proliferation by sodium pyruvate, as discussed above.

There are very few studies concerning amifostine and endothelial cell proliferation, which are controversial. In line with our results are data that show that amifostine increases proliferation of human endothelial cells at the concentration of 4.6 mM.[16] On the other hand, it has been mentioned that amifostine inhibits proliferation of bovine aortic endothelial cells in a concentration dependent manner, at concentrations ranging between 0.5 and 4 mM.[17] In the latter case, amifostine was added to the cells only for 2 h and then washed away, while proliferation was estimated after 24 or 48 h. Therefore, a difference in the culture conditions or the different origin of cells (bovine vs. human) may be the reason(s) for the controversial results.

Amifostine significantly inhibited HUVEC migration in a concentration dependent manner, in line with previous studies using bovine aortic endothelial cells.[18] The decrease in migration was not affected by catalase or sodium pyrouvate and was reversed by L-valine. As discussed above, WR-1065 seems to increase spermidine synthesis in HUVEC, which does not affect migration.[34] Maybe amifostine enhances the degradation of L-arginine through the arginase pathway, thus reducing the amounts that are available for nitric oxide (NO) synthase and NO formation. This hypothesis is supported by the facts that exogenous L-arginine reversed amifostine-induced decrease in HUVEC migration, amifostine had an inhibitory effect on cGMP levels in HUVEC and L-NAME decreased migration of HUVEC (Polytarchou and Papadimitriou, unpublished data). It has also recently been shown that amifostine inhibits lipopolysaccharide-induced NO production in mouse macrophages[35] and the expression of the iNOS gene *in vivo*.[12] The decrease in endothelial cell migration is also in line with the decreased tube formation on matrigel *in vitro* (this study) and the inhibition of angiogenesis *in vivo* by amifostine.[12]

In line with our results on migration are also data showing that treatment of murine sarcoma Sa-NH cells with WR-1065 inhibited the ability of cells to migrate through matrigel membranes.[9] In the same study, WR-1065 inhibited in a concentration- and time-dependent manner the enzymatic activity of MMP-2 and MMP-9. We also found that the amounts of these MMPs released into the culture medium of HUVEC were decreased after amifostine application and this decrease was concentration-dependent and unaffected by catalase. These results agree with our previous *in vivo* studies, where we found that amifostine decreased the gene expression of pro-MMP-2.[12]

Finally, we studied the effect of amifostine on the tyrosine nitration of actin and α-tubulin. We have previously shown that under routine culture conditions, both actin and α-tubulin are tyrosine nitrated in HUVEC[21] and their tyrosine nitration is decreased by L-NAME (data not shown). In the present work, we found that amifostine decreased 3-nitrotyrosine levels of actin and α-tubulin, in line with our previous data *in vivo*.[10] In line with our previous data is also the fact that we observed a differential effect, depending on the cytoskeletal protein studied. Amifostine decreased tyrosine nitration of actin up to 24 h after drug application, while the decrease in tyrosine nitration of α-tubulin had two phases: an initial decrease up to 1 h after drug application, which was reversed at later time points and became significant again at 24 h after amifostine addition into the culture medium. The reasons for these differences are not clear. Concerning α-tubulin tyrosine nitration, the first decrease may be due to a direct free radical scavenging effect of amifostine, while the decrease at 24 h could be secondary to another effect of amifostine, such as increase in the expression of superoxide dismutase observed after 12 h.[36] 3-Nitrotyrosine detection has been used as an index of peroxynitrite formation.[37] Therefore, the decreased levels observed after amifostine application on HUVEC may be due to the decreased production of NO, as discussed above.

In conclusion, the effects of amifostine on endothelial cells seem to depend, at least to a significant degree, on pathways that affect the redox status of the cells, either by producing H_2O_2 or by modulating production of NO.

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