Impact of therapeutically induced reactive oxygen species and radical scavenging by α-tocopherol on tumor cell adhesion

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Abstract. Many tumor treatment modalities such as ionizing radiation or some chemotherapy induce reactive oxygen species (ROS) resulting in therapeutic cell damage. The aim of this study was to analyze whether such ROS induction may affect the mechanical stability of solid tumor tissue by degradation of the extracellular matrix proteins or by a loss of cell adhesion molecules. Additionally, the protective impact of α-tocopherol treatment on these processes was studied. Experimental DS-sarcomas in rats were treated with a combination of localized 44˚C hyperthermia, inspiratory hyperoxia and xanthine oxidase in order to induce pronounced oxidative stress. A second group of animals were pretreated with α-tocopherol. The *in vivo* expression of E- and N-cadherin, α-catenin, integrins αv, ß3 and ß5 as well as the expression of the integrin dimer αvß3 were assessed by flow cytometry. The activity of the matrix metalloproteinases MMP-2 and -9 and the activity of the urokinase-type plasminogen activator (uPA) were determined by zymography. The expression of E-cadherin, the αv-, ß3-integrin and the αvß3-integrin dimer was significantly reduced by ROS induction, an effect which was at least partially reversible by α-tocopherol. N-cadherin, α-catenin and the ß5-integrin expression was not affected by ROS. In addition, MMP-2, MMP-9 and uPA activities were markedly reduced immediately after hyperthermia. Whereas 24 h later the effects on MMP-2 and -9 were no longer evident, for uPA the impact of oxidative stress became even more pronounced at this time. These results show that several processes responsible for the structural stability of the tumor tissue are affected by therapeutic ROS generation. Changes in some of the markers assessed suggested a decrease in tissue stability upon ROS induction, whereas others indicated changes which could lead to a more stable tumor cell cluster. Depending on the individual tumor entity ROS may therefore influence the mechanical stability of solid tumors and by this affect metastatic behavior.

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Abbreviations: ECM, extracellular matrix; HT, localized hyperthermia; i.p., intraperitoneal; i.v., intravenous; MMP, matrix metalloproteinase; ROS, reactive oxygen species; uPA, urokinase-type plasminogen activator; XO, xanthine oxidase

Key words: adhesion molecules, matrix metalloproteinases, urokinase-type plasminogen activator, cadherins, integrins, oxygen radicals

Introduction

The integrity and stability of tumor tissue is dependent on the contact between the tumor cells (e.g., via intercellular adhesion molecules) and on the cell-matrix-interaction which is formed between the cytoskeleton of the cells and extracellular matrix proteins. The structure of these connections is responsible for the stability of the tissue. For instance, down-regulation of cadherins and catenins as elements of the cell-matrix-interaction result in an increased shedding of tumor cells into the blood and have therefore been thought to be responsible for an enhanced metastatic spread of tumor cells (1,2).

The extracellular connecting tissue cannot however be considered as a stable framework. Especially during tumor growth when angiogenesis is taking place, the extracellular matrix (ECM) is subject to continuous remodeling. Matrix-degrading enzymes (matrix metalloproteinases; MMPs) degrade connecting tissue fibers, whereby the enzyme activity is under the control of MMP-inhibitors (TIMPs; tissue inhibitors of matrix metalloproteinases). For this reason, the activity of matrix-degrading enzymes plays an important role in the metastatic behavior of tumors (3), although these enzymes also seem to be of importance for other cellular mechanisms such as induction of apoptosis and deregulation of cell division (4). The expression of various MMPs as well as of adhesion molecules is under the control of local factors such as cytokines, oncogene products and growth factors (5,6).

Besides cytokines, a number of parameters related to the metabolic microenvironment of tumors have been identified, which affect the expression of adhesion molecules and MMPs *in vitro*. Hypoxia for instance, which is a common phenomenon in solid-growing tumors, can increase the expression of MMPs and down-regulates a number of adhesion molecules

(7,8). Additionally, an *in vitro* study has shown that oxidative stress might affect the expression of integrins (9); in normal tissues, the generation of reactive oxygen species (ROS) was shown to lead to the increased activity of MMPs (10-13).

Since many non-surgical tumor treatment modalities such as ionizing radiation, chemotherapy with alkylating agents or hyperthermia induce ROS which in turn are responsible for therapeutic cell damage, the question arises as to whether therapeutically induced oxidative stress may affect cell-cell- or cell-matrix-interactions in tumors and in turn may lead to changes in the metastatic behavior. For this reason, the aim of the present study was to analyze whether therapeutic ROS induction can alter the stability of solid tumor tissue through either degradation of the extracellular matrix proteins or through the loss of the cellular proteins responsible for cell-cell- and cell-matrix-contact *in vivo*. The expression of various adhesion molecules as well as the activity of different matrix-degrading enzymes after a ROS-generating treatment was therefore assessed. At the same time, if ROS was found to play a role in these processes, the question arises as to whether scavenging of oxygen radicals by α-tocopherol may present a possibility of counteracting this potentially unfavorable effect.

Materials and methods

Materials. All chemicals were obtained from Sigma (Deisenhofen, Germany) unless indicated otherwise.

Animals. Male Sprague-Dawley rats (Charles River Wiga, Sulzfeld, Germany; body weight 150 to 200 g) housed in the animal care facility of the University of Mainz were used in this study. Animals were allowed access to food and acidified water *ad libitum* before the investigation. All experiments had previously been approved by the regional animal ethics committee and were conducted in accordance with the German Law for Animal Protection and the UKCCCR Guidelines (14).

Tumors. Solid DS-sarcomas were induced by injection of DS-sarcoma cells (0.4 ml, ~10^4 cells/μl) subcutaneously into the dorsum of the hind foot. Tumors grew as flat, spherical segments and replaced the subcutis and corium completely. Volumes were determined by measuring the three orthogonal diameters (d) of the tumors and using an ellipsoid approximation with the formula: $V = d_1 \times d_2 \times d_3 \times \pi/6$.

ROS-generating treatment. A previous study demonstrated that 44˚C hyperthermia (HT) for 60 min caused a marked generation of reactive oxygen species, an effect which was even more pronounced when HT was combined with xanthine oxidase application and the animals were allowed to breathe pure oxygen (15). For this reason, the same treatment protocol was used to induce ROS in tumors *in vivo* in the present study. Tumors were treated when they reached a volume of 0.5 to 1.0 ml, ~7 to 10 days after implantation.

HT was performed by heating the tumor in a saline bath (9 g/l NaCl) set to a temperature of 44.3˚C. For this, animals were anesthetized with pentobarbital (40 mg/kg, i.p., Narcoren™ , Merial, Hallbergmoos, Germany) and placed on a polystyrene board in a ventral position above the saline bath. The tumor-bearing legs were immersed into the saline through holes in the polystyrene layer so that the tumors were completely submerged for 60 min. The saline bath temperature was set at 44.3˚C which resulted in a temperature of 44.0˚C in the center of the tumor as confirmed in previous studies (15).

Fifteen minutes prior to HT, animals received an i.v. injection of 15 units/kg body weight of xanthine oxidase (XO). XO was previously dissolved in distilled water at a concentration of 7.5 units/ml. During HT, animals additionally breathed pure oxygen spontaneously (respiratory hyperoxia) which was flushed around the nose and mouth of the animal at a flow rate of 2 l/min using a loosely fitting face mask. Control animals were also anesthetized, but not treated with HT, XO or oxygen breathing.

α-tocopherol treatment. One group of animals received α-tocopherol (vitamin E emulsion, Fresenius Kabi, Bad Homburg, Germany) prior to ROS-generating therapy. Tocopherol was injected i.v. at a dose of 10 mg/kg. To find the optimal application course for a significant increase in tocopherol levels in tumor tissue, two different schedules were tested: (a) one dose of α-tocopherol; 2 mg, 24 h prior to hyperthermia and (b) two doses; 2x2 mg, 48 and 24 h prior to HT. Both schedules significantly increased the vitamin E concentration within the tumor. However, the effect of two applications was markedly greater (tocopherol concentration 0.288±0.035 μmol/g protein) than the single dose (0.215±0.013 μmol/g protein) (16). For this reason, the schedule involving the application of two 2 mg doses, 48 and 24 h prior to the HT treatment, was used in the present study.

Treatment groups. The experimental groups can be described as follows: Group 1 (control): Animals were neither treated with ROS-inducing HT nor with α-tocopherol. Group 2 (oxidative stress): Animals underwent ROS-inducing treatment (HT+XO injection + oxygen breathing), but received no vitamin E. Group 3 (vitamin E): Animals were treated with two injections of α-tocopherol, but no HT was applied. Group 4 (oxidative stress + vit. E): Animals received two i.v. injections of vitamin E, 48 and 24 h prior to ROS-inducing treatment.

MMP-zymography. Approximately 6 cryosections (40 μm) of each tumor were lysed in 200 μl HEPES buffer (10 mM HEPES, pH 7.2, 250 mM sucrose, 0.5% Triton X-100) and centrifuged at 20,000 x g (4˚C, 10 min). Protein content was assessed using the RC DC protein assay (BioRad, Munich, Germany). Protein (12 μg) was mixed with Laemmli buffer and applied to a 10% SDS polyacrylamide gel containing 1 mg/ml gelatine. The electrophoretic separation was performed at 100 V over 75 min. Afterwards, the gel was washed using 50 mM Tris (pH 7.5) with 2.5% Triton X-100 and incubated overnight in a buffer containing 50 mM Tris pH 7.5, 150 mM NaCl, 10 mM $CaCl_2$, 0.1% Triton X-100. Subsequently, gels were stained with Coomassie (0.5% Coomassie, 30% methanol, 10% acetic acid) and the MMP-2 and MMP-9 bands were made visible by destaining (30% methanol, 10% acetic acid). The densitometric analysis of the bands was performed using E.A.S.Y. Win32-software (Herolab, Wiesloch, Germany).

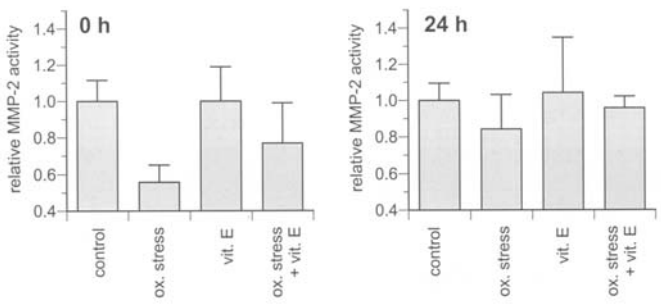

Figure 1. Relative MMP-2 activity in DS-sarcoma cells measured by zymography immediately (0 h) and 24 h after ROS-inducing localized hyperthermia treatment (oxidative stress), after α-tocopherol application (vit. E) or a combination of both treatments (ox. stress + vit. E). Values of 6 tumors are expressed as means ± SEM.

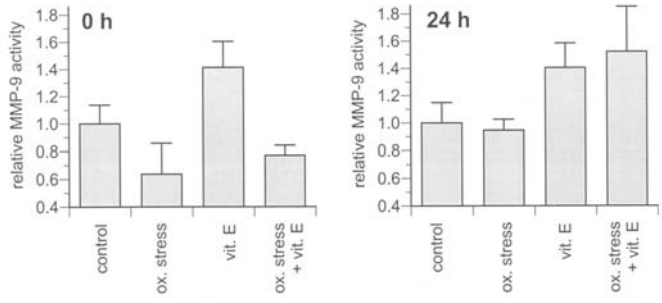

Figure 2. Relative MMP-9 activity in DS-sarcoma cells measured by zymography immediately (0 h) and 24 h after ROS-inducing hyperthermia treatment (oxidative stress), after α-tocopherol application (vit. E) or a combination of both treatments (ox. stress + vit. E). Values of 6 tumors are expressed as means ± SEM.

uPA-zymography. The detection of the urokinase-type plasminogen activator (uPA)-activity was performed by MMP-zymography as described above but with the following modifications (17): the 10% SDS gel contained 0.3% α-casein and 0.25 units/ml plasminogen instead of gelatine. For each sample, 6 μg of protein was applied. The washing buffer contained 100 mM Tris pH 8.1/2.5% Triton X-100 and the incubation buffer 50 mM Tris pH 8.1/10 mM $CaCl_2$. Since gels without the addition of plasminogen do not show uPA-bands, the identification of specific uPA-bands was achieved by the comparison of gels with and without plasminogen.

Measurements of in vivo expression of adhesion molecules. The expression of various adhesion molecules was determined by flow cytometry after staining extra- or intracellular antigens. The expression of the following proteins was analyzed: E-cadherin (antibody Clone G-10; all antibodies were supplied by Santa Cruz Biotechnology, Santa Cruz, CA, USA), N-cadherin (clone D-4), α-catenin (clone H-297), integrin αv (clone N-19), integrin ß3 (clone F-11), integrin ß5 (clone E-19). In addition, the expression of the integrin dimer αvß3 (clone 23C6) was analyzed. For measurements of the expression *in vivo*, tumors were excised 24 h after treatment and mechanically disaggregated to produce a single cell suspension. Following lysis of erythrocytes, DS-sarcoma cells were incubated with the primary antibody. For intracellular

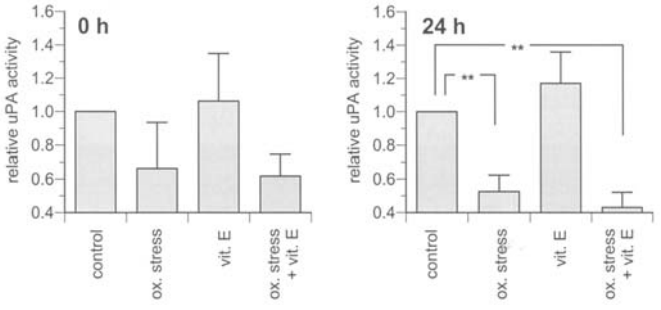

Figure 3. Relative activity of the urokinase-type plasminogen activator (uPA) in DS-sarcoma cells immediately (0 h) and 24 h after ROS-inducing hyperthermia treatment (oxidative stress), after α-tocopherol application (vit. E) or a combination of both treatments (ox. stress + vit. E). Values of 6 tumors are expressed as means ± SEM, $^{**}p<0.01$.

antigens (e.g., α-Catenin), cells were initially fixated and permeabilized (Fix & Perm-Kit, ADG, Kaumberg, Austria). In instances where unlabeled primary antibodies were used, the cells were subsequently incubated with a fluorochrome-labeled secondary antibody and the fluorescence intensity was determined using a flow cytometer (Epics, Coulter, Hialeah FL, USA). Cells marked with unspecific primary antibodies (isotypic negative control) served as references. The values measured in each tumor were normalized to those of the control group.

Statistical analysis. Results are expressed as means ± SEM. Differences between groups were assessed by the two-tailed Wilcoxon test for unpaired samples. The significance level was set at α=5% for all comparisons.

Results

MMP- and uPA-activity. The activity of MMP-2, MMP-9 and uPA was determined immediately after ROS-inducing treatment and 24 h later in order to also study late effects resulting from changes in protein expression. Both MMP-2 and MMP-9 showed reduced activity (however, statistically not significant) in the 'oxidative stress' group and in the group receiving 'oxidative stress + vitamin E' (Figs. 1 and 2). These changes were only detectable immediately after hyperthermia, being no longer evident 24 h after treatment.

In contrast to the short-term changes observed with the MMPs, the activity of the urokinase-type plasminogen activator was affected by the reactive oxygen species for at least 24 h. In both groups receiving hyperthermia with or without additional vitamin E supplementation, the uPA activity was seen to be reduced by 40% immediately after treatment (Fig. 3). This effect was even more pronounced 24 h later when the activity in both groups was reduced by more than 50% indicating a long-term impact of ROS on this proteolytic system.

Expression of adhesion molecules. The expression of several adhesion molecules was markedly affected by the ROS-inducing hyperthermia treatment. The expression of these molecules was measured only 24 h after treatment since it was expected that changes in protein expression would require

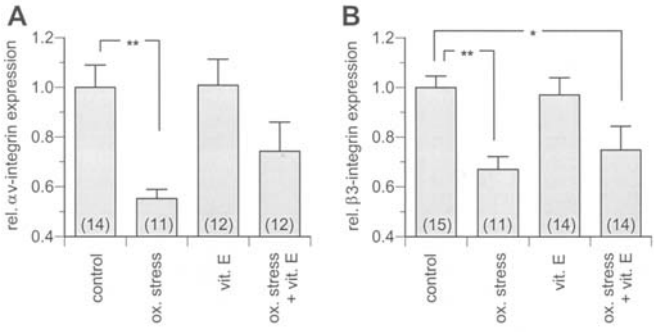

Figure 4. Relative expression of (A) αv- and (B) ß3-integrin in DS-sarcoma cells 24 h after ROS-inducing hyperthermia treatment (oxidative stress), after α-tocopherol application (vit. E) or a combination of both treatments (ox. stress + vit. E). Values are expressed as means ± SEM. Number of tumors investigated in brackets, $^{*}p<0.05$, $^{**}p<0.01$.

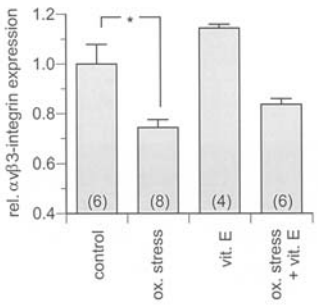

Figure 5. Relative αvß3-integrin expression in DS-sarcoma cells 24 h after ROS-inducing hyperthermia treatment (oxidative stress), after α-tocopherol application (vit. E) or a combination of both treatments (ox. stress + vit. E). Values are expressed as means ± SEM. Number of tumors investigated in brackets, $^{*}p<0.05$.

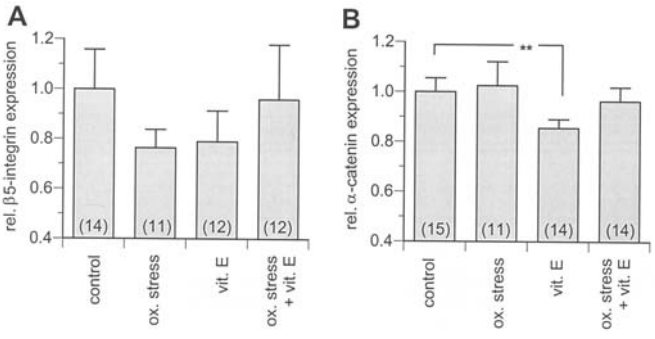

Figure 6. Relative expression of (A) ß5-integrin and (B) α-catenin in DS-sarcoma cells 24 h after ROS-inducing hyperthermia treatment (oxidative stress), after α-tocopherol application (vit. E) or a combination of both treatments (ox. stress + vit. E). Values are expressed as means ± SEM. Number of tumors investigated in brackets, $^{**}p<0.01$.

several hours to take place. Oxidative stress led to a significant reduction in the expression of the αv- and the ß3-integrin by 45 and 33%, respectively (Fig. 4). Upon pre-treating tumors with α-tocopherol, this decreased expression was still detectable. Vitamin E however, reduced the impact of ROS as indicated by a gradual increase in integrin expression. Vitamin E itself had almost no effect on expression (Fig. 4).

Since integrins form heterodimers in order to anchor cells at the matrix proteins, a second experimental series was performed to assess whether the αvß3-integrin dimer was also affected by ROS or whether both integrins were expressed differentially together with other partners. It was found that the αvß3-dimer was also down-regulated by the oxidative stress and that vitamin E pre-treatment also slightly reduced this effect (even though the impact of α-tocopherol was not statistically significant) (Fig. 5).

Both ß5-integrin and intracellular α-catenin were almost unaffected by ROS-induction or vitamin E application (Fig. 6). Only the group treated with α-tocopherol but without subsequent hyperthermia showed a slight decrease in α-catenin. Since this change in expression was not seen in the group receiving vitamin E + oxidative stress treatment, it is not clear whether the observed tocopherol-induced catenin decrease is of any specific importance.

Finally, the expression of E-cadherin was also lowered by ~30% due to oxidative stress, an effect which was found to be partially reversible by pre-treatment with vitamin E (Fig. 7). The expression of N-cadherin was independent of all treatments used in the cell line investigated.

Discussion

Several non-surgical tumor treatment modalities induce oxidative stress to obtain therapeutic cell damage. The question arises as to whether the induction of reactive oxygen species (ROS) might affect the stability of solid tumor tissue either through degradation of extracellular matrix proteins or by a loss of the cellular adhesion molecules. If ROS play a role in these processes, then the scavenging of oxygen radicals (e.g., by α-tocopherol) may present a possibility of counteracting this effect. Previous studies have shown that two applications of vitamin E (2 mg each) 48 and 24 h prior to the ROS-inducing treatment led to a marked increase in tocopherol levels in tumor tissue by a factor of 2 (16).

One important parameter for the metastatic potential of a tumor is the stability of the cell cluster which is mainly dependent on the cell-cell- and the cell-matrix-interaction. In many tumors, the cell-cell contact is based on various cadherins which are connected to the cytoskeleton by intracellular catenins. Down-regulation of cadherins and catenins but also mutations of the respective genes can lead to an increased shedding of tumor cells into the blood and subsequently to a higher metastatic spread as has been shown in a number of studies (1,2,18,19).

In the present study, E-cadherin expression was significantly reduced by ~30% upon oxidative stress (Fig. 7A). In animals pretreated with α-tocopherol, this effect was found to be at least partially reversible although E-cadherin expression in this group was still markedly lower than in control animals. Similar results have been observed in normal tissues. The implantation of superoxide dismutase (SOD)-deficient mammary cells was found to lead to defects in the development of mammary glands due to a lack of E-cadherin expression (20), which the authors attributed to an E-cadherin down-regulation induced by oxidative stress in these experiments. However, other studies have shown no or only a marginal reduction in the cellular E-cadherin content

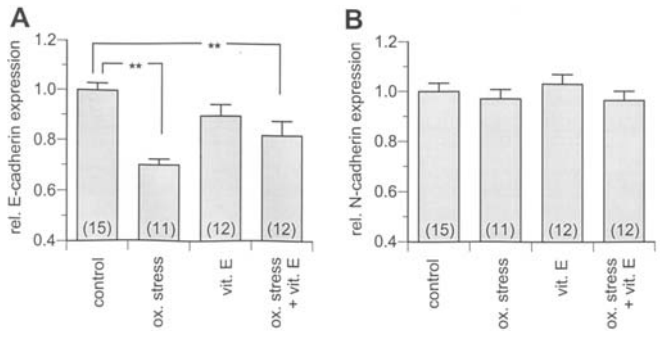

Figure 7. Relative expression of (A) E- and (B) N-cadherin in DS-sarcoma cells 24 h after ROS-inducing hyperthermia treatment (oxidative stress), after α-tocopherol application (vit. E) or a combination of both treatments (ox. stress + vit. E). Values are expressed as means ± SEM. Number of tumors investigated in brackets, $^{**}p<0.01$.

following oxidative stress (9,21,22). However, these studies were able to show that ROS led to a translocation of E-cadherin from the outer cell membrane to intracellular compartments which leads to a reduction in tumor cell adhesion (9,22). Since in the present study E-cadherin expression was measured only at the outer cell membrane using flow cytometry, the findings shown in Fig. 7A are in good accordance with the results of these cell culture studies. Oxidative stress may indeed loosen the tumor cell cluster by a loss of E-cadherin which is otherwise necessary for cell-cell-contacts.

Besides cell-cell-interaction, the adhesion of cells at the extracellular matrix as mediated by integrins is of importance for the stability of tumor tissue. It has been found that tumor cells which have been shed into the blood stream often show reduced integrin expression (23). On the other hand, integrins play an important role not only in the loosening of the tumor cell cluster but also in the adhesion and migration of circulating tumor cells in new host tissue, i.e., metastasis. Several studies have found that tumors with a decreased expression of the αv-integrin or in which the αv-integrin was blocked showed a reduced metastatic spread (24-26). In addition, it has been demonstrated that integrins affect tumor angiogenesis and the activity of MMPs and by this may modulate metastatic behavior (27,28).

In the present study, ROS-inducing hyperthermia led to a significant reduction of the αvß3-integrin which could be at least partially reversed by the application of α-tocopherol (Figs. 4 and 5). Due to the different effects of these integrins in the formation of metastases, it is not possible to predict *a priori* whether this leads to an increase in metastatic behavior (due to the loosening of cells) or to a reduced formation of metastases (as a result of a decreased adhesion of circulating tumor cells in the new host tissue). One other study has analyzed the impact of reactive oxygen species on integrin expression. Mori *et al* (9) showed that long-term exposure of cells to H_2O_2 *in vitro* led to an increased expression of the integrins α2, α6 and ß3. The differences in the findings of the present study concerning ß3-integrin remain unclear. It may be that the duration of the oxidative stress plays a pivotal role since in our experiments it was applied for only 1 h whereas in the study of Mori *et al* the cells were exposed to oxidative stress for 48 to 96 h. At the same time, the cell type investigated may be of importance: in the study of Mori *et al* a mammary gland epithelial cell line (exposed to ROS *in vitro*) was investigated whereas in the present study a tumor cell line was used (exposed to ROS *in vivo*).

Another important parameter for tumor growth, angiogenesis and metastatic behavior is the activity of matrix-degrading enzymes. For this reason, the activity of the matrix metalloproteinases (MMP) 2 and 9 as well as of the urokinase-type plasminogen activator (uPA) were measured. Previous studies in normal tissue cells (fibroblasts, endothelial cells) indicate that the expression and activity of MMP-2 and 9 are increased by oxidative stress (10-13). For hepatic stellate cells it has been demonstrated that ROS (induced by the application of xanthine + xanthine oxidase) increase the activity of MMP-2 and by this the invasiveness of the cells, a phenomenon that could be counteracted by antioxidants (29). However, changes in the MMP-2 and 9 expression induced by ROS show large differences between various cell lines and a pronounced dependency on the dose of oxidative stress (30). In contrast to the findings in normal cells, the MMP-2 and 9 activities in the DS-sarcoma cell line used in the present study were markedly reduced immediately after ROS-inducing treatment. Since in the present study a massive oxidative stress was induced, this may lead to a degradation of the MMPs rather than an activation. It is known that peroxynitrite activates pro-MMPs in the presence of glutathione (31). However, with the localized hyperthermia treatment used in the present study a rapid glutathione depletion takes place (32), possibly resulting in an inability to activate MMP due to the lack of glutathione. Finally, the activity of matrix metalloproteinases depends on the expression of MMP inhibitors (TIMPs). It has been shown that besides an induction of MMP expression, ROS are also able to induce TIMP expression and activity (33). The decrease in MMP activity found in the present study can therefore also be the result of a TIMP induction by oxidative stress.

Tissue remodeling by the enzyme plasmin is dependent on the activity and expression of the plasminogen activator, the expression of the plasminogen activator receptor and the activity of specific inhibitors (PAI). In experimental studies, all of these components have been shown to be affected by reactive oxygen species (34-36). However, since these parameters act antagonistically on the formation of plasmin, the overall effect of ROS on the matrix degradation activity (as measured by zymography) cannot be predicted. In the present study, therapeutic ROS induction led to a marked decrease in this enzymatic pathway which was most pronounced 24 h after treatment (Fig. 3). From the present results it is not possible to elucidate whether ROS decreased the urokinase-type plasminogen activator expression or decreased the inhibitor's activity.

The results of the present study clearly show that various parameters relevant for the stability of the tumor tissue are affected by reactive oxygen species. The possibility that ROS induction is responsible for these effects on tumor cell adhesion is supported by the observation that for several parameters (e.g., MMP-2 activity, αv-integrin and E-cadherin expression), the impact of ROS is at least partially antagonized by pretreatment with the ROS scavenger α-tocopherol (Figs. 1,

4A and 7A). Considering these findings, the question arises as to whether α-tocopherol also reduces the efficacy of ROS-generating treatment modalities. In previous experiments however, in which the same tumor and treatment model were used, no reduction in the therapeutic cytotoxicity of the ROS treatment was seen following the administration of α-tocopherol (37).

In conclusion, the results of the present study clearly show that many of the processes responsible for the stability of tumor tissue (cellular expression of cadherins, catenins and integrins, activity of matrix-degrading enzyme systems) are affected by a pronounced therapeutic ROS generation. In some cases, reactive oxygen species decrease the mechanical stability of the solid tumor (e.g., by down-regulation of intercellular adhesion molecules) although changes leading to a more stable tumor cell cluster (e.g., by down-regulation of matrix-degrading enzyme activity) were also found. For this reason, it can be concluded that ROS *per se* do not seem to have a purely pro- or anti-metastatic impact. Instead, depending on the individual tumor entity, ROS may influence the structural stability of the solid tumor and by this affect the metastatic behavior.

Acknowledgements

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