Antioxidants Modify the Effect of X Rays on Blood Vessels

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Abstract. Background: It was recently shown, with the chicken embryo chorioallantoic membrane (CAM) model, that X rays decrease the number of blood vessels within the first hours after irradiation. In the present study, the possible role of reactive oxygen species (ROS) and nitric oxide (NO) in this effect of X rays was evaluated. Materials and Methods: An area of 1 cm² of the CAM, restricted by a plastic ring, was irradiated at room temperature, in the presence or absence of the tested agents. The number of vessels was measured 48 h after irradiation of the tissue. Results: Superoxide dismutase and tempol, which are superoxide ion scavengers and catalase, a hydrogen peroxide scavenger, had additive effects, while dimethylsulfoxide, a hydroxyl radical scavenger, reversed the vascular targeting effect of X rays. The combination of X rays with W1400, a selective inducible NO synthase (NOS) inhibitor, had an additive effect on the decrease in number of CAM blood vessels. In contrast, L-NAME, a non-selective NOS inhibitor and D-NAME, its inactive analog, reversed the vascular-targeting effect of X rays, possibly due to their ability to act as potent hydroxyl radical scavengers. Conclusion: The above data collectively suggest that hydroxyl radicals mediate the damaging effects of X rays on CAM blood vessels, while antioxidants against other ROS do not protect against the vascular-targeting effect of X rays.

Ionizing radiation is thought to damage the endothelium (1-3) and thus decrease the number of blood vessels (4). Although the exact mechanisms of action of ionizing radiation on tissues remain unclear, it is well known that X rays cause the production of reactive oxygen species (ROS), leading to the manifestation of tissue oxidative stress that involves cytotoxic effects (5). Major ROS are superoxide ion, hydroxyl radical and hydrogen peroxide.

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Key Words: X rays, reactive oxygen species, hydroxyl radicals, blood vessels, nitric oxide synthase.
The CAMs of at least five eggs were dissected, cut into small pieces and washed five times with sterilized phosphate-buffered saline (PBS) pH 7.4. They were then placed in 24-well plates (each well contained approximately 1 mg protein) in Ham’s F10 medium that did not contain phenol red. 1400W was added at the indicated concentrations and the CAMs were incubated for 24 h at 37°C and 5% CO₂. After completion of the incubation, the samples were collected and centrifuged at 7,000 xg for 2 min in a microcentrifuge. Nitrites were measured in the supernatant with the use of the Griess reagent, as previously described (8). Total protein amounts were measured in the tissue pellets using the Bradford assay. The results are expressed as nmol NO₂⁻/mg total protein.

Figure 1. Effect of ROS scavengers in combination with X rays (10 Gy) on the number of CAM blood vessels. The tested agents in the same final volume of 20 µl were applied on a CAM area of 1 cm² restricted by a plastic ring on day 9, 15 min before irradiation of the tissue, as described in Materials and Methods. After 48 h of incubation at 37°C, the CAMs were fixed, excised from the eggs, photographed and the total length of the vessel network was measured using image analysis software. The results are expressed as mean ± S.E.M. of the % change of the number of vessels in treated compared with untreated, non-irradiated tissue (control). IR, irradiation; SD, SOD 300 u/cm²; TE, tempol 580 nmol/cm²; CT, catalase 100 u/cm²; DS, DMSO 25 nmol/cm². Asterisks denote a statistically significant difference from the control. *p<0.05, **p<0.01, ***p<0.001.

Statistical analysis. The significance of variability between the results from each group and the corresponding control was determined by unpaired t-test or ANOVA. Each experiment included at least triplicate measurements for each condition tested and all the results are expressed as mean±S.E.M. from at least three independent experiments.

Results

Effect of ROS scavengers in combination with X rays on the number of blood vessels in vivo. In order to evaluate the possible involvement of ROS in the effect of X rays on the number of CAM blood vessels, the influence of different antioxidants were studied in combination with irradiation. As shown in Figure 1, the combination of SOD, tempol and catalase with X rays caused a greater decrease in the number of CAM blood vessels, compared with the effect of X rays alone. In contrast, DMSO completely reversed the effect of X rays.

Effect of X rays on iNOS protein levels. The effect of X rays, on iNOS protein levels was also investigated. As shown in Figure 2, the iNOS protein levels decreased 6 and 24 h after irradiation. This effect was reversed at later time-points, similarly to the pattern observed at the mRNA level (15).
Effect of NOS inhibitors in combination with X rays on the number of blood vessels in vivo. The combination of dexamethasone, which inhibits iNOS mRNA transcription and activity in the CAM (8), with X rays, caused a greater decrease in the number of CAM blood vessels compared with the effect of X rays alone (Figure 3). In contrast to the effect of dexamethasone, the combination of the non-selective NOS inhibitor L-NAME with X rays completely reversed the effect of the latter (Figure 3). D-NAME produced results similar to those observed for L-NAME (Figure 3), although it had no effect on CAM iNOS activity (8). In order to explain this, 1400W, a selective iNOS inhibitor, was employed. 1400W decreased both iNOS activity and the number of CAM blood vessels in a dose-dependent manner (Figure 4). This effect was not due to toxicity, as verified on CAM paraffin sections stained with eosin-hematoxylin (data not shown). The combination of 1400W with X rays caused a greater decrease in the number of CAM blood vessels, compared with the effect of X rays alone (Figure 3).

Discussion

We have previously shown that X rays initially decrease the number of CAM blood vessels due to increased apoptosis within the first hours after irradiation (4, 12). In the present study, it was shown that this effect of X rays seems to be due to the production of hydroxyl radicals, since it was reversed by the hydroxyl radical scavenger DMSO. This notion is in line with our previous results, showing that the effect of X rays on CAM blood vessels was reversed by amifostine (16), a well established hydroxyl radical scavenger (17) and is in agreement with data suggesting that the production of hydroxyl radicals by X rays correlated with radiation-induced apoptosis (18).

Hydroxyl radicals have also been reported to inhibit NOS activity (19) and may further be responsible for the X ray-induced decreases in iNOS expression and activity. The subsequent increase in iNOS expression (this study and 15) may be one of the mechanisms that the tissue utilizes in order to achieve cellular free radical homeostasis (19) and restore normal function.

Inducible NOS is the only NOS isoform detected to date in the CAM (8, 20), and seems to play a significant role in the formation and/or stability of blood vessels under physiological angiogenesis of the tissue (8). The data from this study support this notion, since the specific iNOS inhibitor, 1400W, also inhibited angiogenesis to a similar
degree with L-NAME or dexamethasone (8). The combination of iNOS inhibitors with X rays further decreased the number of CAM blood vessels, although their mechanism of action seems to be different. Inducible NOS inhibitors inhibited CAM angiogenesis without being toxic or inducing apoptosis (this study and 8), while the effect of X rays is probably due to vascular targeting rather than to inhibition of angiogenesis (4, 12, 15).

Scavengers for superoxide or hydrogen peroxide decrease CAM angiogenesis through, at least partly, the down-regulation of iNOS expression and activity (8). In accordance with the discussion above on the role of iNOS in blood vessel formation and stability, as well as the effect of iNOS inhibitors in combination with X rays on the number of CAM blood vessels, the down-regulation of iNOS by superoxide or hydrogen peroxide scavengers could also explain their synergistic effect on the decrease in CAM blood vessels when administered in combination with irradiation.

In contrast to the effect of dexamethasone or 1400W on the X ray-induced decrease in the number of CAM blood vessels, L-NAME completely reversed the effect of irradiation. This effect does not seem to be due to NOS inhibition, since the inactive analog D-NAME had a similar effect at the same concentration, although it did not affect CAM angiogenesis or NOS activity (8). The effect of L-NAME and D-NAME on the X ray-induced decrease in the number of CAM blood vessels is probably due to the ability of both agents to act as potent hydroxyl radical scavengers (21).

In conclusion, the results of the present study suggest that hydroxyl radicals mediate the early effects of X rays on CAM blood vessels in vivo.

References


Received March 7, 2006
Accepted April 28, 2006