Low dose gamma irradiation enhances defined signaling components of intercellular reactive oxygen-mediated apoptosis induction

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Abstract. Transformed cells are selectively removed by intercellular ROS-mediated induction of apoptosis. Signaling is based on the HOCl and the NO/peroxynitrite pathway (major pathways) and the nitryl chloride and the metal-catalyzed Haber-Weiss pathway (minor pathways). During tumor progression, resistance against intercellular induction of apoptosis is acquired through expression of membrane-associated catalase. Low dose radiation of nontransformed cells has been shown to enhance intercellular induction of apoptosis. The present study was performed to define the signaling components which are modulated by low dose gamma irradiation. Low dose radiation induced the release of peroxidase from nontransformed, transformed and tumor cells. Extracellular superoxide anion generation was strongly enhanced in the case of transformed cells and tumor cells, but not in nontransformed cells. Enhancement of peroxidase release and superoxide anion generation either increased intercellular induction of apoptosis of transformed cells, or caused a partial protection under specific signaling conditions. In tumor cells, low dose radiation enhanced the production of major signaling components, but this had no effect on apoptosis induction, due to the strong resistance mechanism of tumor cells. Our data specify the nature of low dose radiation-induced effects on specific signaling components of intercellular induction of apoptosis at defined stages of multistep carcinogenesis.

1. Introduction
Activation of membrane-associated NADPH oxidase (NOX-1) is functionally connected to oncogenic transformation [1-5]. Extracellular superoxide anions generated by NOX-1 and their dismutation product hydrogen peroxide are crucial for the control of proliferation, maintenance of the transformed state and tumor progression [1-3,5-8]. However, superoxide anions also drive specific elimination of transformed cells through reactive oxygen species (ROS)-mediated intercellular induction of apoptosis [9-15]. [In this paper the term ‘ROS’ comprises classical reactive oxygen species like superoxide anions, HOCl, hydrogen peroxide or hydroxyl radicals as well as reactive nitrogen species like nitric oxide (NO) or peroxynitrite]. During ROS-mediated intercellular induction of apoptosis, superoxide anion-generating transformed cells represent the ‘target cells’, whereas nontransformed ‘effector cells’ contribute to intercellular induction of apoptosis through the release of a novel peroxidase (POD) and nitric oxide. As transformed cells also exhibit effector cell functions (release of POD and/or NO), the interaction of target and effector functions of transformed cells can establish autocrine, ROS-mediated apoptotic signaling [16-18]. The interaction of nontransformed and transformed cells, as well as the autocrine interaction of transformed cells establishes the same four ROS signaling pathways that cause
Figure 1: Intercellular ROS-mediated apoptotic signalling. 

**A** Intercellular ROS signaling between nontransformed and transformed cells and autocrine apoptosis induction in transformed cells. **B** Catalase protects tumor cells against intercellular and autocrine ROS-mediated apoptosis signaling. Tumor cells show membrane-bound catalase on their surface [16,18]. Catalase, in its classical reaction, decomposes hydrogen peroxide (H$_2$O$_2$) and thus blocks HOCl signaling (as shown in the figure), as well as the nitryl chloride pathway and the metal-catalyzed Haber-Weiss reaction (not shown in the figure). Catalase also efficiently decomposes peroxynitrite (ONOO$^-$) and thus blocks the NO/peroxynitrite pathway. In addition, compound I of catalase can oxidize NO to NO$_2$ and thus counteract peroxynitrite formation.
selective apoptosis in transformed target cells (for review see [13-15]). Thereby, the HOCl [11] and the NO/peroxynitrite signaling pathway [4, 11] are the dominant pathways, whereas the nitryl chloride pathway [19] and the metal-catalyzed Haber-Weiss reaction [20] seem to be of minor importance. The details of ROS signaling via the HOCl and the NO/peroxynitrite pathway are shown in figure 1A.

Transformed target cells exhibit activated NADPH oxidase (NOX-1) in their membrane and generate extracellular superoxide anions (#1). Superoxide anions dismutate spontaneously to hydrogen peroxide and oxygen (#2). Transformed as well as nontransformed effector cells release duox-coded peroxidase (POD) (#3). This release is triggered by TGF-beta (not shown in the figure). POD utilizes hydrogen peroxide and chloride anions to synthesize HOCl (#4). HOCl then interacts with superoxide anions in the vicinity of the transformed target cell membrane, thereby yielding hydroxyl radicals (#5) that cause lipid peroxidation in the membrane (#6). Lipid peroxidation is followed by ceramide generation, activation of the mitochondrial pathway of apoptosis, activation of caspases 9 and 3, followed by apoptosis (details are not shown in this figure). In the case of excess hydrogen peroxide compared to POD, hydrogen peroxide and HOCl interact in the consumption reaction shown under (#7). This consumption reaction inhibits HOCl signaling. NO synthase (NOS; either iNOS, nNOS or eNOS, depending on the cell system) in target and effector cells utilizes the substrate arginine to synthesize NO (#8). NO readily permeates cellular membranes. It may be consumed by hydrogen peroxide in a complex consumption reaction (#9) [52] that will be described in detail elsewhere. Alternatively, NO interacts with superoxide anions to form peroxynitrite (#10). Peroxynitrite can be protonated to peroxynitrous acid that spontaneously decomposes into NO and apoptosis inducing hydroxyl radicals (#11). For clarity of the graph, the two minor signaling pathways, i.e. the nitryl chloride pathway and the metal-catalyzed Haber-Weiss reaction have not been included. The nitryl chloride pathway utilizes HOCl that interact with nitrite to form apoptosis-inducing nitryl chloride. The metal-catalyzed Haber-Weiss reaction depends on hydrogen peroxide that reacts with ferrous ions to yield hydroxyl radicals and hydroxyl anions. The resulting ferric ions are then reduced by superoxide anions back to ferrous ions that undergo the next cycle.

Whereas transformed cells are regularly sensitive for intercellular ROS signaling, ex vivo tumor cells exhibit resistance against this process [16-18,21]. Their resistance was shown to be based on interference of catalase located at the outside of the cell membrane with intercellular ROS signaling [16-18] (figure 1B). Expression of membrane-associated catalase was found to be a regular feature of ex vivo tumor cells and seems to be required for tumor progression [22,23, Bauer et al., in preparation].

Intercellular effects during oncogenesis may either contribute to tumor formation [24-26] or they may alternatively cause selective elimination of transformed cells through ROS-mediated intercellular induction of apoptosis [9,11,13,27]. Interestingly, both types of intercellular effects seem to be affected by radiation [24,28-30]. Radiation affects carcinogenesis in complex ways and at multiple steps [31]. There is an ongoing discussion on the effects of low dose radiation during carcinogenesis [31,32]. Potential controlling effects of low dose radiation on transformation and tumorigenesis have been well established in the literature [33-44]. Low dose radiation has been shown to induce a decrease in observable transformation frequency [33-36,41-43]. Removal of transformed cells through apoptosis has been discussed as a conceivable mechanism for nontargeted antioncogenic low dose radiation effects [30,45,46]. The report by Portess et al. [30] gives a mechanistic explanation for selective elimination of transformed cells by low dose radiation. Their results show that low dose radiation (both of high and low linear energy transfer (LET)) of nontransformed cells caused a marked increase in apoptosis induction in transformed cells [30]. Doses as low as 0.2 mGy alpha particles and 2 mGy gamma ray were sufficient to show distinct effects. Low dose radiation–induced enhancement of intercellular induction of apoptosis was due to the utilization of the same ROS-mediated intercellular pathways as in classical intercellular induction of apoptosis, i.e. primarily the HOCl/hydroxyl radical and the NO/peroxynitrite signaling pathway. At this point of the study, the analysis did not allow to define which signaling components were actually enhanced through low dose
radiation. The use of neutralizing antibodies to TGF-beta-1 revealed a central role of this cytokine during low dose radiation enhancement of intercellular induction of apoptosis. As only a minority of nontransformed cells had been actually hit by radiation and as TGF-beta shows the ability for autoinduction, Portess et al. suggested a model in which nontransformed cells hit by low dose radiation respond both by release of effector molecules and TGF-beta. TGF-beta then triggers neighbouring cells (that had not been hit by radiation) to propagate TGF-beta signaling and effector molecule release.

The decrease in detectable transformation frequency and removal of transformed cells through low dose radiation effects (frequently termed “protective apoptosis mechanism (PAM)”) has become a central element in models for the mathematical simulation of multistage oncogenesis [47-51]. In this paper we present data on the specific effects of low dose gamma irradiation on intercellular induction of apoptosis and autocrine apoptotic selfdestruction and the resultant consequences for the survival of cells at defined stages of multistep oncogenesis.

2. Materials and Methods
Nontransformed murine 208F, transformed 208Fsrc3 and the murine fibrosarcoma cell L929 have been described in reference [16]. The human gastric carcinoma cell line MKN-45 has been described in reference [18]. Media, culture conditions and assays for the quantitation of intercellular and autocrine ROS-mediated apoptosis induction have been described in references [16,18]. These references also describe the sources, concentrations of stock solutions and concentrations of inhibitors used for the characterisation of specific signaling pathways.

Cells (200 000 cells/ml in suspension) were irradiated with a Compagnie Oris (Gif-sur-Yvette, France) IBL 437 Cs137 gamma source. The dose rate was between 35 and 40 mGy/sec for the experiments shown in this study. Immediately after irradiation, the cells were centrifuged and resuspended in fresh medium. Cells were then either used directly in the experiments or incubated for an additional hour at 37 °C in a humidified incubator, centrifuged and resuspended in fresh medium. All other methods are described in the respective figure legends.

3. Results
A novel peroxidase plays a central role for intercellular ROS signaling (Heinzelmann et al., in preparation). Modulation of its release by low dose radiation might explain the recently published effect of low dose radiation on the effector function during intercellular induction of apoptosis [30]. For a direct test of this hypothesis, a new peroxidase test that is based on competition between the catalase/peroxidase mimetic EUK-8 and peroxidase was utilized. This test has been evaluated with myeloperoxidase (MPO) as gold standard and will be published elsewhere. As can be seen in figure 2 A-C, untreated nontransformed fibroblasts, transformed fibroblasts as well as fibrosarcoma cells only released minor concentrations of POD. Nontransformed 208F cells (A), src-oncogene transformed 208Fsrc3 cells (B) and fibrosarcoma cells L929 (C) were irritated with 40 mGy of gamma radiation or not. Cells were then seeded in Costar 24 well tissue culture clusters (20 000 cells/well) in 0.5 ml complete medium. Some cultures received 20 ng/ml TGF-beta or 10 µM of the matrix metalloprotease inhibitor galardin (Sigma-Aldrich, Deisenhofen, Germany), as indicated in the figures. After 24 hours incubation at 37 °C, supernatants were collected, centrifuged for the removal of cells and stored at -20 °C until they were tested for the presence of POD. For testing the POD content, increasing concentrations of the supernatants were added to 12 500 208Fsrc3 cells in 100 µl complete medium in the presence of 20 ng/ml TGF-beta and 75 µM EUK-8 (Calbiochem). Control assays either were kept free of EUK-8 or received 150 µM of the peroxidase inhibitor aminobenzoyl hydrazide (ABH). All assays were performed in duplicate. Assays were incubated for 7 hours (A, B) or 4.5 hrs (C) at 37 °C and then the percentage of apoptotic cells was determined by the classical apoptosis criteria nuclear condensation, nuclear fragmentation and membrane blebbing, using inverted phase contrast microscopy. A minimum of 200 cell was monitored for each individual assay. Controls without EUK-8 showed less that 7 % apoptotic cells (data not shown). EUK-8 induced between 30 and 50 %
apoptotic cells. This reaction depends on HOCl synthesis by EUK-8 followed by HOCl interaction with 208Fsrc3 cell-derived superoxide anions, resulting in hydroxyl radical mediated apoptosis induction. Natural peroxidase present in the supernatants has been found to interfere with EUK-8-mediated apoptosis induction in a complex way of interaction that will be described elsewhere (Ophoven and Bauer, in preparation). 5-10 mU/ml myeloperoxidase (MPO) caused 50% competition. Therefore, competition with EUK-8-mediated apoptosis was indicative for the presence of peroxidase and allowed quantitation. The specificity of the competition reaction was assured by abrogation of competition by the mechanism-based peroxidase inhibitor ABH. This control reaction was performed in all assays. For reasons of simplicity of the graphs, this control is only shown under C.

Treatment of the cells with TGF-beta caused a massive increase in POD release in all three cell systems tested, which resulted in substantial competition. Low dose radiation had the same effect as TGF-beta and proved to be a massive inducer of POD release. In the presence of the matrix metalloprotease inhibitor galardin, no radiation-induced release was observed, indicating that the peroxidase is removed through protease action. The competition by cell-derived peroxidase was abrogated by the mechanism-based peroxidase inhibitor ABH, thus confirming the specificity of the competition reaction.

![Figure 2](image-url)

**Figure 2.** Low dose radiation triggers POD release from nontransformed and transformed cells.

For the evaluation of the significance of radiation-induced effector cell stimulation, a coculture system between transformed cell clumps and dispersely seeded effector cells was used. In this system, a clump of 2000 transformed cells at high local density causes an optimal steady state local concentration of hydrogen peroxide, but the peroxidase concentration is too low for execution of the
HOCl signaling pathway. When normal or transformed effector cells at high number, but low local density, are added to the clumps, these cells compensate for peroxidase but do not undergo apoptosis induction initially, as they show no or only suboptimal hydrogen peroxide production. As shown in figure 3, a clump of src oncogene-transformed cells did not undergo apoptosis even in the presence of TGF-beta. When dispersely seeded nontransformed (figure 3A) or transformed (figure 3B) effector cells had been irradiated before addition the target cell clumps, a dose-dependent increase in apoptosis induction of the transformed target cells was observed. This effect seemed to depend on establishment of intercellular HOCl signaling, as it was efficiently blocked by the peroxidase inhibitor aminobenzyol hydroazide (ABH.)

![Figure 3. Low dose radiation enhances the effector function.](image)

2000 transformed 208Fsrc3 cells in 5µl of medium were spotted as cell clumps into 24 well tissue culture clusters. The cells were incubated at 37 °C in a humidified incubator for about one hour and were overlaid with 0.5 ml complete medium. Nontransformed 208F cells (A) or transformed 208Fsrc3 cells (B) had been irradiated with the indicated low doses of gamma irradiation or had remained irradiated. These cells served as effector cells and were seeded to the clumps of unirradiated transformed cells (8000 effector cells per assay). After the effector cells had attached, they received either no addition, 20 ng/ml TGF-beta or 150 µM of the peroxidase inhibitor ABH. After 21 hours (A) or 28 hours (B) the percentage of apoptotic target cells was determined in duplicate assays.

In order to test for the impact of low dose radiation on extracellular superoxide anion generation, inhibition of superoxide anion-dependent apoptosis induction by Cu/Zn-SOD was used for quantitation. Inhibition by Cu/Zn-SOD usually leads to a bell-shaped inhibition curve that allows the quantitation of relative increases or decreases in superoxide anions. Nontransformed cells, both unirradiated or irradiated, did not show significant extracellular superoxide anion generation, as they did not drive peroxynitrite-dependent apoptosis after addition of an NO donor (data not shown). In contrast, transformed 208Fsrc3 cells responded to the fast decaying NO donor DEA NONOate by efficient (peroxynitrite-mediated) apoptosis induction (figure 4). This reaction was blocked by an SOD optimum of 9 U/ml. When half of the standard number of cells had been applied, and therefore half the concentration of superoxide anions was in the system, the SOD concentration for maximal inhibition was only half that of the standard test, indicating that the test system was suitable to monitor relative...
changes in superoxide anion concentrations. The observed bell-shaped curve allowed easy comparison of the tests. When the 208Fsrc3 cells had been irradiated with 40 mGy of gamma radiation before the test, an eightfold higher concentration of SOD was needed for maximal inhibition, indicating an eightfold enhancement of extracellular superoxide anion production by low dose radiation. Similarly, the inhibition of autocrine apoptosis induction in the gastric carcinoma cell line MKN-45 in the presence of the catalase inhibitor 3-aminotriazole (3-AT) was blocked by SOD in a characteristic way. Low dose irradiated tumor cells required a 32 fold higher concentration of SOD for maximal inhibition of apoptosis. This finding demonstrates the strong impact of low dose radiation on extracellular superoxide anion production by tumor cells.

Cells were seeded in 96 well tissue culture clusters (12 500 cells per well, 100 µl medium). 208Fsrc3 cells received 0.5 mM of the NO donor DEA NONOate for the induction of NO/peroxynitrite-mediated apoptosis induction, in the presence of 20 ng/ml TGF-beta. MKN-45 cells received 150 mM of the catalase inhibitor 3-AT for induction of autocrine ROS-mediated apoptotic selfdestruction. The indicated concentrations of Cu/Zn-SOD were added and the percentages of apoptotic cells were determined after 3.5 hrs (A) or 4 hrs (B) hours. Under (A), half amount of cells was used in a parallel experiment for demonstration of the quantitation of superoxide anion concentration. Additional calibration experiments confirmed a strict linearity between the concentration of superoxide anions and the concentration of SOD necessary for maximal inhibition (data not shown). Control experiments, using the peroxynitrite decomposition catalyst FeTPPS (25 µM) ensured that the reaction used in experiment A was dependent on peroxynitrite formation, control experiments under (B) confirmed autocrine ROS-mediated apoptosis induction through the use of inhibitors of the HOCl pathway [18] (data not shown). 208Fsrc3 cells in the absence of the NO donor and MKN-45 cell in the absence of the catalase inhibitor showed less than 5 % of apoptotic cells at all SOD concentrations (data not shown).

To test for the consequence of low dose radiation-enhanced superoxide anion production by transformed cells, src-transformed cells remained unirradiated or were irradiated with 40 mGy of gamma irradiation. The cells were then placed in small clumps with increasing cell number (figure 5). After the cells had attached, medium in excess and myeloperoxidase (MPO) were added. For control purposes, some of the assays received the HOCl scavenger taurine. As shown in figure 5, MPO-dependent establishment of HOCl signaling and apoptosis induction increased with the density of the

Figure 4. Low dose radiation enhances extracellular superoxide generation in transformed and tumor cells. Transformed 208Fsrc3 cells or human gastric carcinoma cells MKN-45 were irradiated with 40 mGy gamma irradiation or remained untreated.
clumps in unirradiated target cell assays. 500 cells per clump were too low to allow a detectable apoptosis induction. When the target cells had been irradiated before the experiment started, a strong enhancing effect, especially in the previously suboptimal cell density range was observed. This result is well explained by increased superoxide anion generation by the irradiated transformed cells and subsequent increase in hydrogen peroxide generation, the limiting substrate of HOCl synthesis by MPO. When clumps of irradiated src-transformed cells, again at increasing cell densities, were overlayed with nontransformed dispersely seeded 208F effector cells (figure 5B), a reaction analogous to that shown for MPO (figure 5A) was seen. TGF-beta-treated nontransformed cells induced apoptosis in transformed target cells, thereby showing a stringent requirement for sufficient cell density of the target cells. When the target cells had been irradiated before the beginning of coculture, an increased apoptosis induction, indicating an enhanced contribution of the target cells to intercellular signaling, was seen. The specificities of the signaling reactions were confirmed by inhibition mediated by the HOCl scavenger taurine.

Figure 5. Low dose radiation enhances the target cell function.
Transformed 208Fsrc3 cells were irradiated with 40mGy of gamma irradiation or remained unirradiated, and were then seeded as small clumps of the indicated cell number per 5 µl of medium in 24 well tissue culture clusters. After the cells were attached (within less than one hour), 0.5 ml of medium, containing either no addition (“0 mGy”, “40 mGy”) or 200 mU/ml myeloperoxidase (MPO) (“0 mGy + MPO”; “40 mGy + MPO”) were added. In addition, cells irradiated with 40 mGy received 200 mU/ml MPO plus 50 mM taurine (A). In experiment B, 8000 nontransformed 208F cells were added to irradiated or unirradiated transformed cells and the assays received 20 ng/ml TGF-beta or TGF-beta plus 50 mM of the HOCl scavenger taurine, as indicated. Control assays did not receive 208F cells, but TGF-beta. After 31 hours (A) or 28 hours (B) the percentages of apoptotic cells were determined in duplicate assays.

The experiments shown so far demonstrate that low dose gamma radiation strongly enhances POD release from nontransformed, transformed and tumor cells. It also positively modulates superoxide anion generation by transformed and tumor cells, but not by nontransformed cells. There was no effect of low dose radiation on NO release detectable by our test system (data not shown). The experiments shown in figures 3 and 5 demonstrate that removal of a relative small number of transformed cells by neighbouring nontransformed cells is enhanced, when the system is irradiated. Our next question aimed to elucidate the effect of low dose irradiation on a homogeneous population of transformed or tumor cells under conditions, where autocrine apoptosis induction should be possible in principle. Low dose irradiation of transformed 208Fsrc3 cells caused an enhancement of autocrine apoptosis, when the cells had been seeded at suboptimal cell density, but inhibition, when the optimal density had been
used (data not shown). When the gastric carcinoma cell line MKN-45 was subjected to low dose radiation and then brought to conditions suitable for autocrine apoptosis induction, low dose radiation alone had no apoptosis–inducing effect (figure 6). When, in addition, the protective catalase of the tumor cells was inhibited by the catalase inhibitor 3-AT, low dose radiation enhanced apoptosis induction at suboptimal 3-AT concentrations, but inhibited apoptosis at 3-AT concentrations that had been found to be optimal in the absence of low dose radiation treatment. This shift of the optimum curve due to the action of low dose radiation was reversed when a very small concentration of the catalase mimic EUK-134 removed excess hydrogen peroxide. The analysis of autocrine signaling pathways in MKN-45 cells, without and after low dose radiation revealed that there was not only a shift with respect to the 3-AT concentration, but also a qualitative change in signaling (figure 6). In accordance with previously published work [18], unirradiated MKN-45 cells showed NO/peroxynitrite signaling at low 3-AT concentrations and then a gradual relative increase in HOCl signaling at higher 3-AT concentrations.

Figure 6. Low dose radiation affects autocrine signaling in tumor cells after catalase inhibition. Gastric carcinoma cells MKN-45 were irradiated with either 75 mGy of gamma irradiation or remained untreated. After irradiation, the cells were incubated for 1 hour at 37 °C and then centrifuged, washed and resuspended in fresh medium. 12 500 cells per 100 µl medium were placed in 96 well tissue culture clusters and received the indicated concentrations of the catalase inhibitor 3-AT, both in the absence or presence of 0.5 µM of the catalase mimic EUK-134. Some assays were treated in addition with 50 mM of the HOCl scavenger taurine or 25 µM of the peroxynitrite decomposition catalyst FeTPPS. The percentages of apoptotic cells were determined after 3 hours. Note, that in the absence of catalase inhibitor, low dose radiation had no apoptosis-inducing effect on the tumor cells.
The same signaling was observed when small concentrations of EUK-134 had been added to nonirradiated cells. Removal of excess hydrogen peroxide partially reversed the supraoptimal inhibition at very high 3-AT concentrations. When MKN-45 cells had been irradiated at low dose before the experiment, HOCl signaling was dominant at all 3-AT concentrations, without any indication of NO/peroxynitrite signaling. After addition of 0.5 µM EUK-8, the pattern of signaling pathways was completely reversed to the situation of the unirradiated cells, together with a backshift of the optimum curve. These findings indicate that low dose gamma radiation a) has a stimulating effect on suboptimal signaling via the HOCl pathway; b) causes inhibition of the NO/peroxynitrite pathway, which can be replaced by HOCl signaling, and c) causes inhibition of optimal HOCl signaling, especially under conditions of autocrine apoptosis induction.

4. Discussion

This study determines the effect of low dose radiation on intercellular signaling chemistry exerted by nontransformed, transformed and tumor cells. Without irradiation, the nontransformed cells contribute to intercellular ROS signaling (figures 7, 8) through release of effector molecules like POD or NO (please see figure 1 for details), but show no target cell function due to lack of extracellular superoxide anion generation. Transformed cells contribute both by the release of effector molecules and extracellular superoxide anion generation. They thus drive the efficiency and selectivity of intercellular ROS-mediated as well as autocrine apoptosis induction. Tumor cells exhibit target and effector cell functions but block intercellular signaling through interference by a membrane-associated catalase that removes hydrogen peroxide, destroys peroxynitrite and oxidizes NO [18]. Tumor cells can be converted back experimentally to the state of transformed cells when their catalase is inhibited. This experimental approach allows to study the specific signaling chemistry of tumor cells after abrogation of their catalase-mediated protection.

Figure 7. Multistep carcinogenesis and intercellular ROS signaling without irradiation. Nontransformed cells require several distinct steps to reach the transformed state [15,18,53]. Transformed cells generate extracellular superoxide anions that drive the efficiency and selectivity of intercellular ROS signaling between nontransformed effector cells and transformed target cells (please see signaling details in figure 1) as well as autocrine ROS-mediated signaling. This causes apoptosis selectively in transformed cells and may represent a natural control step in oncogenesis. TGF-beta is a central mediator to trigger peroxidase release (not included into the schematics). During tumor progression, tumor cells establish resistance against intercellular and autocrine apoptotic signaling through expression of membrane-associated catalase [16,18].
Low dose radiation induces peroxidase release from nontransformed, transformed and tumor cells with comparable efficiency (figure 8). In this figure, low dose gamma irradiation leads to the release of peroxidase (POD) from nontransformed (left), transformed (middle) and tumor cells (right) with similar efficiency. Low dose gamma irradiation causes a 8-32 fold increase in extracellular superoxide anion generation in transformed and tumor cells, but not in nontransformed cells. There is no indication on an increase in NO release from our experiments (data not shown). The increase of signaling components may have different effects on apoptosis induction, dependent on density and nature of the cells. Nontransformed cells remain unaffected, as they do not generate extracellular superoxide anions that are necessary for apoptotic signaling. In nontransformed cells, the increase in superoxide anion and peroxidase production may enhance apoptosis (+), especially under previously suboptimal conditions for apoptosis induction (e.g. low target cell density). The increase in superoxide anion generation by transformed cells may have a negative effect on intercellular induction of apoptosis when the resultant excess hydrogen peroxide (after dismutation of superoxide anions) consumes HOCl (please see figure 1 for details of this consumption reaction). Also, intercellular signaling based on NO/peroxynitrite signaling is inhibited by excess hydrogen peroxide. The increase of superoxide anion generation by tumor cells does not lead to apoptosis induction per se, as the resultant potential increase in ROS signaling is efficiently counteracted by tumor cell membrane-associated catalase. Therefore, low dose radiation affects primarily transformed (premalignant) cells, however in different possible ways. The effects of low dose radiation on tumor cells require parallel modulation of catalase activity, which bring the tumor cells back to the signaling stage of transformed cells.

![Figure 8. Low dose gamma radiation enhances defined signaling components of intercellular reactive oxygen-mediated apoptosis induction](image)

This activity of low dose radiation resembled that of TGF-beta. As the release of peroxidase was inhibited by galardin, an inhibitor of matrix metalloproteases, peroxidase seems to require the action of proteases in order to be set free. The release of peroxidase by irradiated cells had direct consequences for neighbouring transformed cells in which apoptosis was induced. Therefore, the release of POD by irradiated cells explains the effects reported by Portess et al. [30] Ongoing work defines TGF-beta as a central modulator during radiation induced release of POD and the peroxidase domain of duox as the responsible enzyme (Heinzelmann et al., in preparation). Peroxidase release is necessary for the effector cell action of nontransformed against transformed target cells. For establishment of autocrine apoptosis induction within a population of transformed target cells,
membrane-associated peroxidase, not released by matrix metalloprotease can efficiently exert HOCl signaling, but has a negative effect on NO/peroxynitrite signaling due to a destructive effect on peroxynitrite due to high local concentrations of the peroxidase (Bauer, unpublished result).

Low dose radiation also has a marked effect on extracellular superoxide anion production by transformed and tumor cells, whereas nontransformed cells do not show such a response. An ongoing study, using nontransformed cells with an inducible RAS oncogene shows that only cells that already express RAS and generate superoxide anions at a measurable extend are enhanced in their superoxide anion generation by low dose radiation. Therefore, the enhancement of the target cell function superoxide anion release maintains the selectivity of this feature with respect to the malignant phenotype. An obvious consequence of the enhancement of superoxide anion generation is an enhancement in intercellular and autocrine apoptosis induction under conditions that are suboptimal for apoptosis induction without radiation. Such situations are given when a low number or low local density of target cells is present. Under these conditions, a clear positive effect of low dose radiation on the removal of these cells is observed in vitro and can be predicted for the situation in vivo. Thereby, the enhancement of superoxide anion production and POD release can be expected to interact in a synergistic way, establishing optimal HOCl signaling. However, our data also show that opposite effects can be induced by low dose radiation. Irradiation of cells at substantial density and optimal conditions may lead to an excess of hydrogen peroxide (derived from stimulated superoxide anion production) in relationship to available POD. Under these conditions, the consumption reaction between HOCl and hydrogen peroxide may cause a massive or even complete removal of HOCl and thus inhibit the HOCl signaling pathway. In this situation, low dose radiation would have a protective rather than a destructive effect on malignant cells. These findings are in agreement with a recent discussion of the potential impact of low dose radiation on intercellular signaling [15]. The study of catalase-inhibited MKN 45 cells (that had been experimentally brought back to the stage of transformed cells) showed that low dose radiation not only shifted the optimum curve with respect to catalase inhibition, but that it also changed the quality of signaling. Low dose radiation caused the disappearance of NO/peroxynitrite signaling that was observed in unirradiated tumor cells at low concentrations of the catalase inhibitor. The negative effect on NO/peroxynitrite signaling is explained by the increased consumption of NO in the presence of an enhanced hydrogen peroxide concentration and by the obvious lack of low dose radiation to enhance NO generation in a detectable way. Therefore it was not unexpected that signaling in a catalase-inhibited Ewing sarcoma cell line, that was completely and solely dependent on NO/peroxynitrite signaling, was inhibited by low dose radiation to a significant degree (data not shown).

Taken together, our experimental data provide rational explanations for the effects observed in a previous paper [30] and demonstrate the potential of low dose radiation to remove malignant cells in the course of tumor establishment and progression. They thus might represent the basis for the protective low dose radiation effects observed in vitro and in vivo [32-44]. However, it is also shown in this paper that cells with certain specific signaling features, ratios between specific signaling components as well as certain situations with respect to the density of cells may lead to protection of malignant cells rather than to their destruction. Which of the two alternative effects dominates in vivo is not predictable from the data obtained in vitro. Therefore, we suggest not to use the term “protective apoptosis mechanism (PAM)” as synonym for intercellular induction of apoptosis, as this would imply that intercellular ROS signaling always favours removal of malignant cells. Our data indicate that this must not necessarily be always the case. These aspects require further experimentation. It will be a challenge to find out, whether a signaling phenotype restricted to NO / PON signaling is advantageous to transformed cells that exhibit this phenotype, as these cells might be protected from low dose radiation effects through negative effects of increased hydrogen peroxide on NO/peroxynitrite signaling. The specific NO/peroxynitrite signaling phenotype might represent a phenotype that is associated with the potential to escape control by low dose radiation, even in the absence of protective catalase.
Autocrine and intercellular ROS signaling is abrogated in tumor cells that have established protection against intercellular signaling through catalase expression at the outer surface of the tumor cells. Low dose radiation alone does not abrogate resistance, as seen in figure 6. Therefore, the concentration of superoxide anions and hydrogen peroxide reached after low dose radiation seems not to be in the concentration range that is necessary to overrun protective catalase. In a recent paper [16], it had been shown that excess hydrogen peroxide generated by added glucose oxidase (GOX) can re-establish intercellular ROS signaling. The effect of GOX was markedly enhanced by exogenous MPO. Interestingly and in line with these published observations, addition of MPO to low dose irradiated, but not to unirradiated tumor cells caused strong apoptosis induction (data not shown). This finding indicates the potential of low dose radiation to enhance apoptosis induction in tumor cells, when parallel signaling requirements are also provided. We are presently establishing the signaling conditions that, predicted by the detailed knowledge of intercellular ROS signaling, should allow to define situations under which protective catalase of tumor cells is overrun, inactivated or even destroyed by the concerted action of specific signaling components. This rational approach may be instrumental to utilize low dose radiation effects for specific therapeutic applications in the future.

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