Modulation of Intercellular ROS Signaling of Human Tumor Cells

WIBKE BECHTEL* and GEORG BAUER

Abteilung Virologie, Institut für Medizinische Mikrobiologie und Hygiene, Universität Freiburg, D-79104 Freiburg, Germany

Abstract. *Tumor cells are resistant against apoptosis-inducing intercellular reactive oxygen species (ROS) signaling but can be resensitized by the inhibition of catalase. Hydrogen peroxide exhibits a dual role in the modulation of intercellular ROS signaling. When suboptimal concentrations of the catalase inhibitior 3-aminotriazole (3-AT) are applied, additional exogenous hydrogen peroxide shifts apoptosis induction to its optimum. When hydrogen peroxide is added at optimal concentrations of 3-AT, or when higher concentrations of 3-AT are applied, the subsequent consumption between HOCl and hydrogen peroxide blunts overall apoptosis induction. These supraoptimal conditions can be brought back to the optimum through excess myeloperoxidase (MPO), partial removal of hydrogen peroxide through the catalase mimetic EUK-134 or partial inhibition of NADPH oxidase. Exogenous nitric oxide (NO) interferes with HOCl signaling through consumption of hydrogen peroxide. Site-specific generation of hydroxyl radicals at the cell membrane of tumor cells induces apoptosis, whereas random HOCl- superoxide anion interaction, and ferrous iron-induced Fenton chemistry of HOCl inhibit intercellular ROS signaling.*

Transformed cells are characterized by extracellular superoxide anion generation, which have both beneficial and detrimental effects for the cells. The benefical effects are related to proliferation and the transformed state of the cells (1-5). Detrimental effects of transformed cell-derived superoxide anions are due to the establishment of intercellular, reactive oxygen species (ROS)-mediated apoptosis-inducing effects by intercellular induction of apoptosis (through interaction with neighbouring nontransformed cells) (6-11) or in an autocrine mode, termed 'autocrine apoptotic self-destruction' (12). As intercellular induction of apoptosis and autocrine apoptotic self-destruction are based on the same signaling chemistry (except for the source of peroxidase and NO), we have suggested to use the term 'intercellular ROS signaling' for the signaling chemistry involved in both processes. This intercellular ROS signaling has been suggested by us to represent a hitherto unrecognized control point during oncogenesis (9-15). Tumor cells show resistance against intercellular ROS-mediated signaling through interference with intercellular signaling. In another study (12), we demonstrated the central role of a tumor cell catalase for the protection of tumor cells against intercellular ROS-mediated signaling. Enhancement of specific signaling components or inhibition of catalase re-established intercellular ROS signaling and caused apoptotic death of otherwise resistant tumor cells. Protective catalase of tumor cells seems to be located at the outside of the membrane, as it interferes efficiently with extracellular ROS signaling. The location at the outside of the membrane is further substantiated by work in progress, as i) it can be inactivated by extracellular singlet oxygen and ii) can be inhibited by cell-impermeable catalase-specific monoclonal antibodies (Bauer, in preparation). In this study the impact of the balance of the central signaling components of the HOCl signaling pathway for the overall outcome of apoptosis induction were investigated. The aim of these studies is to enable intercellular ROS signaling of tumor cells to be optimized with respect to their specific apoptosis induction after catalase inhibition.

**Present address:* Harvard Medical School, BIDMC, Department of Matrix Biology, Boston, MA 02215, U.S.A.

Correspondence to: Georg Bauer, Abteilung Virologie, Institut für Medizinische Mikrobiologie und Hygiene, Hermann-Herder Strasse 11, D-79104 Freiburg, Germany. e-mail: georg.bauer@uniklinik-freiburg.de

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Materials and Methods

Materials. The sources for the inhibitor of NADPH oxidase activation 4-(2-aminoethyl-benzenesulfonyl fluoride (AEBSF) (15), the mechanism-based peroxidase inhibitor 4-aminobenzoyl hydrazide (ABH) (17, 18), the catalase inhibitior 3-aminotriazole (3-AT) (19), NaOCl/HOCl, the hydroxyl radical-specific scavenger mannitol (20), the HOCl-specific scavenger taurine (21), glucose

oxidase (GOX); myeloperoxidase (MPO) (22), manganese-containing superoxide dismutase (Mn-SOD) (23, 24) and the preparation and storage of their stock solutions has been described in another paper (12).

The catalase mimetic EUK-134 (chloro[[2,2'-[1,2-ethanediyl*bis*[(nitrilo-κN)methylidyne]]*bis*[6-methoxyphenolato-κO]]]-manganese) was obtained from Cayman chemicals, Ann Arbor, Michigan, USA. EUK-134 is a cell-permeable synthetic salen-manganese complex which exhibits catalase activity and has been reported to show superoxide dismutase (SOD) activity. EUK-134 was dissolved in DMSO to reach a concentration of 1 M and was then immediately diluted in Eagle's minimal essential medium (EMEM) containing 5% fetal bovine serum (FBS) to a final concentration of 10 mM. This stock solution was stored at –20˚C. EUK-134 was used in this study in the concentration range between 0.25 and 25 μM. Control assays ensured that the residual DMSO concentrations (between 0.000025 and 0.0025%) had no interfering effect on ROS signaling. It should be noted that DMSO above 0.1% shows hydroxyl radical-scavenging activity. EUK-134 concentrations above 25 μM show apoptosis-inducing activity in many cell systems through complex signaling and thus mask the catalase mimetic activity (Bauer, in preparation). Whereas the catalase mimetic activity of EUK-134 represented an excellent tool in our studies, the reported SOD activity of the compound was without relevance in the cellular system studied. The nitric oxide (NO) donor sodium nitroprusside (SNP) was obtained from Sigma Aldrich (Schnelldorf, Germany). It was kept as a 200 mM stock solution in EMEM at –20˚C. SNP represents a classical and slowly decaying NO donor, used in numerous experimental and clinical studies (24). Xanthine oxidase (XO) and xanthine were obtained from Sigma Aldrich. When xanthine oxidase was applied in the experiments, the medium contained 0.4 mM xanthine. Xanthine oxidase generates superoxide anions.

Media for cell culture have been described in another paper (12).

Cells. The source and culture of the gastric carcinoma cell line MKN-45 has been described in the preceding paper (Bechtel and Bauer). The human B cell tumor cell line Gumbus (25) was a gift from Dr. G. Doelken, Greifswald, Germany. Gumbus cells grow in suspension in RPMI-1640 medium containing 10% FBS and supplements. Maintenance of an optimal density around 5×10^5 cells is critical for low background apoptosis induction.

Autocrine apoptotic self-destruction. The central assay used in this study was autocrine apoptotic self-destruction based on intercellular ROS signaling after inhibition of tumor cell catalase (Bechtel and Bauer, preceding paper). MKN-45 cells were seeded at at density of 12,500 cells/100 μl RPMI-1640 medium containing 10% FBS and supplements in 96-well tissue culture clusters. Gumbus cells were seeded at a density of 25,000 cells/100 μl RPMI-1640 medium containing 10% FBS, supplements and 20% conditioned medium (*i.e.* cell-free supernatant from a optimally growing Gumbus culture of a density around 5×10^5 cells/ml). The concentration of the catalase inhibitor 3-AT and other additions are described in the figure legends. The percentage of apoptotic cells was determined by inverted phase-contrast microscopy based on the classical criteria for apoptosis, *i.e.* nuclear condensation, fragmentation and membrane blebbing as described in another paper (12). All assays were performed in duplicate. The key experiments have been repeated at least ten times with similar results. Statistical analysis has been described in the another paper (12).

Results

In another paper (12), we have shown that tumor cells interfere with intercellular ROS signaling through catalase expression. Ongoing work indicates that the inhibitory catalase is associated on the outside of the cell membrane of tumor cells (Bauer, in preparation). Apoptosis-inducing intercellular ROS signaling was restored in these cells when an excess of hydrogen peroxide (steadily produced by GOX) was added or when hydrogen peroxide-consuming tumor cell catalase was inhibited. Hydrogen peroxide therefore seems to represent a central modulator of intercellular ROS signaling. As hydrogen peroxide, besides driving the HOCl signaling pathway, has also the potential to induce apoptosis directly (27, 28), we endeavoured to define the borderline between hydrogen peroxide-driven HOCl signaling and its direct apoptosis-inducing effect.

Figure 1A confirms our previous finding that GOX up to 40 mU/ml mediated apoptosis induction in tumor cells through re-establishing the HOCl signaling pathway, as the reaction was completely blocked by the catalase mimetic EUK-134 and by the HOCl scavenger taurine. When catalase was inhibited by 25 mM or 100 mM 3-AT (Figure 1B, C), GOX below 20 or 10 U/ml mediated apoptosis induction exclusively through HOCl signaling, as seen by the tight inhibition by taurine. At higher concentrations of GOX and in the presence of 3-AT, apoptosis induction shifted from a HOCl signaling-dependent process to direct apoptosis induction by hydrogen peroxide, as seen by the strong inhibition mediated by the catalase mimetic EUK-134 and diminished inhibition by taurine. In this experiment, the analysis was performed at an relatively early time point, when hydrogen peroxide produced by the cells did not yet contribute to apoptosis induction in the presence of inhibited catalase. At this time point, the reaction measured rather depended completely on hydrogen peroxide produced by GOX.

The experiment demonstrated in Figure 2 measured autocrine apoptosis induction after catalase inhibition by 3-AT. In this setup, no exogenous hydrogen peroxide source was added and measurement was taken at a later time point compared to Figure 1. Apoptosis induction increased with increasing concentrations of 3-AT up to 100 mM, where it reached its maximum. When 3-AT was added at concentrations higher than 100 mM, a sharp decrease in overall apoptosis induction was observed. Based on the assumption that an increase in the concentration of the catalase inhibitor leads to increasing concentrations of free hydrogen peroxide in the test system, this finding indicates that hydrogen peroxide is rate limiting at suboptimal concentrations of catalase inhibitor but that an excess of catalase inhibition, leading to an excess of hydrogen peroxide, has a negative effect on intercellular signaling. This scenario might be explained by the well-known interaction of hydrogen peroxide with HOCl, leading to the generation of water, chloride and oxygen, resulting in

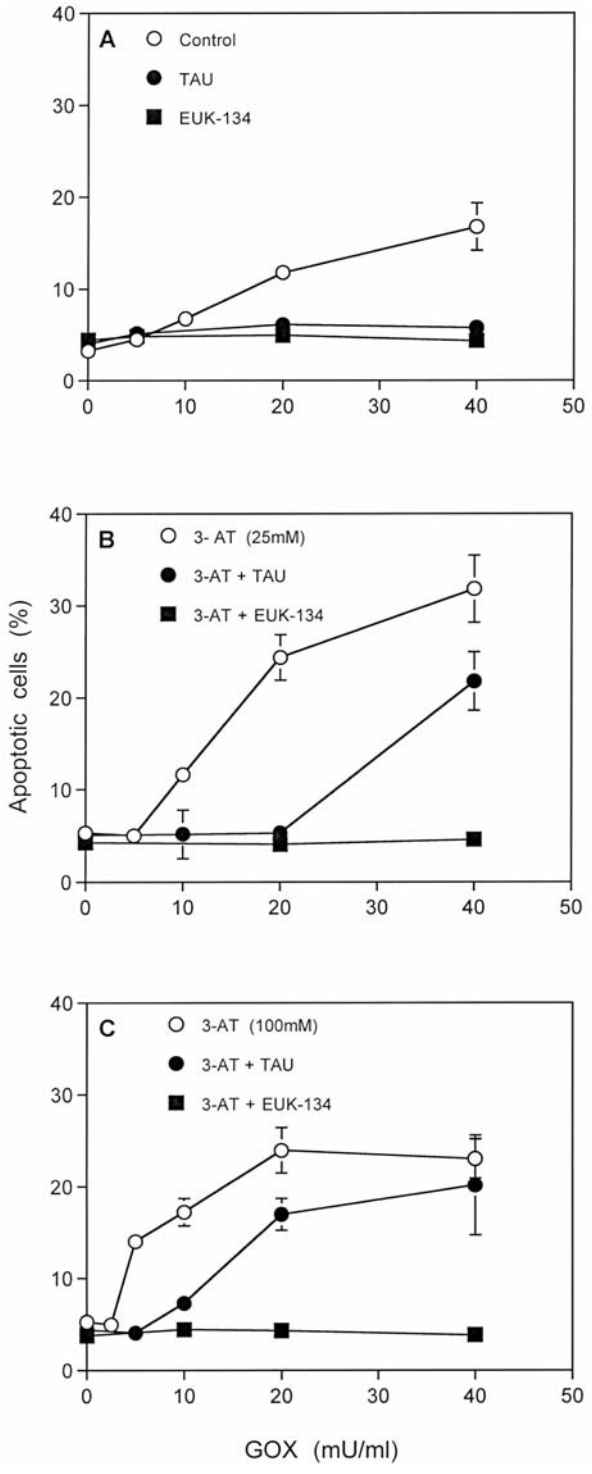

Figure 1. *Apoptosis induction in tumor cells mediated by GOX: from restoration of HOCl signaling to direct hydrogen peroxide action. MKN-45 cells were tested under the conditions for measurement of apoptotic self-destruction in the absence (A) or presence of 25 mM (B) or 100 mM 3-AT (C). Assays received 50 mM taurine or 25 μM EUK-134, where indicated. All assays were performed in duplicate. After 2 h, the percentage of apoptotic target cells was determined by phase-contrast inverted microscopy.*

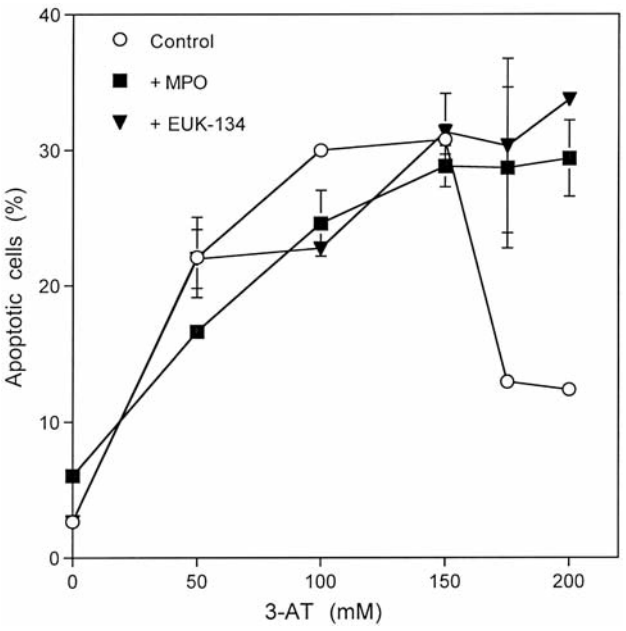

Figure 2. *Addition of MPO or limited removal of hydrogen peroxide shift supraoptimal apoptosis induction at high 3-AT concentration to optimal conditions. MKN-45 cells were tested under the conditions for measurement of apoptotic self-destruction in the presence of increasing concentrations of 3-AT. Assays received no further addition (Control), 200 mU/ml MPO or 0.25 μM EUK-134. All assays were performed in duplicate. After 6 h, the percentage of apoptotic target cells was determined by phase contrast inverted microscopy. Note that the EUK-134 concentration used in this experiment is 100 times lower than that used in the experiment described in Figure 1.*

attenuation of HOCl signaling. Excess free hydrogen peroxide would be present if the concentration of peroxidase was suboptimal relative to the available hydrogen peroxide concentration. If this concept was correct, destruction of excess hydrogen peroxide or addition of peroxidase should counteract the supraoptimal inhibition seen at high 3-AT concentrations. As shown in Figure 2, this was indeed the case. The addition of a very low concentration of the catalase mimetic EUK-134 or alternatively the addition of excess MPO shifted apoptosis induction back to its optimum.

This finding demonstrates the importance of a balance between available hydrogen peroxide and peroxidase in the system. As hydrogen peroxide is derived from dismutation of superoxide anions generated by the tumor cells, its available concentration should be easily modulated by modification of the cell density. Lowering the cell density results in fewer superoxide anions generated per time and volume and also in a lower chance of superoxide anions interacting with superoxide anions from neighbouring cells in the dismutation reaction. As can be seen in Figure 3, the decrease in cell density by 50% caused, as expected, a reduction of apoptosis

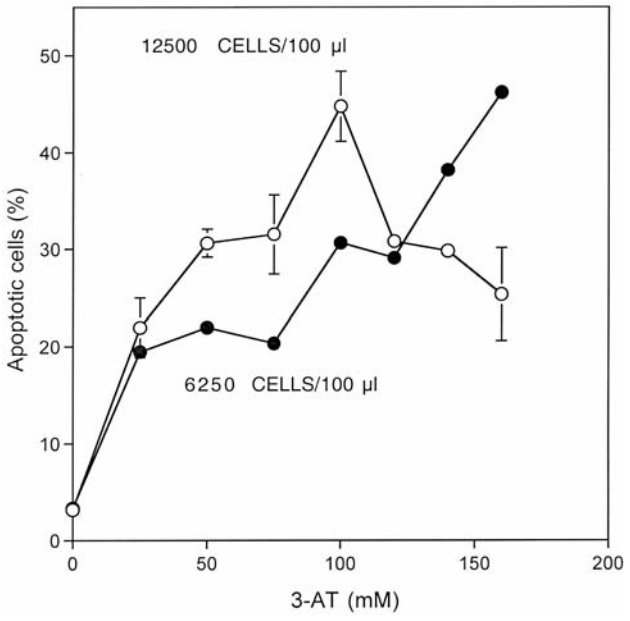

Figure 3. *Effect of cell density on 3-AT-mediated apoptosis induction in MKN-45 tumor cells. MKN-45 cells were tested under the conditions for measurement of apoptotic self-destruction (12,500 cells/100 µl) and at lower cell density (6,250 cells/100 µl) in the presence of the indicated concentrations of 3-AT. All assays were performed in duplicate. After 21 h, the percentage of apoptotic target cells was determined by phase-contrast inverted microscopy.*

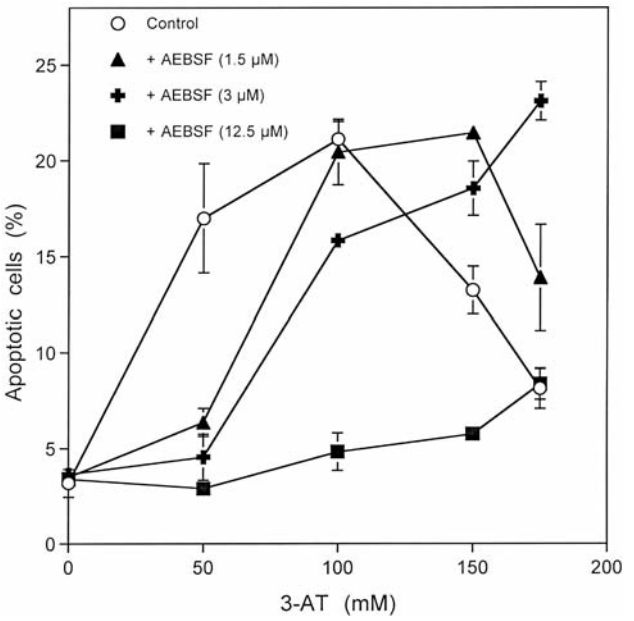

Figure 4. *Effect of gradual inhibition of NADPH oxidase activity on 3-AT-mediated apoptosis induction in human tumor cells. MKN-45 cells were tested under the conditions for measurement of apoptotic self-destruction in the presence of increasing concentrations of 3-AT and the indicated concentrations of AEBSF. All assays were performed in duplicate. The percentage of apoptotic cells was determined after 6.5 h by inverted phase-contrast microscopy.*

induction in the suboptimal and optimal concentration range of 3-AT and a relative increase at higher concentrations of 3-AT, compared to the standard density of cells.

An additional way to modify the available hydrogen peroxide concentration can be achieved by lowering the rate of superoxide anion generation through partial inhibition of NADPH oxidase. Therefore, MKN-45 cells were treated with increasing concentrations of 3-AT in the presence of increasing concentrations of the NADPH oxidase inhibitor AEBSF. The range of the inhibitor concentration was chosen so that only partial inhibition of the oxidase was achieved. This ensured that there were still sufficient superoxide anions for the HOCl/superoxide anion interaction that is necessary for hydroxyl radical formation and apoptosis induction. As can be seen in Figure 4, partial inhibition of NADPH oxidase by 1.55 µM AEBSF caused a shift of the optimum curve to higher 3-AT concentrations, thereby maintaining the typical shape of the curve. This finding demonstrates that lowering superoxide anion generation causes a subsequent decrease in the available hydrogen peroxide concentration. Therefore, in the presence of 1.55 µM AEBSF, the tumor cell catalase requires stronger inhibition, necessitating higher 3-AT concentrations, in order to allow optimal HOCl synthesis as the basis for HOCl-mediated apoptosis signaling. When 3.1 µM AEBSF was applied, a further shift of the curve was observed. In addition, the shape of the curve change from a optimum curve towards a plateau curve. This indicates that the inhibition superoxide anion generating NADPH oxidase by 3.1 µM AEBSF prevented excess hydrogen peroxide generation despite maximal catalase inhibition and therefore attenuation of the HOCl signaling pathway by excess hydrogen peroxide was no longer of relevance. Finally, addition of 12.5 µM AEBSF caused strong inhibition of apoptosis induction over the whole concentration range of 3-AT.

The overt modulating effect of the interplay between hydrogen peroxide and tumor cell catalase during intercellular apoptosis induction allowed the following predictions for the effects of directly added hydrogen peroxide: When inhibition of catalase was suboptimal, exogenously added hydrogen peroxide should enhance apoptosis induction. When catalase inhibition was, however, optimal, addition of exogenous hydrogen peroxide should inhibit apoptosis induction. Therefore, the next experiments were focused on studying the impact of low concentrations of exogenously added hydrogen peroxide on intercellular ROS signaling in tumor cells after inhibition of their protective catalase. The significance of these experiments was to test the predictions from the established signaling concept and to define how the system would be modulated by a variable total hydrogen peroxide concentration. Tumor cells received either no catalase inhibitor

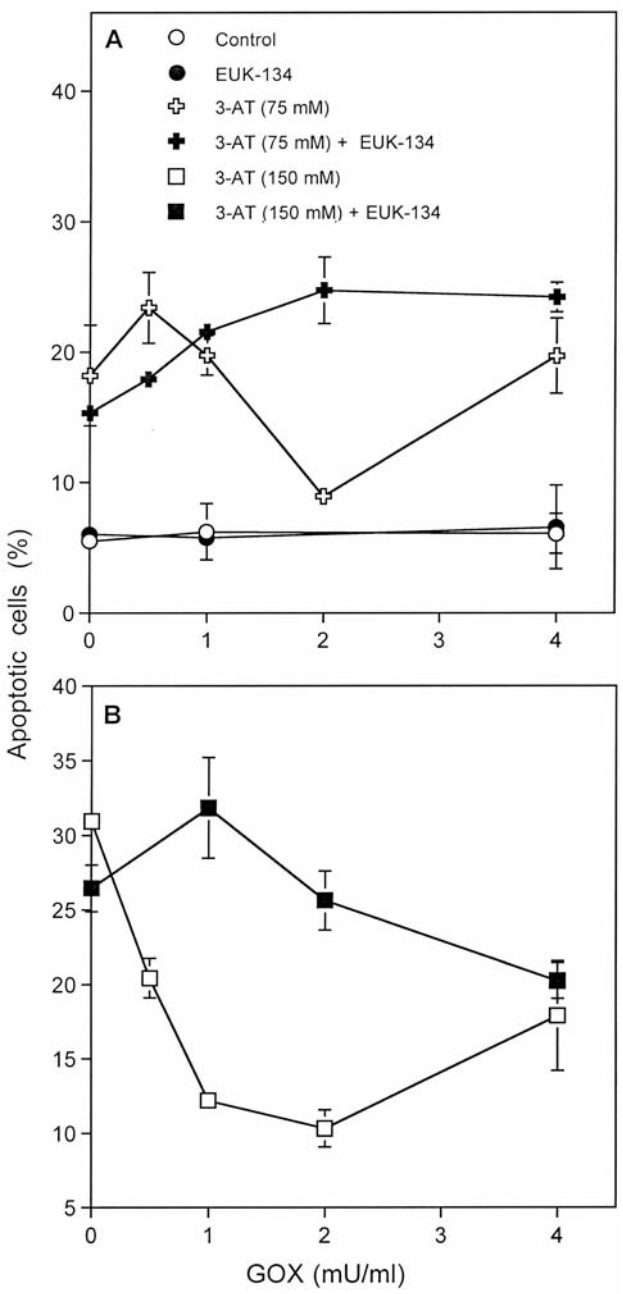

Figure 5. *Adverse effects of exogenous hydrogen peroxide on 3-AT-mediated apoptosis induction in human tumor cells. MKN-45 cells were tested under the conditions for measurement of apoptotic self-destruction in the presence of the indicated concentrations of 3-AT. Where indicated, assays received 0.25 μM EUK-134. All assays were performed in duplicate. The percentage of apoptotic cells was determined by inverted phase-contrast microscopy after 3.5 hours.*

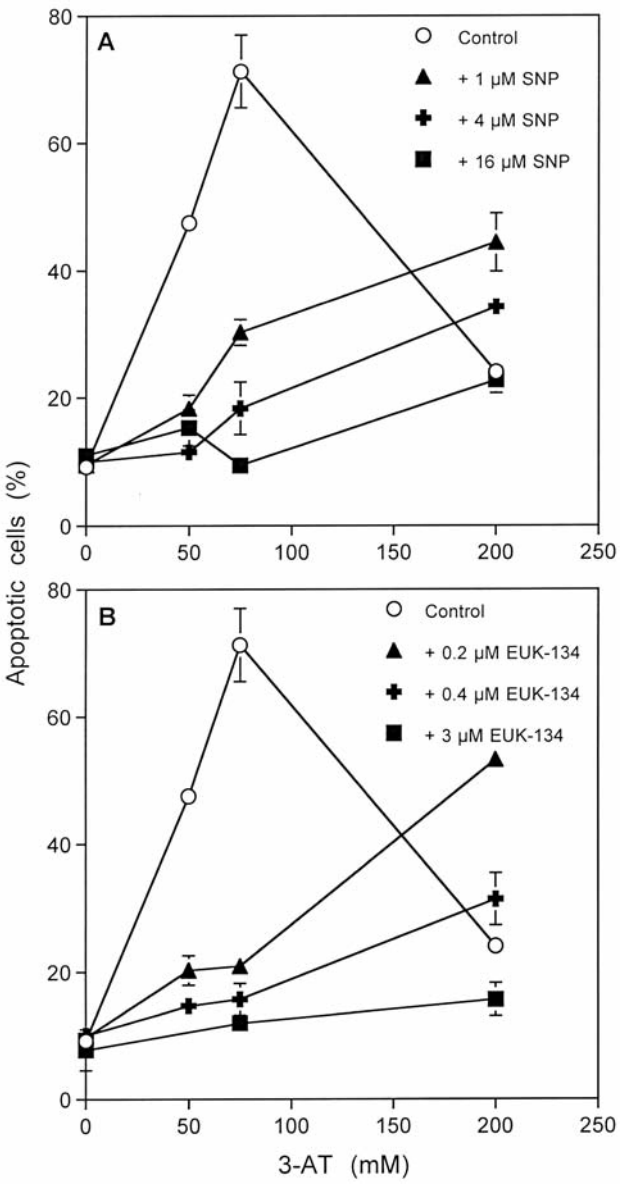

Figure 6. *Interference of nitric oxide with HOCl signaling. Gumbus cells were tested under the conditions for measurement of apoptotic self-destruction in the presence of the indicated concentrations of 3-AT. Assays remained free of further additions (Control) or received the indicated concentrations of SNP (A) or EUK-134 (B). All assays were performed in duplicate. After 6 h, the percentage of apoptotic cells was determined.*

or 3-AT concentrations that had been found to be suboptimal (75 mM) or optimal (150 mM) in preceding experiments. GOX was added at concentrations between 0 and 4 mU/ml for a low to moderate generation of hydrogen peroxide. Figure 5 demonstrates that the cells showed only background apoptosis induction without 3-AT treatment, but there were 17% apoptotic cells in the presence of 75 mM 3-AT and 30% in the presence of 150 mM 3-AT. As expected, in the absence of 3-AT, addition of GOX in the indicated concentration range did not induce significant apoptosis. In the presence of the suboptimal concentration of 3-AT (75 mM), GOX at lower

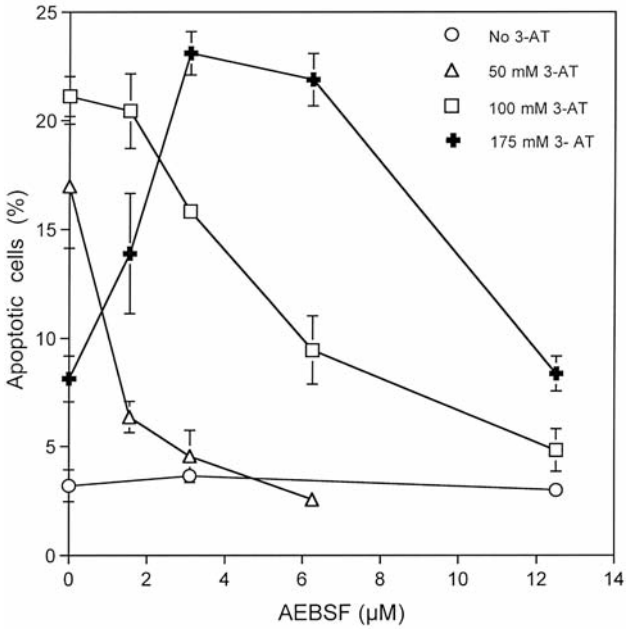

Figure 7. *Differential effects of NADPH oxidase inhibition on 3-AT-mediated apoptosis induction in human tumor cells. MKN-45 cells were tested under the conditions for measurement of apoptotic self-destruction in the presence of the indicated concentrations of 3-AT and AEBSF. All assays were performed in duplicate. The percentage of apoptotic cells was determined after 6.5 h by inverted phase-contrast microscopy.*

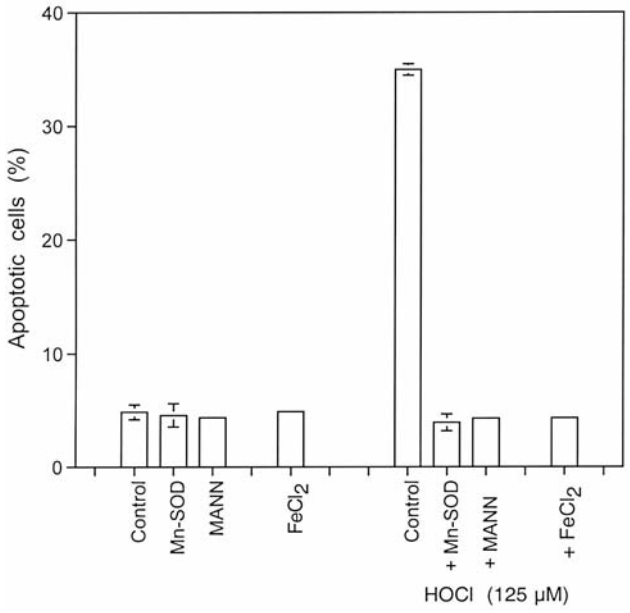

Figure 8. *Site-specific hydroxyl radical formation is required for HOCl-mediated apoptosis induction in human tumor cells. MKN-45 cells were tested under the conditions for measurement of apoptotic self-destruction without inhibitor (Control), 100 U/ml Mn-SOD, 10 mM mannitol (MANN) and 20 μM $FeCl_2$. The assays received either no or 125 μM HOCl. All assays were performed in duplicate. After 2 h, the percentage of apoptotic cells was determined by inverted phase-contrast microscopy.*

concentrations enhanced overall apoptosis induction and then inhibited apoptosis induction at higher GOX concentrations. Addition of GOX to assays with the optimal concentration of 3-AT (150 mM) caused an immediate inhibition of apoptosis, depending on the concentration of GOX. Both the stimulatory and inhibitory effects of GOX were abrogated by 0.25 μM EUK-134, confirming that the effects were due to excess hydrogen peroxide.

We recently showed that NO-mediated apoptosis induction was inhibited by excess hydrogen peroxide, *e.g.* at high cell density (28). This interaction has been interpreted as being indicative of a consumption reaction between hydrogen peroxide and NO. Based on this assumption, the reverse approach, *i.e.* addition of an NO donor to a hydrogen peroxide-driven process should also modulate this process. Therefore, apoptosis was induced in a human tumor cell line (in this case, the lymphoma cell line Gumbus that shows a similar reaction pattern as MKN-45 cells after catalase inhibition) at a suboptimal, optimal and supraoptimal concentration of 3-AT. Parallel assays received either no NO donor or the NO donor SNP at varying concentrations. As shown in Figure 6, all concentrations of the NO donor inhibited apoptosis induction at the suboptimal and the optimal 3-AT concentration. The degree of inhibition was proportional to the concentration of SNP applied. At the supraoptimal concentration of 200 mM 3-AT, the lower concentrations of SNP (1 and 2 μM) brought the reaction closer to the optimum, whereas the higher concentrations caused inhibition again. This finding is best explained by consumption of hydrogen peroxide by NO, leading to inhibition of signaling, when hydrogen peroxide is limiting and stimulatory. When, however, excess hydrogen peroxide attenuates the HOCl pathway through HOCl consumption, consumption of excess hydrogen peroxide by NO restores optimal signaling. If the NO concentration is increased further to this optimal condition, finally inhibition of HOCl signaling occurs. In line with these assumptions, the catalase mimetic EUK-134 showed the same effects as the NO donor. Taken together, these findings show that the HOCl signaling pathway has an optimum with regard to the concentration of available hydrogen peroxide. An increase of the available hydrogen peroxide in the system can therefore bring the reaction from suboptimal conditions to the optimum or from the optimum to supraoptimal conditions, whereas a decrease of hydrogen peroxide brings apoptosis induction from the optimum to suboptimal conditions but also from the supraoptimum back to the optimum. According to our data and our proposed model for intercellular HOCl signaling, superoxide anions

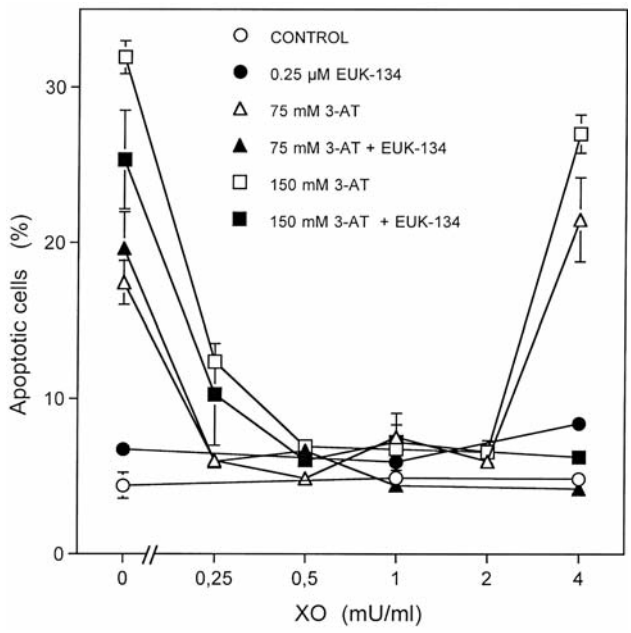

Figure 9. *Exogenous superoxide anion generating attenuates apoptotis induction by the HOCl signaling pathway. MKN-45 cells were tested under the conditions for measurement of apoptotic self-destruction in the presence of the indicated concentrations of xanthine oxidase (XO) and 0.4 mM xanthine. Assays received either no, 75 mM or 150 mM 3-AT and 0.25 µM EUK-134, where indicated. All assays were performed in duplicate. The percentage of apoptotic cells was determined after 3.5 h by inverted phase-contrast microscopy. The horizontal axis is exponential, in order to achieve better resolution of the effects of low xanthine oxidase concentrations.*

exhibit several roles in this process: their concentration determines the rate of dismutation and hydrogen peroxide formation, thus controling the rate of HOCl formation by peroxidase as well as the rate of a conceivable consumption reaction between HOCl and hydrogen peroxide. In addition, free superoxide anions are required for the generation of ultimately apoptosis-inducing hydroxyl radicals through their interaction with HOCl.

In order to dissect the dependence of these distinct processes on the concentration of superoxide anions, apoptosis induction by varying concentrations of 3-AT in the presence of increasing concentrations of the NADPH oxidase inhibitor AEBSF was analyzed with respect to the concentration of AEBSF (Figure 7). At the optimal concentration of 3-AT (100 mM), AEBSF caused a concentration-dependent direct proportional inhibition of apoptosis with a IC_{50} at 4 µM. When catalase inhibition was less pronounced in the presence of 50 mM 3-AT, apoptosis showed a small decrease from 22 to 18% in the absence of AEBSF. However, even the lowest concentration of AEBSF caused a marked decrease of apoptosis, indicating that under conditions of partial catalase inhibition a slight inhibition of NADPH oxidase has a marked effect. Finally, the analysis of the effects at supraoptimal inhibition allowed the two superoxide anion-driven processes, *i.e.* dismutation to hydrogen peroxide and superoxide anion/HOCl interaction to be dissected. When supraoptimal conditions were applied (150 or 175 mM 3-AT), inhibition of NADPH oxidase first caused a reestablishment of optimal conditions. When much higher concentrations of AEBSF were applied, the overall reaction was blocked. This finding demonstrates that the inhibition of NADPH oxidase by 6 µM AEBSF must have a strong influence on the available hydrogen peroxide concentration, as it prevents excess hydrogen peroxide generation and thus allows optimal apoptosis induction despite very high catalase inhibitory. As the reaction reaches a maximum under these conditions, despite the partial inhibition of NADPH oxidase by 6 µM AEBSF, sufficient superoxide anions still seem to be present for the conversion of HOCl to hydroxyl radicals.

Taken together, the impact of NADPH oxidase inhibition seems to be more dominant with respect to generation of hydrogen peroxide and less pronounced with respect to the final reaction of the superoxide anions.

One central aspect of our model is the site-specific reaction of superoxide anions with HOCl close to the membrane of the target cells, leading to the formation of hydroxyl radicals at the desired site of action. This site specificity, based on the small free diffusion path length of superoxide anions generated at the membrane and on the extreme small free diffusion pathlength of generated hydroxyl radicals has been proposed to be the basis for the observed selectivity of intercellular apoptosis induction. If the model was correct, addition of HOCl to superoxide anion-generating tumor cells should result in apoptosis that was blocked by removal of superoxide anions through SOD or by scavenging of hydroxyl radicals. In contrast, generation of hydroxyl radicals without site specificity through ferric ion-catalyzed overall Fenton-like reaction of HOCl (29) should not lead to apoptosis. As can be seen in Figure 8, the results of the experiment confirm the prediction made. Site-specific generation of hydroxyl radicals through interaction of HOCl with tumor cell-derived superoxide anions caused apoptosis, prevented by the hydroxyl radical scavenger mannitol, whereas ferric ions destroyed the site-specific apoptosis inducing potential of HOCl, most likely through Fenton chemistry that shows no site-specificity. In a second approach for verification or falsification of the site specificity of HOCl signaling, superoxide-generating xanthine oxidase plus xanthine were added to tumor cells with inhibited catalase. The overall production of superoxide anions in the medium was predicted to enable HOCl interaction with superoxide anions distant from the cell membranes. The resultant hydroxyl radicals, due to their small free diffusion path length, then should have no chance of hitting the cell membranes and apoptosis induction should thus be inhibited.

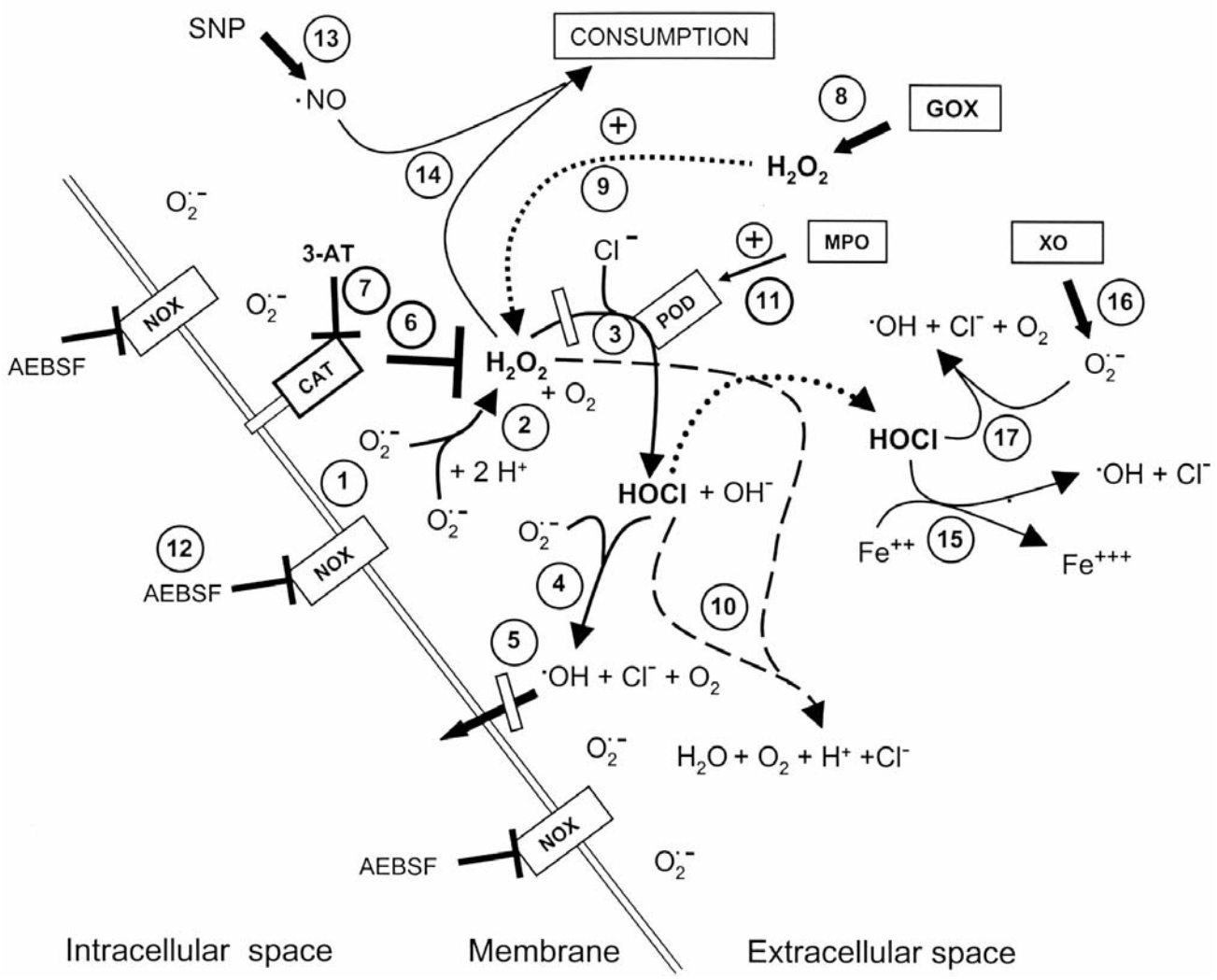

Figure 10. *Modulation of intercellular ROS signaling of tumor cells. Tumor cells (like transformed cells, but unlike nontransformed cells) exhibit membrane-associated NADPH oxidase (NOX) (step 1). NOX generates superoxide anions that spontaneously dismutate to form hydrogen peroxide (step 2). Tumor cells possess membrane-assiociated catalase that efficiently removes hydrogen peroxide (step 6). It thus prevents synthesis of HOCl by a novel peroxidase (step 3) and the subsequent interaction of HOCl with superoxide anions to form apoptosis-inducing hydroxyl radicals (steps 4, 5). Inhibition of catalase by 3-AT (step 7) restores HOCl signaling and apoptosis induction (steps 3-5). When exogenous GOX generates hydrogen peroxide (steps 8, 9) in the presence of suboptimal concentrations of the catalase inhibitor 3-AT, the HOCl signaling pathway is enhanced. When GOX is however added under conditions of optimal catalase inhibition or when 3-AT is present at very high concentrations, excess hydrogen peroxide interacts with HOCl (step 10) and thus attenuates the HOCl signaling pathway. This supraoptimal condition can be reverted to optimal conditions when MPO is added (step 11) and thus excess hydrogen peroxide is converted to HOCl. Alternatively, excess hydrogen peroxide can be removed by low concentrations of the catalase mimetic EUK-134, which is not affected by 3-AT (not shown). Alternatively, excess hydrogen peroxide formation can be prevented by gradual inhibition of NOX through AEBSF (step 12). The central modulator hydrogen peroxide is consumed by NO that can be generated by the exogenous NO donor SNP (steps 13, 14). This consumption reaction inhibits the HOCl pathway at suboptimal and optimal catalase inhibition and partially restores the optimum at supraoptimal catalase inhibition. Ferrous ions interact with HOCl in a Fenton type reaction, leading to the formation of hydroxyl radicals (step 15). As HOCl is a far-ranging molecular species in this system, the majority of the hydroxyl radicals generated in this reaction do not reach the cell membrane, due to the very small free diffusion path length of hydroxyl radicals. The reaction rather attenuates HOCl signaling. Similarly, the overall generation of superoxide anions through soluble exogenous xanthine oxidase (step 16) generates hydroxyl radicals distant from the cell membrane and thus inhibits the HOCl pathway. These effects are contrasted by site-specific hydroxyl radical formation at the membrane when cell-derived superoxide anions and HOCl interact (steps 4, 5) and generate hydroxyl radicals at the right site. When tumor cell catalase is inhibited by 100 mM 3-AT (step 7) and GOX is added at concentrations higher than 5 mU/ml, signaling through the HOCl pathway is gradually displaced by the well-known direct apoptosis-inducing effect of hydrogen peroxide. This process seems to depend on intracellular Fenton chemistry (not shown in the picture).*

This experimental approach has, however, one conceivable pitfall: although xanthine oxidase primarily generates superoxide anions, their dismutation product hydrogen peroxide might inhibit apoptosis induction through HOCl consumption. To cirumvent this problem, three measures were taken: i) relatively small concentrations of xanthine oxidase were used in order to minimize subsequent hydrogen peroxide generation, ii) parallel assays with the catalase mimetic EUK-134 were run for the detection of potential hydrogen peroxide-directed effects and iii) apoptosis was induced by optimal as well as suboptimal concentrations of the catalase inhibitor 3-AT. The latter two approaches allowed the clearest differentiation between known hydrogen peroxide effects and conceivable effects of superoxide anions on the signaling system. If the effect of added xanthine oxidase was due to hydrogen peroxide alone, it should be inhibited by EUK-134 and xanthine oxidase should have an inhibitory effect on cells with optimal catalase inhibition, but a stimulatory effect on cells with suboptimal catalase inhibition, as it would bring the reaction to its optimum. If xanthine oxidase-generated superoxide anions should have the proposed inhibitory effect on HOCl signaling, the inhibitory effect of the enzyme should not be influenced by EUK-134 and the inhibitory effect should be seen both at supoptimal and optimal 3-AT concentrations. As can be seen in Figure 9, addition of increasing concentrations of xanthine oxidase up to 2 mU/ml showed no effect on control cells, but exhibited a concentration-dependent inhibitory effect on apoptosis induction in the presence of 75 and 150 mM 3-AT. This inhibitory effect was not abrogated by the catalase mimetic EUK-134, indicating that it was not due to the action of hydrogen peroxide. At 4 mU/ml xanthine oxidase, apoptosis induction was observed in the presence of 3-AT. In contrast to the inhibitory effect of lower xanthine oxidase concentrations, this effect was completely blocked by EUK-134, indicating that at these high concentrations hydrogen peroxide derived from dismutation of xanthine oxidase-generated superoxide anions drives apoptosis induction, provided the catalase of the cells is inactivated.

Discussion

This paper demonstrates multiple ROS interactions during intercellular ROS signaling and the dominant and central control function of membrane-associated catalase, which protects tumor cells from apoptosis induction. Superoxide anions, their dismutation product hydrogen peroxide and HOCl-synthesizing peroxidase thereby play central and partially inverse roles during intercellular ROS-mediated signaling. In addition, nitric oxide (NO) has the potential to modulate ROS signaling. Our other paper (12) and Figure 1 demonstrate that GOX, an exogenous source for hydrogen peroxide, resensitizes tumor cells for intercellular ROS signaling as it overcomes the hydrogen peroxide-removing effect of membrane-associated tumor cell catalase and thus allows reconstitution of the HOCl signaling pathway. As apoptosis mediated by addition of GOX up to 40 mU/ml is completely blocked by the HOCl-scavenger taurine, restored HOCl signaling rather than a direct apoptosis-inducing effect of hydrogen peroxide seems to be the underlying mechanism. This is further confirmed by the inhibitory effects of AEBSF, SOD, ABH, and mannitol (Figure 6 in the preceding paper). This indicates that hydrogen peroxide generated by GOX between 10 and 40 mU/ml can only contribute to tumor cell apoptosis if it is converted into HOCl, which is not attacked by catalase and which can generate apoptosis-inducing hydroxyl radicals when it interacts with superoxide anions generated at the membrane of the tumor cells. The situation changes drastically when tumor cell catalase is inactivated by 3-AT. In the presence of 100 mM 3-AT, due to abrogation of hydrogen peroxide consumption by catalase, 3 mU/ml of GOX are sufficient to mediate apoptosis induction. But still up to 5 mU/ml of GOX this effect is based completely on the HOCl pathway (demonstrated by complete inhibition by taurine) and then the HOCl signaling effect is gradually replaced by a direct apoptosis-inducing action of hydrogen peroxide.

This direct effect of hydrogen peroxide generated by GOX resembles the recently described nonselective direct apoptosis induction in transformed and nontransformed fibroblasts mediated by GOX in the range of 2-4 mU/ml (26). The direct apoptosis-inducing effect of hydrogen peroxide on nontransformed and transformed cells neither required exogenous superoxide anions nor HOCl generation and therefore has been termed ‘direct’ in this context. It is noteworthy, however, that hydrogen peroxide seems to exert its apoptosis induction intracellularly through Fenton chemistry-derived hydroxyl radicals, as the process was inhibited by cell-permeable iron chelators and hydroxyl radical scavengers (Bauer, in preparation). The presence and significance of protective catalase on tumor cells is demonstrated by their increased resistance to the action of GOX when compared to nontransformed or transformed cells and by a similar sensitivity of the three cell systems after catalase inhibition of tumor cells.

The preceding paper defined a general picture for the extracellular ROS status of nontransformed, transformed and tumor cells *i.e.* i) no extracellular superoxide anion generation in nontransformed cells; ii) extracellular superoxide anion generation by transformed and tumor cells; iii) efficient apoptosis induction in transformed, but not in nontransformed cells by intercellular ROS signaling; iv) catalase protection against intercellular ROS signaling in tumor cells, and v) resensitization of tumor cells for intercellular ROS signaling through inhibition of catalase or supplementation with exogenous hydrogen peroxide. High catalase activity despite maximal superoxide anion generation seems to be the protection strategy of tumor cells

against ROS-mediated apoptosis induction, thereby ensuring the benefits of superoxide anion signaling on proliferation for the tumor cells.

The membrane-associated NADPH oxidase seems to be the major, if not the sole, source of extracellular superoxide anions and their dismutation product hydrogen peroxide, as intercellular ROS signaling can be completely blocked by the NADPH oxidase inhibitor AEBSF. The achieved hydrogen peroxide concentration is critical for the rate of apoptosis induction. Therefore, at suboptimal inhibition of catalase, apoptosis induction is suboptimal due to insufficient HOCl synthesis. Addition of exogenous hydrogen peroxide can compensate for this effect. However, an excess of available hydrogen peroxide (either through experimental addition or due to complete inhibition of catalase) shows an inhibitory effect on apoptosis induction by the HOCl pathway. This is explained by the interaction of free hydrogen peroxide with HOCl leading to the generation of water and chloride (30-32). This conclusion is proven correct as i) addition of MPO, or ii) partial removal of hydrogen peroxide, or iii) slight inhibition of superoxide anion synthesis, or iv) a decrease in cell density abrogates the inhibitory effect and shifts the supraoptimum back to a plateau of reaction. In line with this conclusion i) addition of hydrogen peroxide-generating GOX to tumor cells with supoptimal catalase inhibition first enhanced apoptosis induction and caused subsequent inhibition at higher GOX concentrations, and ii) addition of GOX at optimal catalase inhibition attenuates apoptosis induction. The specificity of this reaction is shown through abrogation of inhibition by the catalase mimetic EUK-134.

These findings also stress the importance of a balance between available hydrogen peroxide and peroxidase. At first sight it seems unlikely that the slower reaction between HOCl and hydrogen peroxide ($k=10^5$ $M^{-1}s^{-1}$) (30, 31) can efficiently outcompete the 100-fold faster reaction between HOCl and superoxide anions ($k=10^7$ $M^{-1}s^{-1}$) (29, 33, 34). This puzzle is resolved when the geometric situation is regarded. Hydrogen peroxide and HOCl are far-ranging molecular species that can also interact in the medium above the cells, distant from the cell membrane, whereas the interaction of the short-ranging superoxide anions with HOCl is confined to the cell membrane where the superoxide anions are generated. Therefore the majority of HOCl seems to be located at a site where only the slower reaction can occur.

The reaction of hydrogen peroxide with HOCl is of great complexity. Under slightly alkaline conditions, the hypochlorous anion and hydrogen peroxide have been shown to generate singlet oxygen (31), whereas there was no indication for singlet oxygen generation under slightly acidic conditions. Under neutral conditions, chlorine, derived from HOCl seems to drive the interaction with hydrogen peroxide. As singlet oxygen can induce lipid peroxidation and apoptosis (32), one might expect that the loss of HOCl/superoxide anion-dependent apoptosis signaling in the presence of excess hydrogen peroxide would be compensated for by the generation of singlet oxygen. As we observed an attenuation of HOCl signaling in the presence of excess hydrogen peroxide, this would not seem to be the case. Several reasons might account for this finding: i) as HOCl and hydrogen peroxide are both far-ranging molecular species, most of the singlet oxygen might be generated distant from the cell membrane and due to its reactivity would interact with medium components before it could reach the membrane; ii) due to its reactivity with azole, singlet oxygen might be scavenged by the catalase inhibitor 3-AT.

Optimal ROS signaling of tumor cells seems to require a perfect tuning of catalase inhibition (protection of hydrogen peroxide), superoxide generation and dismutation (generation of hydrogen peroxide) and peroxidase activity (utilizing hydrogen peroxide for HOCl synthesis). Changes in any of these critical parameters causes predictable changes in the overall outcome of intercellular ROS signaling.

Ongoing work in our group shows that low-dose gamma irradiation enhances superoxide anion generation in transformed and tumor cells (14). This effect enhances tumor cell apoptosis under conditions of suboptimal catalase inhibition/ destruction or low cell density (through an increase in the hydrogen peroxide concentration), but has an attenuating effect under otherwise optimal conditions for tumor cell apoptosis through intercellular ROS signaling. Therefore, it can be concluded that low-dose radiation can influence transformed or tumor cell apoptosis in adverse ways, but that the direction of the outcome should be predictable on the basis of the data presented here. In addition, a strict requirement for site specificity for hydroxyl radical generation and subsequent apoptosis induction is overt.

When HOCl interacts with superoxide anions generated at the membrane of the tumor cells, the resultant hydroxyl radicals are able to attack the membrane, despite their small free diffusion path length. When, however, HOCl is added to cells in the presence of sufficient exogenous ferrous ions to enable Fenton chemistry ($HOCl + Fe^{++} \rightarrow OH^{\bullet} + Cl^{-} + Fe^{+++}$) (29); the resultant hydroxyl radicals do not harm the cells as the majority is generated more distant from the cells than the free diffusion path length. However, this experimental approach has a potential pitfall, as hydroxyl radicals might interact with residual ferrous ions, yielding hydroxyl anions and ferric ions (35). This reaction would also contribute to attenuation of apoptosis induction by the HOCl signaling pathway. However, as in a second approach, random generation of superoxide anions by xanthine oxidase inhibited apoptosis, HOCl would seem to interact with superoxide anions from xanthine oxidase, yielding hydroxyl radicals mostly distant from the cell membrane. This effect of xanthine oxidase seems to be due to the superoxide anions generated by the enyzyme and seems not

to reflect the inhibitory effect of their dismutation product hydrogen peroxide, as i) the catalase mimetic did not counteract the effect of xanthine oxidase and ii) xanthine oxidase also inhibited apoptosis at suboptimal concentrations of 3-AT where hydrogen peroxide would have enhanced the suboptimal reaction. Only at higher xanthine oxidase concentrations did apoptosis-triggering effects of hydrogen peroxide become dominating and were abrogated by EUK-134 (thereby demonstrating the efficacy of the catalase mimetic).

The role of superoxide anions is twofold in our experimental system: they are the source of hydrogen peroxide and in addition represent the reaction partner for HOCl. Reaction of superoxide anions with HOCl has to be site-specific at the cell membrane and therefore this aspect of the signaling system cannot be easily mimicked or modulated through reconstitution. Gradual inhibition of NADPH oxidase by AEBSF demonstrates that partial inhibition of NADPH oxidase has a greater effect on hydrogen peroxide synthesis than on HOCl superoxide interaction. This is easily seen when catalase is inhibited by high 3-AT concentrations and the abundant hydrogen peroxide blocks the HOCl signaling pathway. Partial inhibition of NADPH oxidase under these conditions restores optimal apoptosis induction. Based on the underlying signaling chemistry, this must imply a decrease of available hydrogen peroxide due to reduced concentrations of superoxide anions while there is still sufficient concentration of hydrogen peroxide for interaction their interaction with HOCl. This finding is in line with the lower reaction constant for spontaneous dismutation of superoxide anions compared to HOCl superoxide anion interaction.

The central role of hydrogen peroxide for intercellular ROS signaling of tumor cells and the multiple ways for its modulation is summarized in Figure 10. This figure also includes the consumption of hydrogen peroxide by exogenous NO as recently described (28). Work in progress indicates that this consumption reaction is more complex than originally anticipated (Bauer, in preparation). The consumption of hydrogen peroxide by NO and *vice versa* has been used as an experimental tool in this study. However, the analysis of the complete intercellular signaling pathways of many human tumor cells after inhibition of their catalase has shown that the balance between the hydrogen peroxide and NO determines the relative efficiencies of the resultant signaling pathways, *i.e.* the HOCl and the NO/peroxynitrite signaling pathway. The findings summarized in Figure 10 have been established for tumor cells under conditions of catalase inhibition. They are therefore identical for the modulation of intercellular ROS signaling of transformed cells that have no or much less protective catalase. This identy has been recently confirmed experimentally (Bauer, unpublished results).

This knowledge should allow prediction of the effect of modulation of any of the central signaling elements on the efficiency of apoptosis induction in transformed or tumor cells through intercellular ROS signaling. It is important that the balance of the signaling components determines whether apoptosis induction in tumor cells after catalase inhibition will be prevented, enabled, stimulated or inhibited again.

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