Catalase Protects Tumor Cells from Apoptosis Induction by Intercellular ROS Signaling

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Abstract. *Transformed cells are subject to intercellular induction of apoptosis by neighbouring nontransformed cells and to autocrine apoptotic self-destruction. Both processes depend on extracellular superoxide anion generation by the transformed cells and on the release of peroxidase from both nontransformed and transformed cells. This concerted action results in HOCl synthesis, HOCl-superoxide anion interaction and generation of apoptosis-inducing hydroxyl radicals. In contrast to transformed cells, ex vivo tumor cells are resistant against intercellular induction of apoptosis and autocrine apoptotic self-destruction. Resistance of tumor cells against intercellular ROS signaling depends on interference through catalase expression on the membrane. Intercellular ROS signaling of tumor cells can be restored when i) exogenous HOCl is added; ii) exogenous hydrogen peroxide is supplied, or iii) catalase is inhibited. These findings define the biochemical basis for specific apoptosis induction in tumor cells through re-establishment of intercellular ROS signaling, a potential novel approach in tumor prevention and therapy.*

Multistep oncogenesis is characterized by distinct and interconnected steps such as abrogation of senescence control, oncogene activation, tumor suppressor gene inactivation (1-3), independence of exogenous proliferation signals through autocrine mechanisms (4-6), independence of control by neighbouring cells (7-10), acquisition of new defense mechanisms by the tumor cells (11-13), escape from immune surveillance (2), resistance against hypoxia-induced p53-mediated cell death (14, 15), tumor angiogenesis and others (reviewed in 16-20).

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Generation of extracellular superoxide anions through a membrane-associated NADPH oxidase (Nox1) is associated with oncogene activation and seems to represent one of the hallmarks of the transformed state (21-28). Ras and rac play central roles for the activation of Nox1 (21, 29). The activated NADPH oxidase seems to be required for the control of proliferation and the maintenance of the transformed state (21, 23, 24, 28, 30), changes in the cytoskeleton of transformed cells (31) and induction of the angiogenic switch (32). Nox1 activity also seems to be relevant for tumorigenesis *in vivo* (28, 33, 34).

On the flip side of the coin, extracellular superoxide anions generated by transformed cells drive both the efficiency and selectivity of intercellular induction of apoptosis, a hitherto unrecognized potential control step during multistage oncogenesis (17-20, 25, 26, 35). During intercellular induction of apoptosis, transformed cells are selectively induced to die by apoptosis after a concerted action of transformed cell-derived reactive oxygen species (ROS) and signaling components released by surrounding nontransformed cells (17-19, 25, 26). Four signaling pathways have been elucidated so far: i) the HOCl signaling pathway (25), ii) the nitric oxide (NO)/peroxynitrite signaling pathway (25, 27), iii) the nitryl chloride signaling pathway (36) and iv) the metal ion catalyzed Haber Weiss reaction (37). In many transformed cell systems studied by our group, the HOCl pathway represented the major signaling pathway of intercellular induction of apoptosis. Therefore, this pathway is the focus of the study reported here.

The HOCl signaling pathway depends on the generation of superoxide anions by the transformed target cells (25). Their dismutation product hydrogen peroxide is utilized by a novel peroxidase for the synthesis of HOCl. HOCl in the micromolar concentration range does not affect cells directly (38, 39). However, the interaction of HOCl with superoxide anions (40-42) leads to the generation of hydroxyl radicals, which have a very limited free diffusion path length and have the ability to trigger the onset of apoptosis through lipid peroxidation. As superoxide anions and hydroxyl radicals have relatively short free diffusion path lengths (43, 44), apoptosis induction is selectively directed against the

transformed target cells. As nontransformed cells generate fewer extracellular superoxide anions than their transformed counterparts (25-27, 38), they are not affected by HOCl in the micromolar concentration range.

Ongoing work has demonstrated that intercellular induction of apoptosis does not necessarily require the presence of nontransformed cells, as peroxidase is also released by transformed cells themselves (Bauer, unpublished result). Therefore, the presence of transformed cells at high density and in high numbers allows the establishment of autocrine apoptotic ROS-mediated self-destruction of transformed cells. This process depends on the same signaling chemistry as the interaction between nontransformed and transformed cells (Bauer, unpublished result). Likewise, autocrine apoptotic self-destruction is highly selective for transformed cells. Again, this selectivity is based on extracellular superoxide anion generation by the transformed cells. During autocrine self-destruction of transformed cells, sufficient local density of the cells ensures optimal generation of hydrogen peroxide through dismutation of superoxide anions and using a sufficient total number of cells per assay ensures an optimal supply of peroxidase that is released into the medium.

Autocrine apoptotic self-destruction can be tested in two ways. When clumps of transformed cells (2,000 cells, 300 cells/mm^2) are surrounded by 15,000 dispersely seeded effector cells (40 cells/mm^2), the transformed cells are induced to die by apoptosis, but the effector cells are not, even if they are transformed. This is due to insufficient hydrogen peroxide generation in the dispersely seeded cells and sufficient hydrogen peroxide generation in the densely seeded target cells, followed by the interaction of target cell-derived hydrogen peroxide with effector cell-derived peroxidase. If a clump of target cells is seeded alone, its peroxidase is diluted and therefore apoptosis is not induced despite sufficient hydrogen peroxide. Dispersely seeded effector cells generate sufficient peroxidase but, even if they are transformed, do not generate sufficient hydrogen peroxide due to suboptimal density. Alternatively, autocrine apoptotic self-destruction can be determined when homogeneous populations of transformed cells are seeded at optimal cell density, cell number and volume of overlaying medium (for details please see the Methods section).

Whereas the term 'intercellular induction of apoptosis' has been used for the interaction between nontransformed and transformed cells, the term 'autocrine apoptotic self-destruction' is restricted to the interaction of transformed cells. We suggest using the term 'intercellular ROS signaling' to describe the signaling chemistry of both processes, as it is identical except for the source of effector molecules such as peroxidase or NO. We also suggest to use the operational terms 'target cells' for the superoxide anion generating cells that are subject to apoptosis induction by intercellular ROS signaling and 'effector cells' for the cells that supply intercellular ROS signaling with free peroxidase and NO. The 'target cell function' is strictly dependent on the transformed state of the cells, whereas the 'effector cell function' is not. Whereas cells transformed *in vitro* show sensitivity against intercellular induction of aptoptosis and autocrine self-destruction, independently of the origin of tissue and the transforming principle, *bona fide* tumor cells established from tumors showed resistance against intercellular induction of apoptosis (45, 46). This resistance might be caused by a multitude of different biochemical effects: tumor cells might have defects in their apoptosis machinery, lack superoxide anion generation or generate insufficient concentrations of superoxide anions, have established strong intracellular defense mechanisms against apoptosis-inducing signals or interfere with intercellular ROS signaling through expression of antioxidative enzymes. If the latter scenario were true, it should be possible to resensitize tumor cells for ROS-mediated apoptosis induction through enhancement of signaling components or through the inhibition of the interfering enzyme(s). Thus the aim of the present study was to define the exact biochemical mechanism of tumor cell resistance against intercellular ROS signaling.

Materials and Methods

Materials. 4-(2-Aminoethyl-benzenesulfonyl fluoride (AEBSF), a specific inhibitor of NADPH oxidases (47), was obtained from Sigma-Aldrich (Schnelldorf, Germany) and stored as a stock solution of 10 mM in phosphate-buffered saline (PBS) at –20˚C. 4-Aminobenzoyl hydrazide (ABH), a mechanism-based inhibitor of MPO (48, 49), obtained from Acros Organics (Geel, Belgium) was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 1 M. It was then diluted with medium to a concentration of 1 mM (stock solution). The stock solution was kept at –20˚C. The catalase inhibitior 3-aminotriazole (3-AT) [for review of its action see (50)] was obtained from Sigma - Aldrich. The stock solution (2 M in sterile PBS) was stored at –20˚C. Dimethylthiourea (DMTU), a hydroxyl radical-specific scavenger, was obtained from Sigma Aldrich and kept as a stock solution of 1M in PBS at –20˚C. NaOCl was obtained from Sigma, Schnelldorf, Germany. The stock solution of 860 mM was kept at 4˚C in the dark. As the pKa of OCl^- is 7.64, the majority of the species are present as HOCl at neutral pH. For simplicity, the term 'HOCl' is used through out this paper. HOCl was diluted in cold, sterile PBS and then added to the assays as a single aliquot (10-20 μl per 100 μl assay). Care was taken to add HOCl at a similar speed and from the same distance above the medium in order to avoid differences in local concentration of HOCl immediately after addition. Mannitol, a hydroxyl radical-specific scavenger (51), was obtained from Sigma Aldrich and kept as a stock solution of 1 M in PBS at –20˚C. Taurine (Sigma Aldrich), a HOCl-specific scavenger (52), was kept as a stock solution of 500 mM in sterile PBS at –20˚C and was used at a concentration of 50 mM in our assays. Glucose oxidase (GOX, from *Aspergillus niger*) generates hydrogen peroxide using glucose as substrate. (Glucose is present in abundance in Eagle's minimal essential medium (EMEM) and RPMI-1640 medium.) GOX was obtained from Sigma Aldrich (Schnelldorf, Germany) and kept as 6,000 U/ml stock solution at 4˚C. Myeloperoxidase (MPO, from

human leukocytes) was obtained from Sigma Aldrich. Stock solution (5 U/ml) in EMEM with 5% fetal bovine serum (FBS) was kept at –20˚C and only used once per aliquot. MPO catalyzes the generation of HOCl from H_2O_2 and chloride (53). Manganese-containing superoxide dismutase (Mn-SOD) from *Escherichia coli* (Sigma Aldrich) (stock solutions 30,000 Units/ml in sterile PBS) were kept at –20˚C and only used once per aliquot. Mn-SOD is an efficient scavenger of superoxide anions, in a two step reaction. Mn-SOD is not cellpermeable (54, 55) and therefore allows the functional role of extracellular superoxide anions to be demonstrated. Mn-SOD does not exhibit the sharp bell-shaped inhibition curve that is characteristic for copper-containing SOD and therefore is superior to Cu-SOD in inhibition studies. Transforming growth factor β-1 (TGF-β-1) was purified from human platelets (56) and kept as a stock solution of 1.5 μg/ml in EMEM plus 5% FBS at –20˚C. Caspase-3 inhibitior (Z-DEVD-FMK) and caspase-9 inhibitor (Z-LEHD-FMK) were obtained from R&D Systems (Wiesbaden-Nordenstadt, Germany). The inhibitors were first dissolved in DMSO to reach a concentration of 60 mM and were then diluted with ethanol to a final concentration of 20 mM. These stock solutions were kept at –20˚C and used within the subsequent weeks. Caspase-3 inhibitor was applied at a final concentration of 50 μM, caspase-9 inhibitior at a final concentration of 25 μM. The residual DMSO concentration was below the critical concentration which affects ROS signaling.

Media for cell culture. Cells were either kept in EMEM, containing 5% FBS or in RPMI-1640 medium, containing 10% FBS, as indicated for the respective cell lines. FBS (Biochrom, Berlin, Germany) had been heated for 30 minutes at 56˚C prior to use. Both media were supplemented with penicillin (40 U/ml), streptomycin (50 μg/ml), neomycin (10 μg/ml), moronal (10 U/ml) and glutamine (280 μg/ml). Cell culture was performed in plastic tissue culture flasks. Cells were passaged once or twice weekly.

Cells. 'Nontransformed cells' (208F) are normal rat fibroblasts that do not show criss cross morphology, colony formation in soft agar and are not tumorigenic. They do not show sufficient extracellular superoxide anion generation to be the target of intercellular ROS signaling (25-27). Nontransformed 208F cells exhibit effector function *in vitro*, *i.e*. the release of a novel peroxidase and, to a lesser extent, nitric oxide (25) which both establish apoptosis-inducing ROS signaling in transformed cells. 'Transformed cells' (208Fsrc3, FE-8, fgr413, fms41, raf55) are derived from 208F cells that have been transformed *in vitro* and are defined in the context of this article as having the potential for tumorigenesis without having yet been confronted by the natural antitumor mechanisms of an organism and the resultant selection processes. Transformed cells show criss cross morphology in monolayer, colony formation in soft agar and extracellular superoxide anion generation that drives both the efficiency and selectivity of intercellular ROS signaling (25-27). 'Tumor cells' of murine (L929, CMS-5, SSK, CCL-107) or human origin (BG-1, MKN-45, SIHA) are defined in this article as having been isolated from an *in vivo* tumor. L929, CMS-5, SSK and CCL-107 are fibrosarcomas, BG-1 has been isolated from an ovarial carcinoma, MKN-45 from a gastric carcinoma and SIHA from a cervical carcinoma. They form colonies in soft agar. Despite extracellular superoxide anion generation, they are resistant to intercellular ROS signaling. The resistance mechanism and strategies to resensitize tumor cells for intercellular ROS signaling are the focus of this paper. Nontransformed rat fibroblasts 208F and their derivatives transformed through constitutive expression of v-src (208Fsrc3), HRAS (FE-8), v-fgr (fgr 413), v-fms (fms41) and v-raf (raf-55) were established by and a generous, valuable gift by Dr. C. Sers and Dr. R. Schäfer, Berlin, Germany. The transformed cell lines FE-8, fgr413, fms41 and raf-55 show similar characteristics to 208Fsrc3 cells. 208F cells and their transformed derivatives were cultured in EMEM, 5% FBS and supplemented as indicated above. The murine fibrosarcoma cell line L929 was obtained from Dr. Adam, Kiel, Germany and was cultured in EMEM, 5% FBS and supplements. The murine fibrosarcoma cell lines CMS-5 and SSK and the rat glioblastoma line CCL-107 (C6) have been recently described (46). They were cultured in EMEM, 5% FBS and supplements. The human ovarial carcinoma cell line BG-1 was obtained from Dr. T. Bauknecht, Freiburg, Germany; the cervix carcinoma line SIHA from Dr. L. Gissmann, DKFZ Heidelberg, Germany. The cell lines were cultivated in EMEM, 5% FBS and supplements. The gastric carcinoma cell line MKN-45 was purchased from DSMZ, Braunschweig, Germany. The cells growing in suspension with some cells attaching to the plastic were cultured in RPMI-1640, 10% FBS and supplements. Care was taken to avoid cell densities below 300,000/ml and above 10^6/ml. Under optimal conditions, the percentage of spontaneous apoptosis induction was below 1%.

Apoptosis induction through intercellular ROS signaling.

A. Tissue culture insert system (25, 35). For co-cultivation of cells without cell-to-cell contact, a combination of Falcon 6-well tissue culture clusters with tissue culture inserts (TCI) was used (pore-size of inserts 0.4 μm, distance between cell layers approximately 2 mm, Becton Dickinson, Heidelberg, Germany). Effector cells (*i.e*. the cells to be tested for support of intercellular ROS signaling through release of peroxidase and NO) were seeded into the inserts (4×10^4 cells per insert or as indicated in the respective figure legends). After the cells were attached, they were treated with 20 ng/ml TGF-β for two days (37˚C, 5% CO_2) or not, as indicated in the respective figure legends. Medium was then removed, the inserts were washed with medium and placed above target cells (*i.e*. cells to be tested for their apoptotic response to intercellular ROS signaling, based on their superoxide anion generation) in 6-well plates. Target cells were seeded dispersely (40,000 cells per assay or number as indicated). Tissue culture inserts were placed above target cells within less than a day after the seeding of the latter. After the indicated time of co-culture, the assays were checked for the classical morphological signs for apoptotic cells (membrane blebbing, chromatin condensation and fragmentation) using phase-contrast microscopy as described elsewhere (35, 57). We have recently confirmed (27, 57) that chromatin condensation/fragmentation was paralled by DNA strand breaks, detectable by the TUNEL reaction, following the method described by Gorcyca *et al*. (58).

The percentage of apoptotic cells was determined from at least 200 cells categorized per assay. Care was taken to differentiate apoptotic cells from nonapoptotic rounded cells with intact nuclei.

B. Coculture of clumps of target cells with overlaid dispersed effector cells. Alternatively to the tissue culture insert system, apoptosis induction through intercellular ROS signaling can be measured when target cells are seeded as two clumps (2,000 cells in 5 μl medium) in 12-well tissue culture clusters. After the cells attached, the clumps were overlaid with 1 ml of medium and 15,000 effector cells. TGF-β and inhibitors were added or not. As essential control, clumps of target cells were cultivated in medium

in the absence of effector cells. As further control, dispersely seeded effector cells were cultivated in the absence of target cells. Apoptosis induction in the target cell clumps was determined as described above for the tissue culture insert system. The principle of this assay is based on the support of peroxidase and NO by the dispersely seeded effector cells (which have high number but low local density) to the target cells that are low in total cell number but high in local density. This high local density is required for efficient hydrogen peroxide formation through dismutation of target cell-derived superoxide anions. Therefore, the combination of dispersely seeded effector cells and target cells in clumps leads to apoptosis specifically in the target cells. This assay can be used for the measurement of intercellular induction of apoptosis (when transformed target cells are surrounded by nontransformed effector cells) or autocrine apoptotic self-destruction (when transformed target cells are surrounded by dispersely seeded transformed effector cells).

C. Direct measurement of autocrine apoptotic self-destruction. Cells to be tested were seeded at a density of 25,000 cells in 48 well tissue culture clusters (overlaied by 200 μl of complete medium) (Figures 1 B and 9) or 12,500 cells in 96 well tissue culture clusters (100 μl of complete medium) (Figures 4-8). After attachment of the cells, TGF-β as well as inhibitors were added or not, as indicated in the respective figure legends. The percentage of apoptotic cells was determined at the indicated times according to the criteria described under A.

D. Treatment of tumor cells with specific compounds. Treatment of tumor cells with HOCl, GOX, MPO, 3-AT in the absence or presence of specific scavengers (AEBSF, Mn-SOD, taurine, mannitol, DMTU, ABH) or caspase-3 and caspase-9 inhibitors was performed under the conditions of direct measurement of autocrine apoptotic self-destruction (12,500 cells/100 μl complete medium in 96-well tissue culture clusters). The concentrations of the compounds added and the time of measurement are given in the respective figure legends.

Statistics. In all experiments, assays were performed in duplicate. The empirical standard deviation was calculated and is shown in the figures. Absence of standard deviation bars for certain points indicates that the standard deviation was too small to be reported by the graphic program, *i.e.* that results obtained in parallel were nearly identical. Empirical standard deviations were calculated merely to demonstrate how close the results were obtained in parallel assays within the same experiment and not with the intention of statistical analysis of variance, which would require larger numbers of parallel assays. Standard deviations were not calculated between different experiments, due to the usual variation in kinetics of complex biological systems *in vitro*. The Yates continuity corrected chi-square test was used for the statistical determination of significances.

Results

In order to gain insight into the mechanism of the recently described resistance of tumor cells against intercellular induction of apoptosis (45, 46) and to test whether this finding extends to autocrine apoptotic self-destruction, src oncogene-transformed fibroblasts (208Fsrc3) and the murine fibrosarcoma cell line L929 were subjected to conditions of intercellular induction of apoptosis by nontransformed murine fibroblasts and to autocrine self-destruction. As can be seen in Figure 1, the transformed cells readily underwent intercellular induction of apoptosis and autocrine self-destruction, whereas the tumor cells were resistant against both effects. When nontransformed cells were used as target cells, they showed no sensitivity for apoptosis induction, confirming the selectivity of the process with respect to the transformed state of the target cells (data not shown).

For a direct measurement of the cellular effector function, tissue culture inserts containing either transformed, nontransformed or tumor cells were placed above transformed target cells. Cell-containing inserts had been pretreated with TGF-β or not. As can be seen in Figure 2, all three cell systems (nontransformed, transformed, tumor cells) exhibited a strong apoptosis-inducing effect on transformed target cells when they had been pretreated with TGF-β, and a delayed response without preceding TGF-β pretreatment. In the absence of exogenous TGF-β pretreatment, the tumor cells showed the strongest effect amongst the three cell systems tested. This result demonstrates that nontransformed, transformed and tumor cells show comparable effector function. Therefore, the effector cell function (in contrast to the target cell function, which is restricted to cells with the transformed phenotype) is not specific for a distinct stage of cells during multistage oncogenesis. In addition, the lack of autocrine self-destruction of tumor cells seems not to be due to their lacking effector function. Figure 3 shows that a variety of murine and human tumor cells tested uniformly showed effector function specifically against transformed target cells, while nontransformed target cells remained unaffected. When nontransformed 208F cells and several of its derivatives transformed by different oncogenes were tested as target cells challenged by the tumor cell L929 as effector cells, the nontransformed parental cell 208F showed no apoptotic response, whereas all transformed lines, independent of the oncogene responsible for their transformation, were found to be sensitive to apoptosis induction.

The experiments shown so far indicate that the tumor cells still possess the effector function which is necessary for autocrine self-destruction. Their lacking an apoptotic response in the effector cell-driven and the autocrine assay might therefore be due to a lack of extracellular superoxide anion generation, a defect in intracellular apoptotic pathways, or resistance against intercellular ROS signaling. In order to test for superoxide anion production and a functional apoptotic response, the human gastric tumor cell line MKN-45 was treated with increasing concentrations of exogenous HOCl, which represents the major player in the HOCl signaling pathway (25, 18) and that requires interaction with target cell-derived superoxide anions to allow generation of apoptosis-inducing hydroxyl radicals. As shown in Figure 4, HOCl induced apoptosis in the tumor cells rapidly and in a concentration-dependent manner.

Apoptosis induction was blocked by the HOCl scavenger taurine, verifying that HOCl was indeed the apoptosis-mediating agent. Apoptosis induction by HOCl was inhibited by Mn-SOD (a scavenger of superoxide anions), AEBSF (an inhibitor of the superoxide anion generating NADPH oxidase) and the hydroxyl radical-scavenger mannitol.

First of all, this finding confirms that HOCl does not induce apoptosis directly, but rather that it acts through its reaction with superoxide anions, leading to the formation of hydroxyl radicals. These seem to represent the ultimate apoptosis inducers. Secondly and very importantly, the apoptosis-inducing effect of HOCl and the inhibitor data demonstrate that i) the tumor cells generate sufficient superoxide anions for the interaction with HOCl, ii) NADPH oxidase seems to be the source of superoxide anions, and that iii) the intracellular apoptosis pathways that are induced by intercellular ROS signaling are functional. Therefore, interference with extracellular ROS signaling remains one very reasonable explanation for the resistance of tumor cells to intercellular ROS signaling.

In order to clarify the basis of a potential interference mechanism, the tumor cells were kept under conditions that would allow autocrine self-destruction in sensitive transformed cells. Two major signaling components were added to the tumor cells: GOX to establish continuous hydrogen peroxide generation, MPO, and a combination of both. As can be seen in Figure 5, the tumor cells showed a remarkable insensitivity against hydrogen peroxide generated by GOX. Only relatively high concentrations of GOX induced apoptosis. This apoptosis-inducing effect was, however, not due to the direct apoptosis-inducing potential of hydrogen peroxide (39), as the process was completely inhibited by the HOCl scavenger taurine. Addition of 200 mU/ml of MPO alone had no significant direct effect on apoptosis induction. However, in combination with hydrogen peroxide-generating GOX, MPO exhibited an impressive synergistic effect. This synergistic effect also seemed to be due to the formation and action of HOCl, as it was completely inhibited by taurine.

For a detailed analysis of apoptosis induction in tumor cells after addition of GOX alone or in combination with MPO, specific inhibitors of intercellular ROS signaling as well as caspase inhibitors were added to the system and the effects were measured. As can be seen in Figure 6, both the effect of GOX given alone and its synergistic effect with MPO were inhibited by the superoxide anion scavenger MN-SOD, the NADPH oxidase inhibitor AEBSF, the peroxidase inhibitor ABH, the HOCl scavenger taurine, the hydroxyl radical-scavenger mannitol, as well as by caspase-3 and caspase-9 inhibitors. These findings indicate that the addition of high concentrations of glucose oxidase or the combination of glucose oxidase with MPO restores the HOCl signaling pathway. The inhibition of the process by caspase-3 and -9 inhibitors demonstrates that the cells die by caspase-dependent apoptosis. The strong effect of the caspase-9 inhibitor thereby indicates that the mitochondrial apoptosis signaling pathway is used (59).

The data shown so far indicate that tumor cells can be resensitized for intercellular ROS-induces apoptosis when the HOCl signaling pathway is re-established by the addition of an exogenous hydrogen peroxide source. The best explanation for this finding is a protective role of tumor cell catalase against intercellular ROS signaling. If this assumption were correct, the addition of the specific catalase inhibitor 3-AT should restore autocrine ROS-mediated apoptosis in tumor cells. In analogy to the experiments shown in Figures 5 and 6, this process of resensitization should be further enhanced by the addition of MPO. To address this question, human tumor cells were treated with increasing concentrations of 3-AT in the absence or presence of additional MPO. As can be seen in Figure 7, 3-AT caused a significant increase in apoptosis induction in tumor cells. At 3.5 hours, apoptosis induction by 3-AT alone showed the characteristics of an optimum curve. As expected from our previous findings, MPO added to the cells alone showed no significant effect, but it exhibited a strong synergistic effect with that of 3-AT. The experiments shown in Figure 8 demonstrate that the synergistic effect between MPO and 3-AT, as well as the effect of 3-AT alone, were dependent on intercellular ROS signaling by the HOCl pathway, as scavenging of each one of the components of this signaling pathway caused strong inhibition of apoptosis. As in the previous controls, tumor cells alone or in the presence of MPO but without 3-AT showed no apoptosis induction above background levels. The strong effect of the catalase inhibitor 3-AT indicates the protective role of tumor cell catalase against intercellular ROS signaling. It also points to the potential of catalase inhibition for resensitization of tumor cells. The strong inhibitory effect of caspase-3 and caspase-9 inhibitor demonstrates that tumor cells use the mitochondrial apoptosis pathway when their intercellular ROS signaling is re-established after catalase inhibition.

The final experiments (Table I and Figure 9) define the differential modes of intercellular ROS signaling in cells that represent three consecutive stages of tumor development, *i.e.* nontransformed, transformed and tumor cells. Table I demonstrates that nontransformed 208F cells are insensitive to intercellular induction of apoptosis by nontransformed effector cells both in the absence and presence of the catalase inhibitior 3-AT. Transformed 208Fsrc3 cells responded readily to intercellular induction of apoptosis by nontransformed effector cells. Their reaction was further enhanced by 3-AT. As expected from the preceding experiments, tumor cells showed resistance to intercellular induction of apoptosis but were resensitized by 3-AT. The resultant apoptosis induction

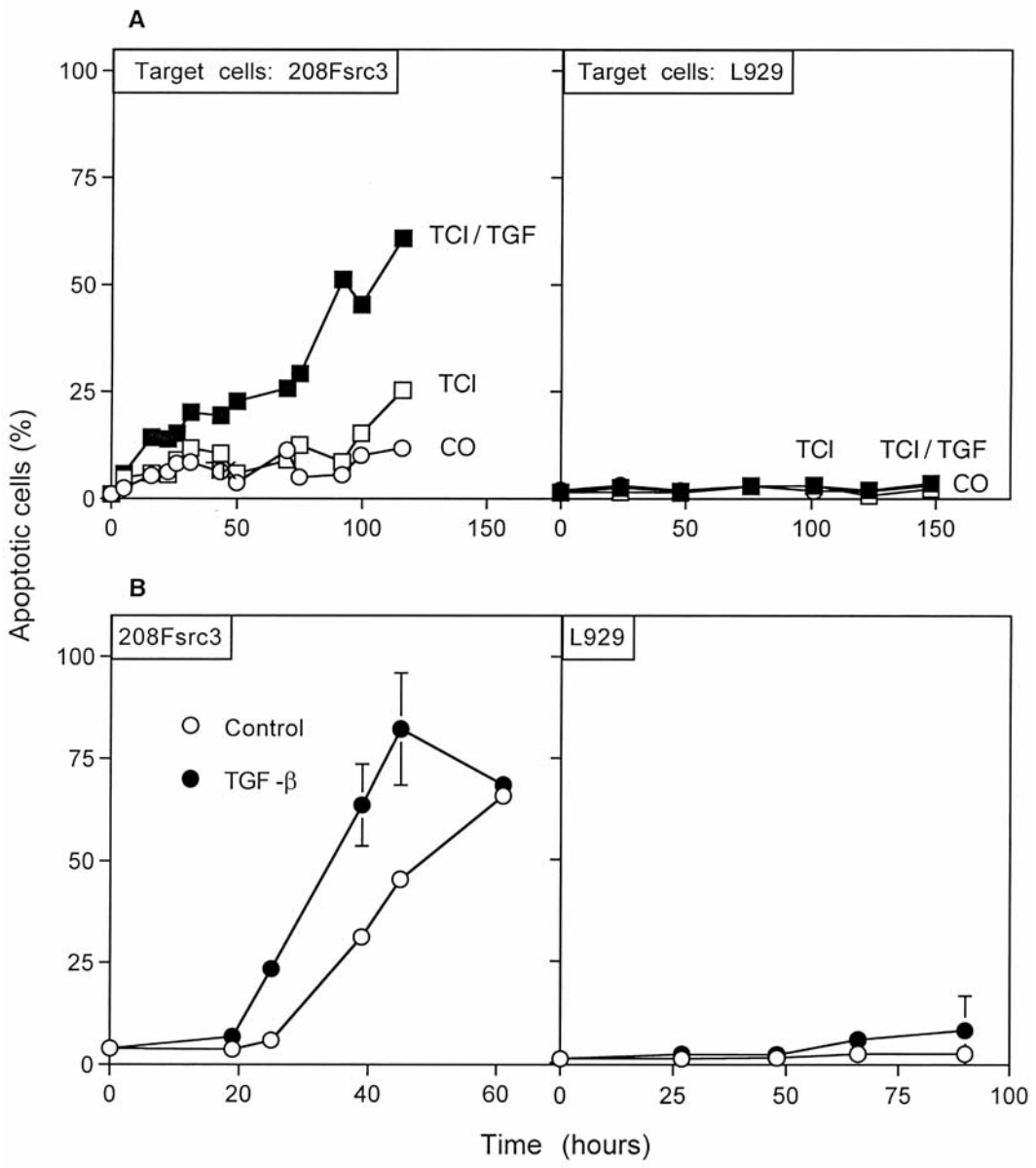

Figure 1. *L929 fibrosarcoma cells are resistant to intercellular induction of apoptosis and autocrine apoptotic self-destruction. A, Intercellular induction of apoptosis. Apoptosis induction through intercellular ROS signaling using the tissue culture insert system (TCI) was performed with the indicated target cells and either 208F effector cells in tissue culture inserts (TCI) or transforming growth factor-β-pretreated 208F effector cells in tissue culture inserts (TCI/TGF). Control assays with target cells remained without tissue culture inserts and effector cells (CO). All assays were performed in duplicate. At the indicated times, the percentage of apoptotic target cells was determined by phase-contrast inverted microscopy, as described under Materials and Methods. B, Direct measurement of autocrine apoptotic self-destruction. 208Fsrc3 or L929 cells were tested under the conditions for direct measurement of apoptotic self-destruction in the absence (Control) or presence (TGF-β) of 20 ng/ml TGF-β. All assays were performed in duplicate. At the indicated times, the percentage of apoptotic target cells was determined by phase-contrast inverted microscopy.*

was dependent on the establishment of the HOCl signaling pathway. When nontransformed cells were omitted as effector cells and the three cell systems were subjected to conditions of autocrine apoptotic self-destruction, the general outcome was analogous to that seen for intercellular induction of apoptosis (Figure 9): nontransformed cells showed insensitivity to autocrine apoptotic self-destruction, transformed cells were sensitive and their reaction was further enhanced by 3-AT, while tumor cells showed resistance, but were sensitized by the catalase inhibitor.

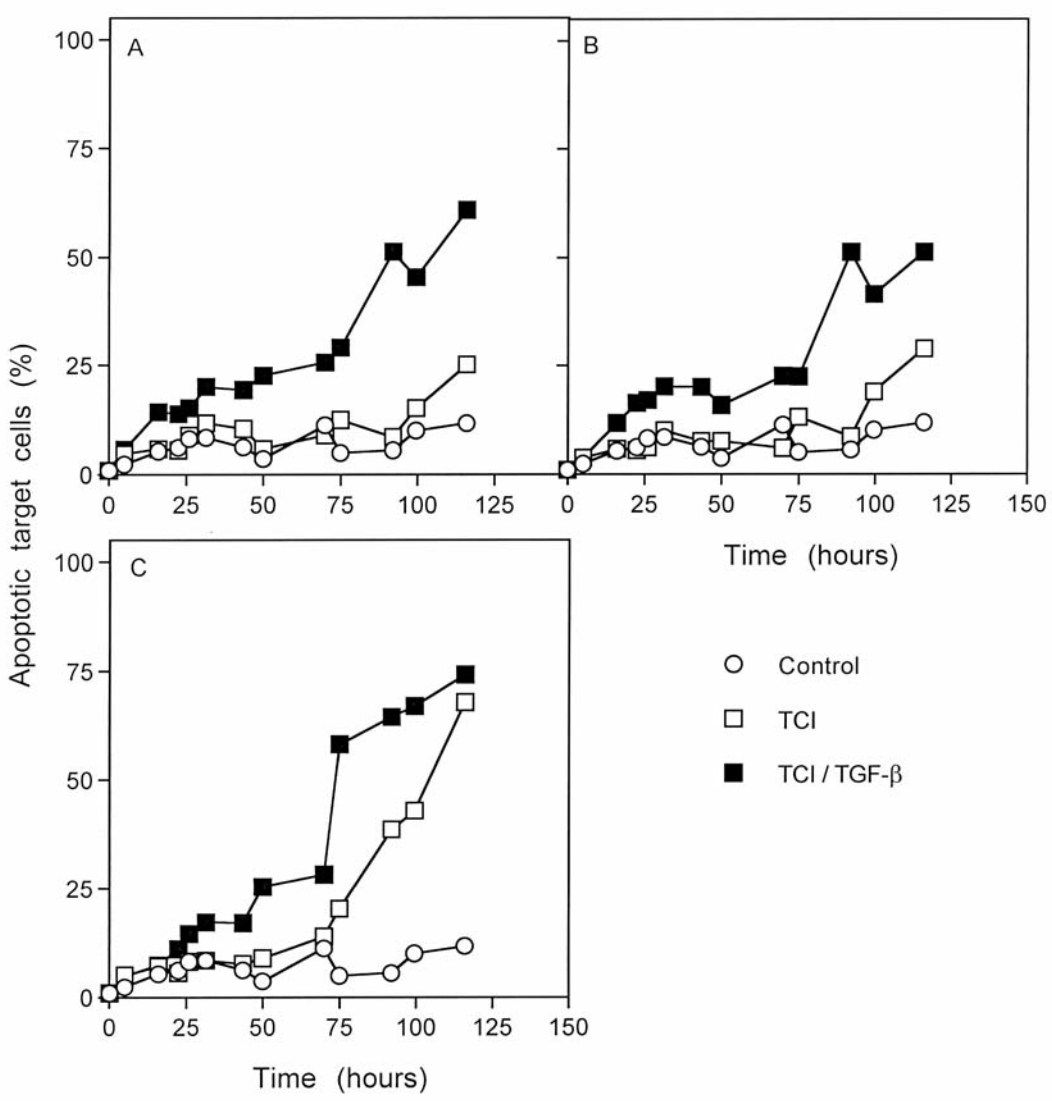

Figure 2. *Nontransformed (A), transformed (B) and tumor cells (C) exhibit effector function. Apoptosis induction through intercellular ROS signaling using the tissue culture insert system was performed with 208Fsrc3 cells as target cells and either 208F cells (A), 208Fsrc3 cells (B) or L929 cells (C) as effector cells. Effector cells were either untreated (TCI) or had been pretreated with 20 ng/ml TGF-β (TCI/TGF-β) Control target cell assays (Control) remained without tissue culture inserts. All assays were performed in duplicate. At the indicated times, the percentage of apoptotic target cells was determined by phase-contrast inverted microscopy.*

Discussion

During intercellular induction of apoptosis (18-20, 25, 35, 57) apoptosis is selectively induced in transformed target cells through interaction with nontransformed effector cells. Extracellular superoxide anion generation by the target cells and release of peroxidase by the effector cells represents the biochemical basis for the HOCl signaling pathway which is of central importance for intercellular ROS signaling (18, 19, 25). This pathway is the focus of this study. The nature and function of the peroxidase will be reported elsewhere (in preparation). Whereas the target cell function is highly specific for cells with the transformed phenotype (21, 25-27), the effector function can be exerted by nontransformed, transformed and also by tumor cells, as shown here. Several murine and human tumor cell lines showed efficient effector function that specifically interacted with target cells transformed by different oncogenes, but not with nontransformed cells. This specificity is based on extracellular superoxide anion generation by transformed cells (21, 22, 25, 26). The release of peroxidase, as shown by the inhibition of apoptosis by ABH, enabled transformed

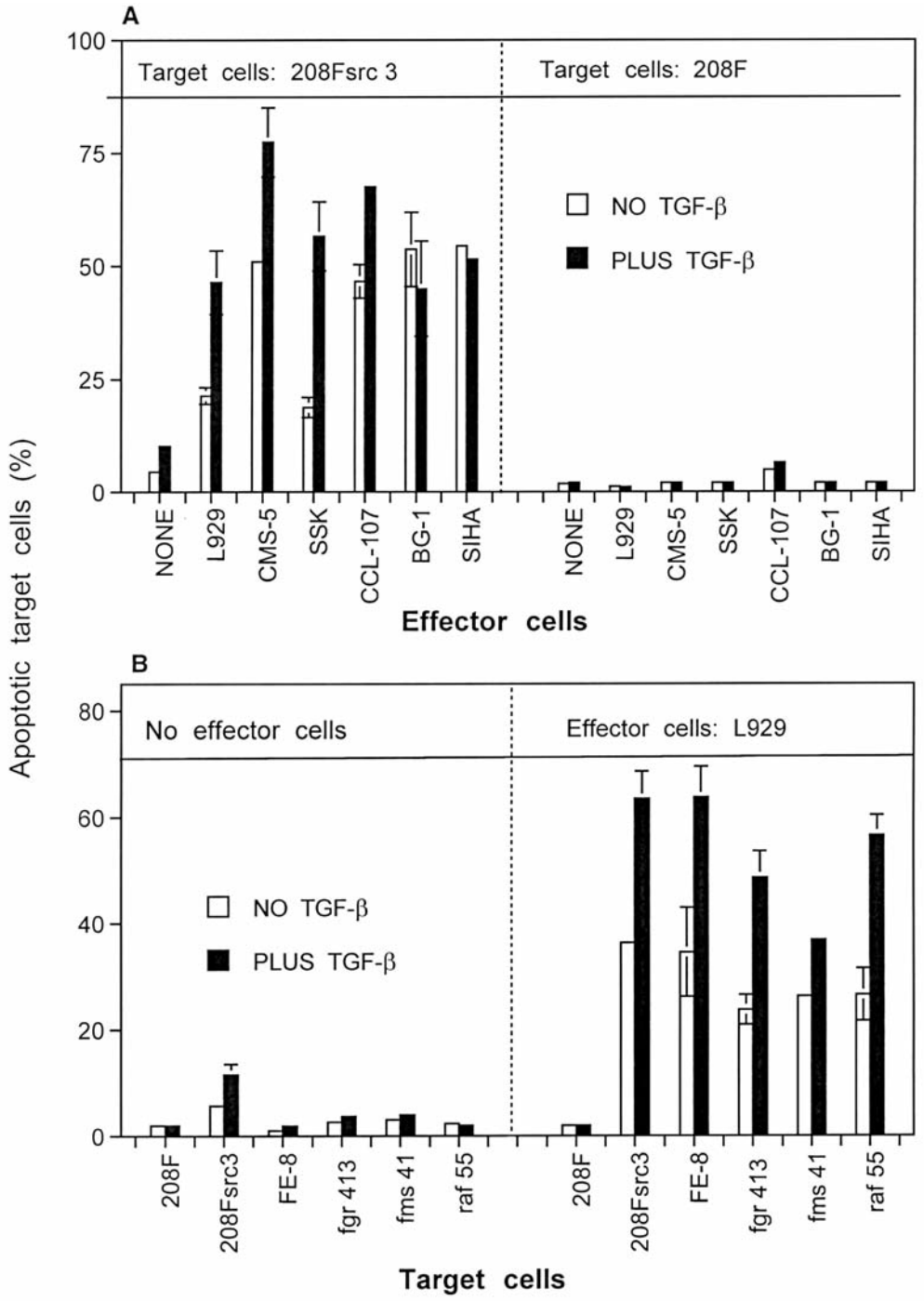

Figure 3. *Tumor cell effector function is specifically directed against the transformed phenotype. A, Effector function of murine and human tumor cell lines is selectively directed against transformed cells. Coculture of clumps of target cells (208Fsrc3 or 208F cells, as indicated) was performed with overlaid indicated disperse effector cells. L929, CMS-5 and SSK are murine fibrosarcoma lines, CCL-107 represents a rat glioblastoma line, BG-1 are human ovarial carcinoma cells and SIHA was derived from a HPV-positive cervix carcinoma. In this experimental setup, the target cells have high local density (ensuring sufficient local hydrogen peroxide production) but are small in total number. Therefore, apoptosis induction depends on exogenously added effector cells for establishment of a sufficient peroxidase concentration. All assays were performed in duplicate. After two days, the percentage of apoptotic cells in the clumps of target cells was determined by phase-contrast inverted microscopy. B, Effector function of L929 cells is directed against cells transformed by various oncogenes but not against nontransformed parental cells. Clumps of the target cell lines 208F (nontransformed fibroblasts) or their derivatives transformed by the v-src (208Fsrc3), HRAS (FE-8), v-fgr (fgr413), v-fms (fms41) or v-raf oncogene (raf 55) were overlaid with L929 cells as effector cells or remained free of effector cells. All assays were performed in duplicate, either in the absence or presence of 20 ng/ml TGF-β. After three days, the percentage of apoptotic target cells in the clumps was determined by phase-contrast inverted microscopy.*

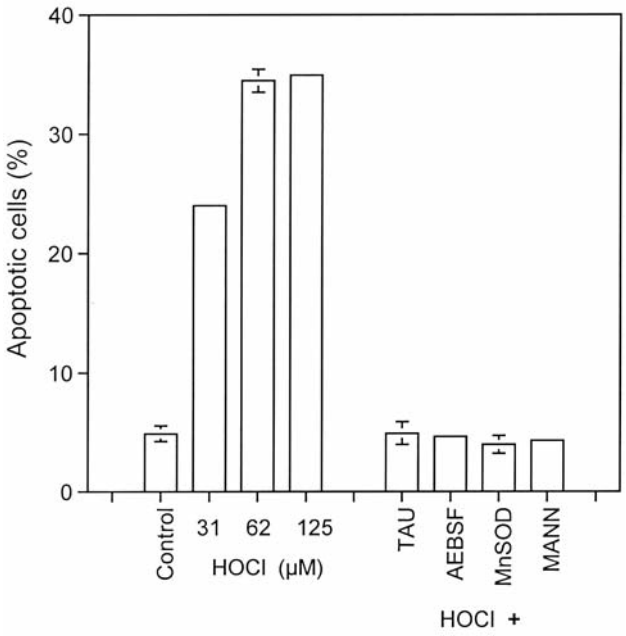

Figure 4. *HOCl induces apoptosis in tumor cells. MKN-45 gastric carcinoma cells were seeded under conditions for direct measurement of autocrine apoptotic self-destruction. Cells received the indicated final concentration of HOCl or 125 μM HOCl in the presence of 50 mM taurine, 100 μM AEBSF, 100 U/ml Mn-SOD, or 10 mM mannitol. Control assays received no HOCl. All assays were performed in duplicate. After 2 h, the percentage of apoptotic target cells was determined by phase-contrast inverted microscopy. Parallel assays ensured that the inhibitors alone had no toxic effect (data not shown).*

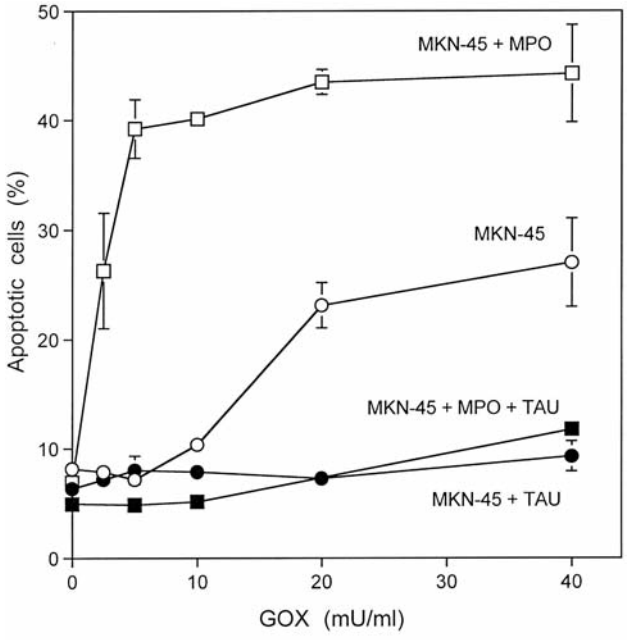

Figure 5. *Apoptosis induction in tumor cells by GOX and GOX/MPO. MKN-45 cells were seeded under conditions for direct measurement of autocrine apoptotic self-destruction The cells received the indicated concentrations of GOX in the absence or presence of additional MPO (200 mU/ml). Assays with GOX or GOX/MPO received 50 mM taurine or not. All assays were performed in duplicate. After 1.5 h, the percentage of apoptotic cells was determined by inverted phase-contrast microscopy.*

cells to establish autocrine apoptotic self-destruction through interaction between their own peroxidase with hydrogen peroxide derived from their extracellular superoxide anions, and subsequent ROS signaling *via* the HOCl signaling pathway. Autocrine self-destruction requires a sufficient cell density for optimal hydrogen peroxide generation and a sufficient cell number for maintaining a sufficient concentration of peroxidase. ROS signaling during intercellular induction of apoptosis and autocrine apoptotic self-destruction are identical (except for the source of the effector molecules). Therefore we suggest that the term 'intercellular ROS signaling' be used for both systems. Nontransformed cells cannot exert autocrine apoptotic self-destruction as they do not generate superoxide anions. As tumor cells are resistant to autocrine apoptotic self-destruction despite efficient effector function (Figure 1) and are also resistant to intercellular induction of apoptosis, their resistance to intercellular ROS signaling might be due to different mechanisms: i) lacking or insufficient extracellular superoxide anion generation, ii) defects in the intracellular apoptosis-related pathways or iii) interference with intercellular ROS signaling. The use of the autocrine experimental setup allowed a direct and experimentally straightforward approach to resolve these questions.

As addition of HOCl readily induced apoptosis in tumor cells and in a process that depended on the interaction of HOCl with superoxide anions and subsequent hydroxyl radical formation, it was immediately ruled out that tumor cells would not generate superoxide anions and that they could not perform apoptosis. Tumor cells responded to HOCl like superoxide anion-generating transformed cells and differently from insensitive nontransformed cells that lack extracellular superoxide anion generation (38). As the addition of exogenous hydrogen peroxide (through GOX as source for steady hydrogen peroxide production) re-established intercellular ROS signaling of tumor cells, interference with ROS signaling or insufficient hydrogen peroxide generation by the tumor cells might account for their resistance. Theoretically, interference might act at any point of the HOCl signaling pathway and would be overcome when the central signaling element hydrogen peroxide was exogenously substituted. Alternatively, despite sufficient superoxide anion generation for efficient HOCl-

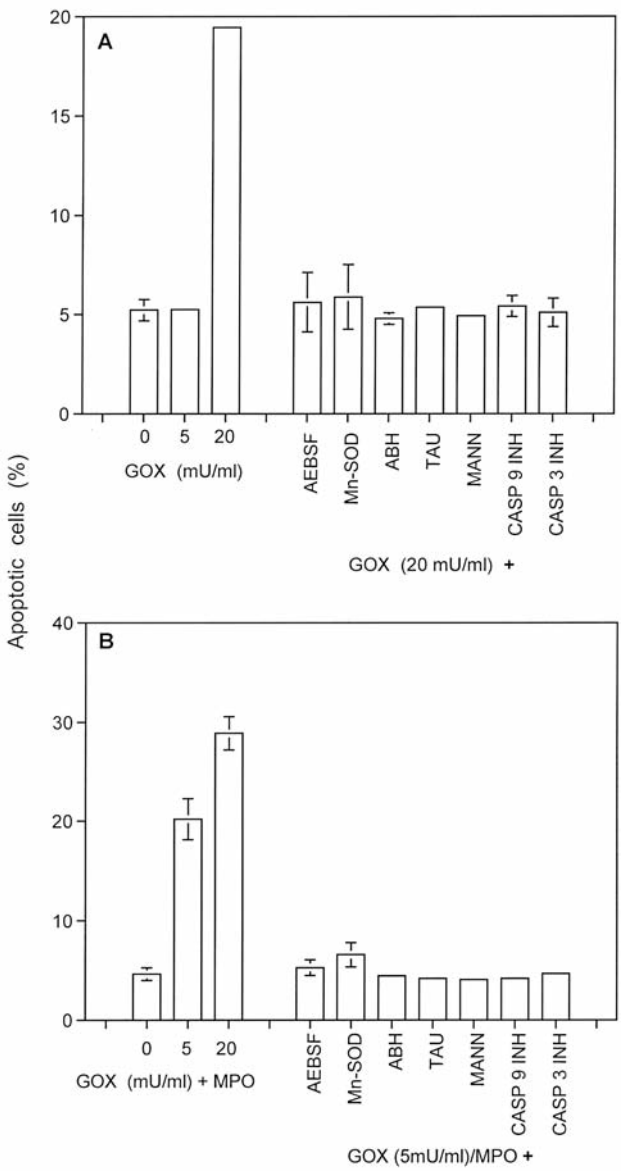

Figure 6. *Apoptosis induction by GOX or GOX / MPO is mediated by intercellular ROS signaling. MKN-45 gastric carcinoma cells were seeded under conditions for direct measurement of autocrine apoptotic self-destruction. A, The cells received the indicated concentrations of GOX, as well as 20 mU/ml GOX in the presence of 100 μM AEBSF (NADPH oxidase inhibitor), 120 U/ml Mn-SOD, 150 μM ABH (mechanism-based peroxidase inhibitor), 50 mM taurine (HOCl scavenger), 10 mM mannitol (hydroxyl radical scavenger), 25 μM caspase-9 inhibitor and 50 μM caspase-3 inhibitor. All assays were performed in duplicate. After 2 h, the percentage of apoptotic cells was determined by inverted phase-contrast microscopy. Control assays ensured that the inhibitors alone had not significant effect on apoptosis induction (data not shown). B, The experiment was performed as described under A, except that the cells received the indicated concentrations of GOX in combination with 200 mU/ml MPO. The inhibitor studies were performed with 5 mU/ml GOX plus 200 mU/ml MPO in the presence of the same inhibitors as described under A. All assays were performed in duplicate. After 2 h, the percentage of apoptotic cells was determined as described under A.*

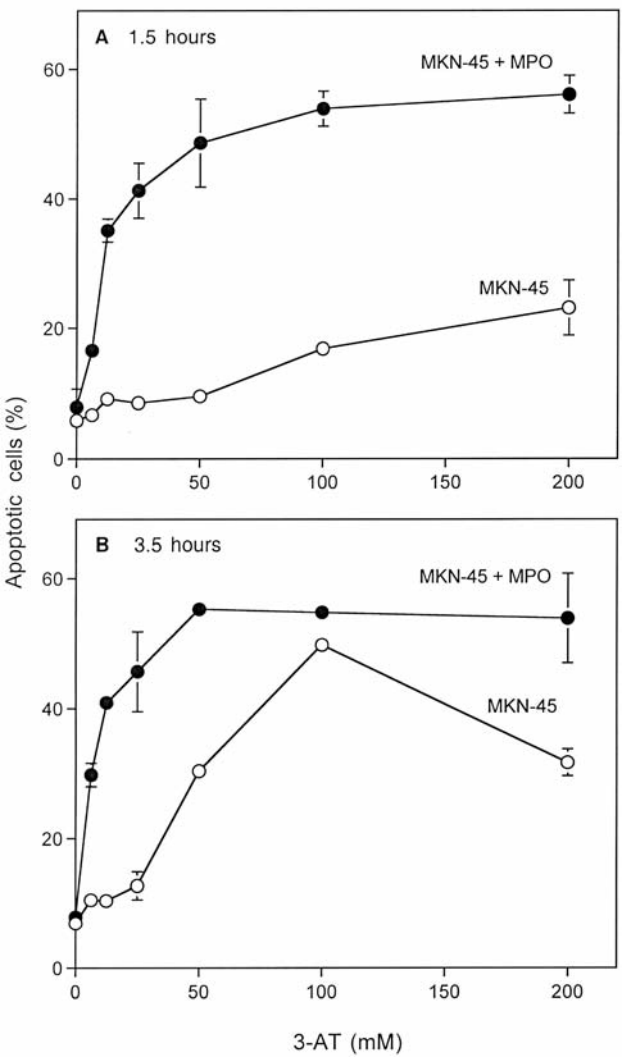

Figure 7. *Apoptosis induction in tumor cells after catalase inhibition by 3-AT. MKN-45 gastric carcinoma cells were seeded under conditions for direct measurement of autocrine apoptotic self-destruction and were treated with the indicated concentrations of 3-AT in the absence or presence of 200 mU/ml MPO. All assays were performed in duplicate. After 1.5 (A) and 3.5 h (B) the percentages of apoptotic cells were determined by phase contrast inverted microscopy.*

superoxide anion interaction, the concentration of superoxide anions generated by the tumor cells might be too low to drive an optimal hydrogen peroxide generation through the dismutation reaction. The inhibitory effects of taurine, AEBSF, Mn-SOD, mannitol and ABH proved that apoptosis induction after GOX addition was not due to the direct apoptosis-inducing effect of hydrogen peroxide (which is not selective with respect to the transformed state) (39), but must be due to the specific re-establishment of the HOCl pathway. The lack of apoptosis induction by exogenous MPO given

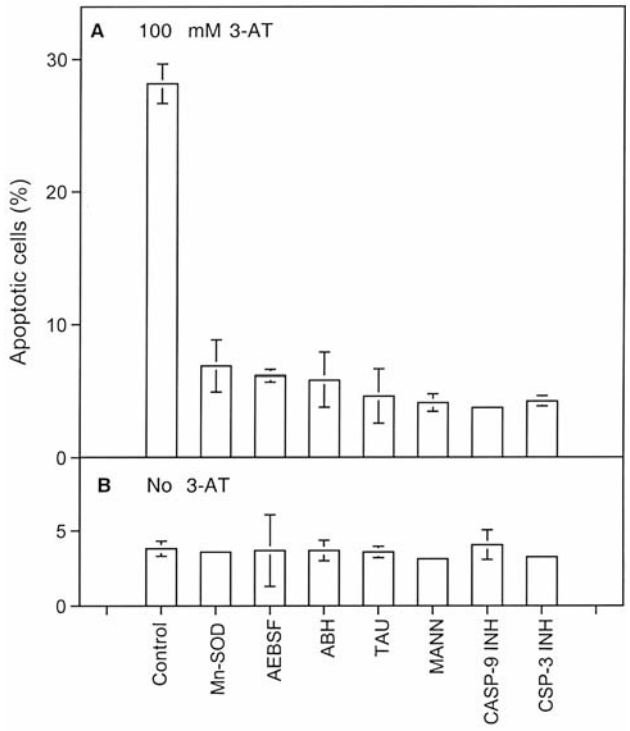

Figure 8. *Catalase inhibition leads to intercellular ROS signaling. MKN-45 gastric carcinoma cells were seeded under conditions for direct measurement of autocrine apoptotic self-destruction. Cells were incubated with the indicated inhibitors (concentration of the inhibitors as described in Figure 6) in the presence of 100 mM 3-AT (A) or without 3-AT (B). All assays were performed in duplicate. After 5.5 hours the percentage of apoptotic cells was determined by phase-contrast inverted microscopy. A parallel assay, using MKN-45 cells in the presence of 100 mM 3-AT and 200 mU/ml MPO in combination with the same inhibitors led to the same profile of inhibition (after 3.5 h) as demonstrated for the assays with 100 mM 3-AT alone (data not shown).*

alone points to there being limiting hydrogen peroxide in the tumor cell population, but does not exclude other modes of interference. The synergistic effect between MPO and GOX is well explained by an efficient use of hydrogen peroxide by MPO, in a situation where hydrogen peroxide is limited due to interference by the tumor cells.

Based on these considerations, it was not unlikely that consumption of hydrogen peroxide by tumor cell catalase was the mechanism for the observed resistance of tumor cells to intercellular ROS-dependent signaling. Re-establishment of autocrine apoptotic self-destruction in tumor cells through the catalase inhibitor 3-AT directly shows this concept to be correct. Therefore, consumption of hydrogen peroxide through catalase and not an insufficient superoxide anion generation seems to be the biochemical basis for tumor cell resistance against intercellular ROS-mediated signaling. Otherwise, inhibition of catalase alone would not have re-established intercellular ROS signaling. Apoptosis induction in tumor cells in the presence of 3-AT was dependent on the elements of the HOCl signaling pathway, *i.e.* on superoxide anions, peroxidase, HOCl and hydroxyl radicals. Parallel experiments (Table I) confirmed that 3-AT also sensitized the tumor cells for intercellular induction of apoptosis by neighbouring nontransformed cells. 3-AT showed no effect on the nontransformed effector cells and apoptosis induction in tumor cells was due to the same signaling as shown for the autocrine system here. Control of signaling by catalase in the presence of high superoxide anion generation by the tumor cells is in line with an increased Nox-1 expression during tumorigenesis *in vivo* (28, 33, 34). The inhibitory effect of caspase-3 and caspase-9 inhibitor confirmed that apoptosis induction in 3-AT-treated tumor cells, as well as in GOX and GOX + MPO-treated tumor cells was mediated by caspases and points to the central role of the mitochondrial apoptosis pathway (59).

Resistance of *ex vivo* tumor cells to intercellular induction of apoptosis and autocrine self-destruction, as shown here for the murine tumor cell line L929 and for the human gastric carcinoma cell line MKN-45, seems to be characteristic of human and murine tumor cells. In a broad survey that will be published elsewhere, more than 50 different human tumor cells were shown to uniformly use catalase as protective mechanism against intercellular ROS signaling. Resistance to ROS signaling through catalase expression therefore seems to be a rather general phenotypic trait of tumor cells. It therefore might also have a central protective function under *in vivo* conditions. This conclusion is in perfect concordance with the pioneering work by Galina Deichman (11-13), who showed that experimental tumor progression is dependent on a phenotype characterized by an increased resistance to hydrogen peroxide and prostaglandin E2 release. Injection of *in vitro* transformed hydrogen peroxide-sensitive cells into syngeneic hamsters caused tumor formation. Tumor cells isolated from these experimentally induced tumors exhibited the hydrogen peroxide-resistant phenotype, in contrast to the originally transformed cells. These data and the finding that all human tumor cells tested so far exhibit catalase-mediated resistance to intercellular ROS signaling supports the hypothesis that intercellular induction of apoptosis and autocrine apoptotic self-destruction represent a hitherto unrecognized control system that selectively eliminates ROS-signaling-sensitive transformed cells. If the transformed cells express protective catalase, the control system might fail and allow the growth of tumor cells that are protected against ROS signaling by their catalase. Work in progress, using the valuable experimental system of Deichman further supports this idea (Bauer and Deichman, in preparation). Protection of tumor cells by catalase has been further substantiated by the work of several other groups (60-62).

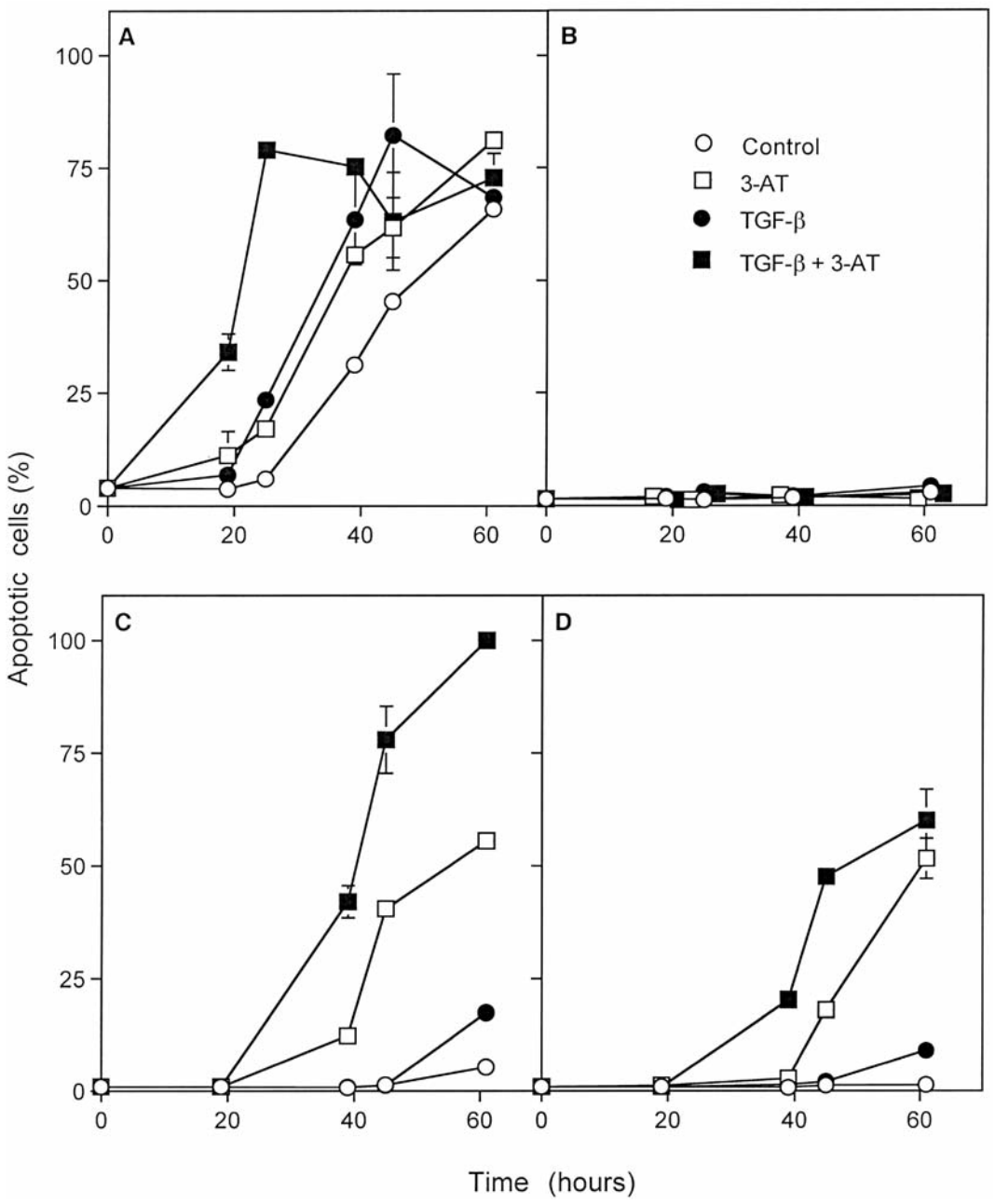

Figure 9. *Nontransformed, transformed and tumor cells: distinct features in autocrine ROS-mediated apoptotic self-destruction. Transformed 208Fsrc3 cells (A), nontransformed 208F cells (B), or murine tumor cells CMS-5 (C) or SSK (D) were seeded under conditions for direct measurement of autocrine apoptotic self-destruction. The assays received either no addition (Control), 25 mM 3-AT, 20 ng/ml TGF-β or 3-AT and TGF-β. All assays were performed in duplicate. At the indicated times, the percentage of apoptotic cells was determined by phase-contrast inverted microscopy. Note that nontransformed cells (B) are insensitive to autocrine apoptosis induction and can not be sensitized by the catalase inhibitor 3-AT. In contrast, their transformed descendants 208Fsrc3 (A) are sensitive, but can be further enhanced in reactivity by 3-AT. The tumor cells tested (C, D) are resistant to autocrine apoptosis induction and only show marginal apoptosis induction without 3-AT. In the presence of the catalase inhibitor they become sensitized for autocrine signaling.*

Our findings are in concordance with the classical work by Clark and colleagues on the cytotoxic effects on tumor cells of the combination of myeloperoxidase or lactoperoxidase with hydrogen peroxide-generating systems, either applied directly or by neutrophils (63-67). It is reasonable to assume that in these experiments, HOCl synthesized by peroxidase in the presence of hydrogen peroxide reacted with superoxide anions and led to the generation of apoptosis-inducing

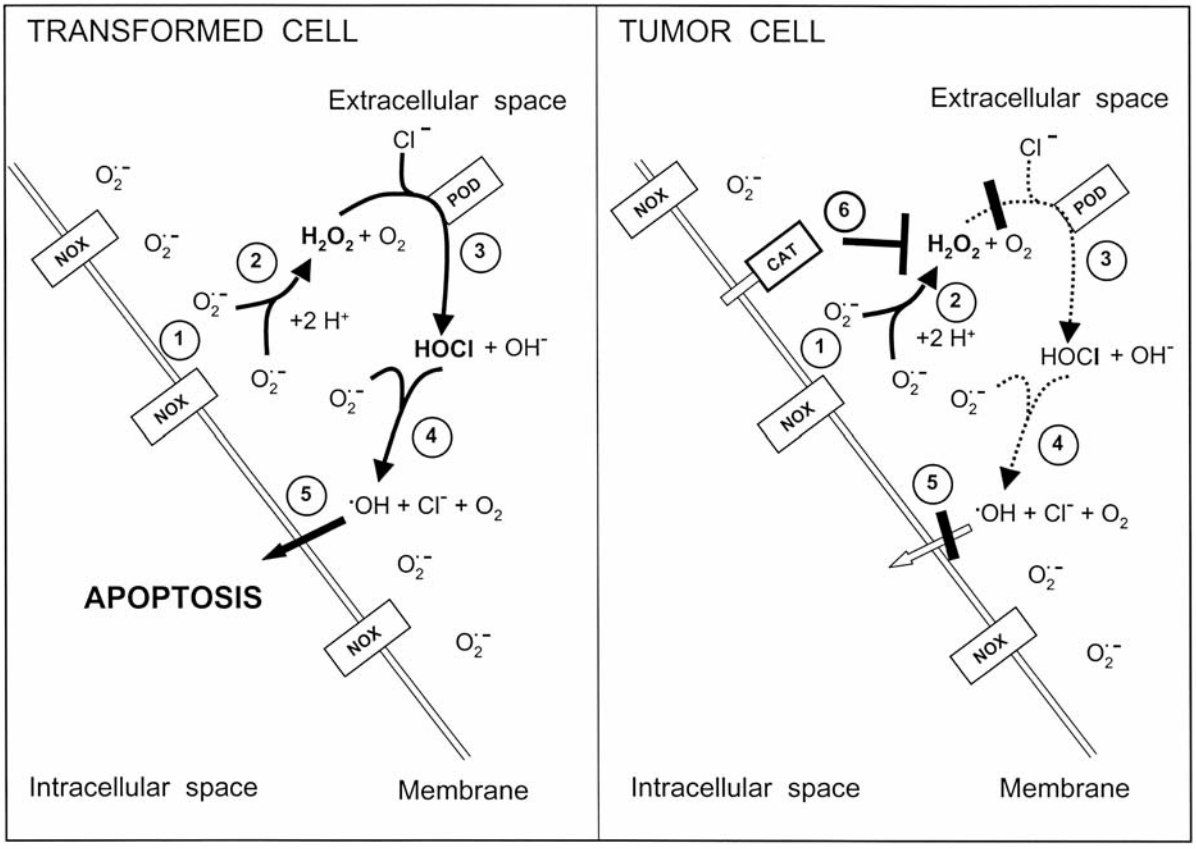

Figure 10. *Catalase protects tumor cells against intercellular ROS signaling. Intercellular ROS signaling of transformed cells. Transformed cells are characterized by activated membrane-associated NADPH oxidase (NOX) that generates superoxide anions (step 1). Superoxide anions dismutate spontaneously and form hydrogen peroxide (step 2). A novel peroxidase, either released by neighbouring nontransformed cells or by the transformed cells themselves in an autocrine mode, uses hydrogen peroxide as substrate for HOCl synthesis (step 3). HOCl then interacts with superoxide anions at the membrane of the transformed cell (step 4), resulting in the generation of apoptosis-inducing hydroxyl radicals (step 5). Apoptosis induction is based on lipid peroxidation and subsequent activation of the mitochondrial pathway, involving caspase-9 (not shown). Finally, caspase-3 becomes involved (not shown). Nontransformed cells lack extracellular superoxide anion generation and therefore cannot be the target of intercellular ROS signaling (not shown). Transformed cells in this context are defined as cells with activated oncogenes and the potential to form tumors. Transformed cells have not been challenged by the natural antitumor systems of a living organism. Catalase protects tumor cells against intercellular ROS signaling. Tumor cells (derived from bona fide tumors of animals or patients) are characterized by resistance to intercellular ROS signaling. Tumor cells exhibit superoxide anion generation and functional apoptosis pathways. However, intercellular ROS signaling is inhibited by a membrane-associated catalase that efficiently destroys hydrogen peroxide (step 6) and thus prevents HOCl synthesis.*

hydroxyl radicals. At the time of these classic studies, it was not known that tumor cells generate extracellular superoxide anions and thus can specifically contribute to ROS signaling. As MPO and hydrogen peroxide generation had to be present to obtain the cytotoxic effect, the experimental situation in these earlier papers seems to resemble the synergistic effect between MPO and GOX shown in Figure 5 of the work presented here. Our own work does not support the finding by Weiss and Slivaka (68) on the independence of HOCl-mediated cytotoxic effects from hydroxyl radical formation. Furthermore, our work is in direct contradiction to the findings by Wagner *et al*. (69) on the role of chloramines for HOCl-mediated cytotoxic effects. Chloramines do not seem to play a detectable role in HOCl-mediated apoptosis in our experiments, as the HOCl effect was inhibited by taurine, despite the interaction of HOCl and taurine resulting in the formation of taurine chloramine.

As large numbers of resistant tumor cells can act as effector cells that establish apoptosis induction in clumps of neighbouring sensitive transformed target cells at high local density (Figures 2 and 3), catalase does not seem to be released by the tumor cells, but rather seems to be adherent to them in a stable mode. Otherwise the effector function of the tumor cells would have been masked by interference of tumor cell-derived catalase with ROS signaling of the transformed target cells. This argument is further strengthened through the

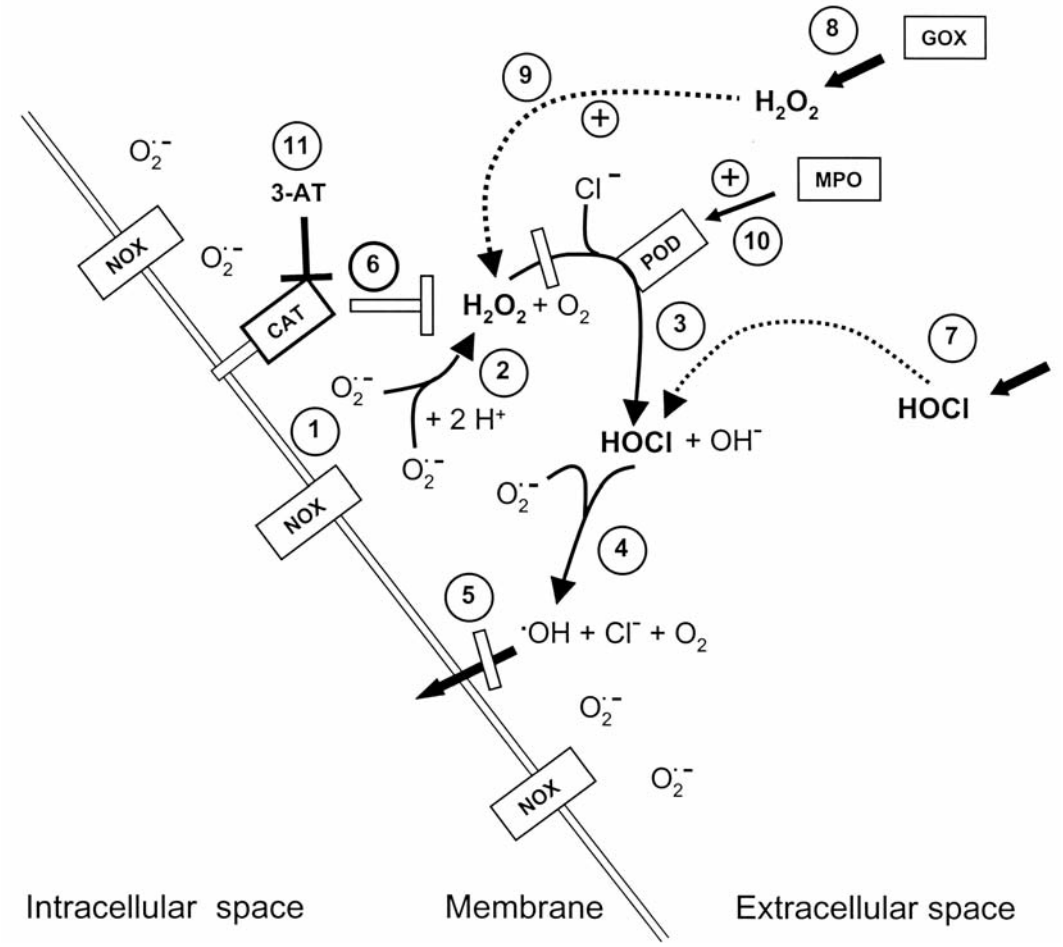

Figure 11. *Sensitization of tumor cells for intercellular ROS signaling through addition of signaling components. As outlined under Figure 10, tumor cells are resistant to intercellular ROS signaling through the expression of membrane-associated catalase (steps 1-6). Addition of exogenous HOCl (step 7) leads to apoptosis induction, as HOCl interacts with superoxide anions generated by the tumor cells and forms apoptosis-inducing hydroxyl radicals (steps 4, 5). Addition of glucose oxidase (GOX) leads to the generation of hydrogen peroxide (steps 8, 9) that eventually overruns the effect of catalase and restores signaling by the HOCl pathway (steps 3-5). Addition of exogenous MPO (step 10) has not detectable effect by itself but interacts synergistically with hydrogen peroxide generated by GOX. Inhibition of catalase by 3-AT restores intercellular ROS signaling (step 11). Addition of GOX and/or MPO interact synergistically with catalase inhibition by 3-AT.*

finding that tumor cells retain their high resistance to exogenous hydrogen peroxide even if they are centrifuged, washed and challenged immediately (data not shown).

When individualized transformed cells were mixed with an excess of tumor cells, ROS signaling of the transformed cells was abrogated as catalase located on neighbouring tumor cells seemed to destroy hydrogen peroxide generated by transformed cells (data not shown). In a reverse experiment, a small number of individualized tumor cells mixed with an excess of transformed cells showed sensitivity to intercellular signaling, as the bound catalase was unable to interfere with ROS signaling of the neighbouring transformed cells present in excess (data not shown). Thus, it was shown that HOCl generated by the transformed cells reaches the tumor cells and induces apoptosis after interaction with superoxide anions and generation of hydroxyl radicals. Catalase responsible for interference with intercellular and autocrine ROS signaling seems to be located on the outside of the tumor cell membrane, as i) its activity can be blocked by monoclonal antibodies against catalase and ROS signaling of tumor cells is then restored (Bauer *et al.*, in preparation), ii) it can be inactivated by extracellular singlet oxygen (Riethmüller and Bauer, in preparation), iii) it can be detected by indirect immunofluorescence and FACS analysis on intact cells (data not shown). Protective catalase of tumor cells seems to be located specifically at the outside of the cell membrane, in addition to classical intracellular catalase. Although the locations are different, both enzyme activities are otherwise indistinguishable, as indicated by siRNA interference experiments (work in progress). The localization of catalase

Table I. *Insensitivity, sensitivity and resistance to intercellular induction of apoptosis. Catalase protects tumor cells against intercellular induction of apoptosis by nontransformed effector cells.*

Target cell	Effector cell in TCI	3-AT	Apoptotic cells (%)
208F	–	–	1.5±0.2
208F	–	+	2.1±0.2
208F	208F	–	2.7±0.2
208F	208F	+	3±1.4
208Fsrc3	–	–	5±0.4
208Fsrc3	–	+	10.9±1
208Fsrc3	208F	–	55.8±10
208Fsrc3	208F	+	81.8±1
L929	–	–	1±0.2
L929	–	+	1.3± 0.2
L929	208F	–	1.5±0.2
L929	208F	+	38.5±0.2
L929 + Mn-SOD	208F	+	10.2±0.4
L929 + Taurine	208F	+	3.3±0.2
L929 + DMTU	208F	+	6.3±0.4

Apoptosis induction through intercellular ROS signaling using the tissue culture insert system (TCI) was performed with nontransformed 208F, transformed 208Fsrc3 and L929 tumor cells as target cells and TGF-β-pretreated 208F cells as effector cells. The assays received 25 mM of the catalase inhibitor 3-aminotriazole (3-AT) or not. In addition, assays with L929 cells as target cells, in the presence of TGF-β-pretreated 208F effector cells and 25 mM 3-AT received either 100 U/ml Mn-SOD, 50 mM taurine or 10 mM dimethylthiourea (DMTU). The percentage of apoptotic cells in duplicate assays was determined after 45 h in the case of 208Fsrc3 target cells and 69 h in the case of 208F and L929 target cells.

at the cell membrane of tumor cells may be of advantage, as this leads to a high local catalase concentration at the site to be protected. Ottaviano *et al*. (70) suggested that intracellular catalase would efficiently counteract extracellular hydrogen peroxide due to the rapid diffusion of hydrogen peroxide through membranes. However, based on our data, it seems that the extracellular location of catalase is favorable in the case of tumor cell protection against extracellular ROS signaling. In line with our data, catalase at the surface of tumor cells has been directly demonstrated by proteomic analysis (71). These findings contrast with but not contradicted by the findings of Gupta *et al*. (72) and Finch *et al*. (73) who demonstrated that an increase in total cellular catalase attenuates or even reverses tumorigenicity. A lower intracellular catalase activity has also been described in lung cancer (74). It will be important to differentiate between the effects of intracellular and cell membrane-associated tumor cell catalase in the future, as these activities are distinct and seem to influence tumorigenesis in opposite ways: a high level of extracellular membrane-associated catalase protects against extracellular ROS signaling, whereas a low level of intracellular catalase allows efficient intracellular signaling by hydrogen peroxide.

Figure 10 summarizes our findings on the protective role of tumor cell catalase against intercellular ROS signaling. The focus thereby is on the HOCl signaling pathway. Work in progress indicates that catalase also protects tumor cells against apoptosis induction by the NO-peroxynitrite and the nitryl chloride signaling pathway, as well as against the metal-catalyzed Haber Weiss reaction (Heinzelmann and Bauer, in preparation). Figure 11 summarizes the experimental approaches taken in this paper to elucidate the mechanism of tumor cell resistance to intercellular ROS signaling. Although already rather complex, this focuses on the major reactions only. Work in progress is elucidating a complicated network of secondary reactions arising from the basic scheme (Bauer, in preparation).

The knowledge of there being protective catalase on the membrane of tumor cells in combination with the potential to exert powerful apoptosis-inducing ROS signaling after catalase inhibition or destruction should allow novel and specific forms of antitumor therapy to be established and enlighten our understanding of tumor prevention. This approach is especially intriguing as the extracellular ROS generation of tumor cells, a specific trait which is linked to their transformed state, drives their selective apoptosis induction. Membrane-associated catalase thereby seems to represent the critical control element. Inhibition or destruction of membrane-associated catalase or prevention of its expression through siRNA might become useful and specific tools to resensitize tumor cells to apoptosis-inducing ROS signaling. Work along these lines may hopefully stimulate novel approaches in tumor prevention, drug development and cancer therapy.

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