Abstract. The lack of extracellular superoxide anion production by non-transformed cells prevents H$_2$O$_2$/peroxidase-mediated HOCl synthesis by these cells, as well as apoptosis induction by exogenous HOCl. In contrast, transformed cells generate extracellular superoxide anions and HOCl, and die by apoptosis after HOCl/superoxide-dependent hydroxyl radical generation at their membrane. Tumor cells prevent HOCl synthesis through expression of membrane-associated catalase, but their extracellular superoxide anions readily react with exogenous HOCl. The interaction between HOCl and H$_2$O$_2$ causes singlet oxygen generation that inactivates superoxide dismutase (SOD) on the surface of the tumor cells and thus enhances HOCl-mediated apoptosis through an increase in free superoxide anions. Higher concentrations of singlet oxygen inactivate membrane-associated catalase and thus lead to partial inhibition of apoptosis induction by exogenous HOCl, due to consumption of HOCl by H$_2$O$_2$. The data presented here show a complex, but coherent picture of interactions between defined reactive oxygen species and protective enzymes on the surface of tumor cells.

HOCl is used by neutrophils for their attack against microbes and tumor cells (1-12). The reaction of HOCl with superoxide anions leads to the generation of hydroxyl radicals (13-15) that efficiently destroy microbial structures or cause induction of apoptosis of malignant cells through lipid peroxidation. Due to the relatively short free-diffusion path length of superoxide anions and the extremely narrow free-diffusion path length of hydroxyl radicals, HOCl allows for site-specific generation of hydroxyl radicals defined by the locus of superoxide anion generation (16-18).

Site-specific generation of hydroxyl radicals through HOCl/superoxide anion interaction also represents an essential and critical step for selective reactive oxygen species (ROS)-driven apoptosis induction specifically in malignant cells, through the HOCl signaling pathway. The HOCl signaling pathway is established during intercellular induction of apoptosis and autocrine apoptotic self-destruction, two recently described potential controlling steps during oncogenesis (19-23). HOCl signaling depends on the generation of extracellular superoxide anions through membrane-associated NADPH oxidase (NOX1) that is characteristic and essential for cells transformed in vitro and for bona fide tumor cells (19, 22-29). Extracellular superoxide anions generated by malignant cells drive both the efficiency and selectivity of intercellular induction of apoptosis and autocrine apoptotic self-destruction (reviewed in 21, 23, 30). In an initial step, superoxide anions dismutate to hydrogen peroxide, which is subsequently used by the peroxidase domain of dual oxidase (DUOX) to generate HOCl. The peroxidase domain of DUOX can be supplied by neighboring non-transformed cells or the population of transformed cells themselves. HOCl then reacts with superoxide anions generated by the membrane-associated NADPH oxidase (NOX1), yielding apoptosis-inducing hydroxyl radicals. Whereas transformed cells are readily induced to die by apoptosis through this mechanism, tumor cells prevent HOCl synthesis through decomposition of hydrogen peroxide by membrane-associated catalase (21, 22). They thus escape the HOCl signaling pathway. However, tumor cells are sensitive to apoptosis induction by exogenously added HOCl or HOCl generated by attacking neutrophils. In contrast, despite the presence of DUOX, non-transformed cells cannot achieve HOCl synthesis as they lack active NOX1 to support generation of hydrogen peroxide. Non-transformed cells are also not sensitive to exogenously added HOCl in the micromolar concentration range, as they lack abundant extracellular superoxide anion generation required for HOCl/superoxide anion interaction.
The experimental design underlying this study conceptually dissects the HOCl signaling pathway into the initial step ‘HOCl synthesis’ and the subsequent step ‘HOCl/superoxide anion interaction’, focusing specifically on the latter. This approach allowed the unraveling of the additional strong potential of HOCl to modulate the apoptotic response through fostering singlet oxygen generation and inactivation of antioxidant enzymes of tumor cells. It, thus, significantly extends our experimentally-derived concept of tumor cell-specific resistance mechanisms directed towards ROS-mediated induction of apoptosis.

Materials and Methods

Materials. The following compounds were obtained from Sigma Aldrich (Schnelldorf, Germany). 4-(2-Aminoethyl-benzenesulfonyl fluoride (AEBSF) is a specific inhibitor of NADPH oxidases (31) and was stored as a stock solution of 10 mM in phosphate-buffered saline (PBS) at −20°C.

The catalase inhibitor 3-aminoatrizole (3-AT) (32) as stock solution (2 M in steril PBS) was stored at −20°C.

NaOCl as stock solution of 860 mM was kept at 4°C in the dark. As the negative base-10 logarithm of the acid dissociation constant (pKa) of NaOCl is 7.64, the majority of the compound is present as HOCl at neutral pH. For simplicity, the term ‘HOCl’ is used throughout this article. HOCl was diluted in cold, sterile PBS and then added to the assays by bolus addition (10–20 μl per 100 μl assay).

The singlet oxygen scavenger histidine (33) was kept as a stock solution of 20 mM in sterile PBS at −20°C.

Mannitol was kept as a stock solution of 1 M in PBS at −20°C. It represents a specific hydroxyl radical scavenger (34).

Manganese-containing superoxide dismutase (MnSOD) from Escherichia coli (stock solution at 30,000 Units/ml in sterile PBS) was kept at −20°C and only used once per aliquot. MnSOD is an efficient scavenger of superoxide anions, in a two-step reaction. MnSOD is not cell-permeable (35, 36) and therefore allows the functional role of extracellular superoxide anions to be demonstrated. MnSOD does not exhibit the sharp bell-shaped inhibition curve that is characteristic of cupper-containing SOD and therefore is superior to CuSOD in inhibition studies.

CuSOD from bovine erythrocytes was kept as a stock solution of 30,000 Units/ml in sterile PBS at −20°C. Each aliquot was frozen/thawed only once. CuSOD was used for the quantitation of extracellular superoxide anion generation.

N-α-Nitro-L-arginine methylester hydrochloride (L-NNAME), an inhibitor of NO synthase, was kept as stock solution (60 mM) in medium at −20°C.

Taurine was kept as a stock solution of 500 mM in sterile PBS at −20°C. Taurine is a specific scavenger of HOCl (37).

Monoclonal antibodies directed towards human catalase (clone CAT-505, mouse, IgG1), towards recombinant human CuSOD (clone SD-G6), towards epidermal growth factor (EGF) receptor (clone 225), as well as non-specific control IgG were kept as stock solutions of 50 μg/ml in PBS at −20°C.

Catalase from bovine liver as stock solution (958,000 U/ml, 20 mg protein/ml) was kept at 4°C and gently homogenized and diluted in medium containing 5% fetal bovine serum (FBS) prior to use.

Peroxynitrite and the peroxynitrite decomposition catalyst (FeTPPS) were obtained from Calbiochem (Merck Chemicals Ltd., Beeston Nottingham, UK). FeTPPS as stock solution of 10 mM was kept at −20°C. Peroxynitrite (synthesized from isoamyl nitrite and hydrogen peroxide) was obtained as a 200 mM stock solution and was kept at −70°C until use.

Caspase-3 inhibitor Z-DEVD-FMK; inhibitor sequence: Z-Asp(Ome)-Glu(Ome)-Val-Asp(Ome)-FMK, caspase-8 inhibitor Z-IETD-FMK; inhibitor sequence: Z-Ile(Ome)-Tyr(Ome)-Asp(Ome)-FMK and caspase-9 inhibitor Z-LEHD-FMK; inhibitor sequence: Z-Leu(Ome)-Glu(Ome)-His(Ome)-Val(Ome)-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 as soon as possible. Caspase-3 inhibitor was applied at a final concentration of 50 μM, and caspase-9 inhibitor at a final concentration of 25 μM. The residual DMSO concentration was below the critical concentration affecting ROS signaling.

The catalase-mimetic EUK-134 (chloro[2,2’-1,2-ethenediylbis ([nitrilo-N-methylidyne][bis[6-methoxyphenolato-O])]-manganese) was obtained from Cayman Chemicals (Ann Arbor, MI, USA) through Biomol (Hamburg, Germany). EUK-134 is a cell-permeable synthetic salen-manganese complex which exhibits catalase activity (38). EUK-134 was dissolved in DMSO to reach a concentration of 1 M and was then immediately diluted in EMEM containing 5% 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 had no interfering effect on ROS signaling.

The photosensitizer photofrin was a product from Axcian Scandipharma Inc. and was obtained from Meduna (Isernhagen, Germany). It was dissolved in sterile PBS at a concentration of 10 mg/ml and kept at −20°C in the dark. Transforming growth factor beta-1 (TGF-β1) was purified from human platelets (39) and kept as a stock solution of 1.5 μg/ml in EMEM plus 5% FBS at −20°C.

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) was 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. Non-transformed 208F rat fibroblasts and their derivative transformed through constitutive expression of v-src (208Fsrc3) (19, 28, 40, 41) were established by and were a generous gift from Drs C. Sers and R. Schäfer, Berlin, Germany. 208F cells and their transformed derivatives were cultured in EMEM with 5% FBS and supplemented as indicated above. The gastric carcinoma cell line MKN-45 was purchased from DSMZ, Braunschweig, Germany and was cultured in RPMI 1640 with 10 % FBS and supplements. Care was taken to avoid cell densities below 3x105/ml and above 106/ml. The metastatic murine melanoma cell line B16F10 was obtained from Dr. P. Aichele, Institute for Immunology, Department of Medical Microbiology and Hygiene, Freiburg, Germany. The cells were kept in EMEM with 5% FBS and supplements. MKN-45 and B16F10 cells had been originally isolated from an in vivo tumor.
They exhibit extracellular superoxide anion production but interfere with intercellular ROS signaling through expression of membrane-associated catalase (22, 23, 41).

**Apoptosis induction by exogenously added HOCl.** Cells were seeded in 96-well tissue culture clusters in 100 μl of complete medium at the following densities: 208F and 208Fsrc3 cells: 5×10^3 cells/100 μl; MKN-45 cells: 1.25×10^4 cells/100 μl; B16F10 cells: 5×10^3 cells/100 μl. When only one concentration of HOCl was applied, 208F and 208Fsrc3 cells were treated with 430 μM HOCl and MKN-45 cells received 125 μM HOCl. Control cells did not receive HOCl. Where indicated, 100 U/ml MnSOD had been added prior to HOCl addition.

MKN-45 cells were treated with increasing concentrations of HOCl (0; 15.6-430 μM) in the presence of the indicated inhibitors (specified in the next paragraph). B16F10 cells were treated with increasing concentrations of HOCl (0; 0.3-250 μM) after they had been pretreated with antibodies directed to SOD or catalase, or with photofrin (details are presented below). In all experiments, assays were performed in duplicate. After 2 h at 37°C and 5% CO₂, the percentage of apoptotic cells was determined by inverted phase contrast microscopy, based on the classical criteria for apoptosis, i.e. nuclear condensation or fragmentation and membrane blebbing (22, 42, 43). At least 200 cells were scored for each point of measurement. Control assays ensured that the morphological features 'nuclear condensation/fragmentation' as determined by inverted phase-contrast microscopy were correlated to intense staining with bisbenzimide and to DNA strand breaks, detectable by the terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) reaction (40, 44, 45). However, the TUNEL reaction was not used for routine quantitation, as the distinct steps during preparation of the samples caused a marked loss specifically of apoptotic cells.

**Inhibitor studies of HOCl-mediated apoptosis induction.** MKN-45 cells remained without inhibitor or received 100 μM of the NADPH oxidase inhibitor AEBSF, 100 U/ml MnSOD, 50 mM of the HOCl scavenger taurine, 10 mM of the hydroxyl radical scavenger mannitol, 2 μM of the catalase mimetic salen manganese complex EUK-134, 2 mM of the singlet oxygen scavenger histidine or 20 μM FeCl₃ before the HOCl (0; 15.6-250 μM) was added.

In a second experiment, MKN-45 cells received no addition of inhibitor, 25 mM of the catalase inhibitor 3-aminoazirazole or 25 mM 3-AT plus 2 μM EUK-134 before HOCl (0; 15.6-250 μM) was added.

In a third experiment, MKN-45 cells received 2 mM of the singlet oxygen scavenger histidine either prior to HOCl addition (HIS 0 min), 10 minutes after HOCl addition (HIS 10 min), or no histidine (control). Parallel assays received 50 mM of the HOCl scavenger taurine, 10 μM of the catalase mimetic EUK-134, 120 U/ml MnSOD, 10 mM of the hydroxyl radical scavenger mannitol, 25 μM of the peroxynitrite decomposition catalyst FeTPPS, 25 μM of caspase-8 or caspase-9 inhibitor or 50 μM caspase-3 inhibitor. Caspase inhibitors were added 15 minutes before HOCl (0; 53-430 μM) was added.

In a fourth experiment, MKN-45 cells received 2 mM of the singlet oxygen scavenger histidine either prior to HOCl addition (HIS 0 min), 10 min after HOCl addition (HIS 10 min), or received no histidine (control). Parallel assays received 10 μM of the catalase mimetic EUK-134, 50 mM taurine, 10 mM of the hydroxyl radical scavenger mannitol, 25 μM of caspase-9 inhibitor or 50 μM caspase-3 inhibitor. Caspase inhibitors were added 15 min before HOCl (0; 53 μM-3.5 mM) was added.

**Quantitation of extracellular superoxide anion generation after HOCl treatment and in controls.** MKN-45 cells (2.5×10^5) in 2 ml complete medium received either 20 mM mannitol (control), 20 mM mannitol plus 250 μM HOCl, 20 mM Mannitol plus 2 mM histidine plus 250 μM HOCl or 20 mM mannitol plus 25 μM caspase-8 inhibitor plus 250 μM HOCl. The assays were incubated for 10 minutes, diluted in 10 ml medium containing 2 mM histidine and centrifuged. The cells were resuspended in 5 ml fresh medium containing 2 mM histidine, centrifuged again and resuspended in fresh medium containing 2 mM histidine and at a cell density of 5×10^3 cells/100 μl medium. Assays (in duplicate) received CuSOD (0; 0.3-37.5 U/ml) and then 250 μM HOCl was added to trigger apoptosis induction. After 2 h at 37°C and 5% CO₂, the percentage of apoptotic cells was determined. The rightward shift of the bell-shaped inhibition curve by CuSOD correlates with an increase in extracellular superoxide anion concentration in a linear mode (46, 47). The signaling chemistry underlying the bell-shaped inhibition curve has been recently outlined (46, 47). The presence of the hydroxyl radical scavenger mannitol during the pretreatment phase prevented apoptosis induction by HOCl and thus allowed focus on singlet oxygen formation by HOCl. The presence of the singlet oxygen scavenger histidine (in the absence of mannitol) during the phase of apoptosis induction by a second HOCl treatment prevented secondary singlet oxygen-mediated effects at this stage, but allowed apoptosis induction by hydroxyl radicals generated by HOCl/superoxide anion interaction.

**Analysis of the effect of anti-SOD on HOCl-mediated apoptosis induction.** B16F10 murine melanoma cells (5×10^3 cells/100 μl medium) were seeded in 96-well tissue culture clusters. After the cells had attached to the surface, all assays received 2 mM histidine and either no further addition (control), or 1 μg/ml control antibody to EGF receptor (EGFR), 1 μg/ml anti-SOD, 1 μg/ml anti-catalase. After 15 min, HOCl (0; 0.48-250 μM) was added and the percentage of apoptotic cells was determined in duplicate assays after 1.5 h. The presence of histidine prevented singlet oxygen generation after addition of the challenging HOCl. In a second experiment, B16F10 cells received 20 mM mannitol and were pretreated with 250 μM HOCl in the absence or presence of 2 mM histidine. After 10 minutes, the medium was removed and all assays were washed twice with medium containing 2 mM histidine. Fresh medium, containing 2 mM histidine was added to all assays. Parallel assays received either no addition or 1 μg/ml anti-SOD. After 15 min, HOCl (0; 0.48-62.5 μM) was added. The percentage of apoptotic cells was determined in duplicate assays after 1.5 hours. The presence of mannitol during HOCl pretreatment prevented immediate apoptosis induction through HOCl/superoxide anion interaction and hydroxyl radical formation. It thus allowed focus on HOCl-mediated effects on cellular targets (e.g. SOD). The presence of histidine during the HOCl challenge avoided secondary singlet oxygen generation.

**Determination of HOCl-dependent, singlet oxygen-mediated inactivation of membrane-associated catalase of tumor cells.** MKN-45 cells (2.5×10^5) in 2 ml complete medium remained either untreated or received 250 μM HOCl, or 2.5 mM HOCl, or 25 μM HOCl plus 20 mM mannitol, or 2.5 mM HOCl plus 20 mM mannitol, or 2.5 mM HOCl plus 20 mM mannitol plus 2 mM histidine. After 5 min, the cells were diluted in 10 ml complete medium, centrifuged, resuspended in 5 ml fresh medium, centrifuged...
again and resuspended in fresh medium containing 100 μM AEBSF at a density of 6x10³ cells/100 μl medium. Peroxynitrite was added (0; 3-25 μM) and the percentage of apoptotic cells was determined after 1.5 hours. This assay takes advantage of the finding that extracellular (but not intracellular) catalase protects against apoptosis induction by exogenous peroxynitrite (22). The presence of the NADPH oxidase inhibitor AEBSF during the peroxynitrite challenge prevented the interaction between peroxynitrite and hydrogen peroxide (derived after dismutation of superoxide anions).

Analysis of the effect of exogenous singlet oxygen on HOCl-mediated apoptosis induction in tumor cells. B16F10 murine melanoma cells (5x10⁵) were seeded in 100 μl complete medium. After attachment of the cells, photofrin was added at 0-3.3 μg/ml and the uncovered plates were illuminated under visible light at room temperature on a sterile working bench for 25 min. Histidine was added to reach a final concentration of 2 mM and then HOCl (0; 0.3-250 μM) was added to duplicate assays. After 1.5 h, the percentage of apoptotic cells was determined.

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 very small. Empirical standard deviations were calculated merely to demonstrate reproducibility between parallel assays within the same experiment, and not with the intention of statistical analysis of variance. The key experiments have been repeated more than three times. The Yates continuity corrected chi-square test was used for the statistical determination of significances. As rather broad concentration ranges are used in our study, the presentation of the data required logarithmic abscissae in the case of Figures 2-4, 6, 7, 9).

Results

Non-transformed rat 208F fibroblasts, src-oncogene-transformed 208Fsrc3 cells (i.e. cells transformed in vitro, exhibiting the potential to form tumors, but without a history of confrontation by the antitumor mechanisms of an organism) and human MKN-45 gastric carcinoma cells (representing bona fide tumor cells isolated from an in vivo tumor) represent cells from three defined steps of oncogenesis. They were treated with HOCl, both in the absence and presence of SOD. Non-transformed cells did not exhibit apoptosis induction after HOCl treatment, whereas the two malignant cell lines, i.e. transformed and tumor cells, responded to exogenous HOCl by rapid apoptosis induction (Figure 1). Inhibition of HOCl-mediated apoptosis induction in these cell lines by SOD indicates that in contrast to the non-transformed cells, the two malignant cell lines generate extracellular superoxide anions at a sufficient concentration for interaction with HOCl, leading to apoptosis induction (13-15). Apoptosis induction by HOCl in tumor cells was dependent on the HOCl concentration, showing the mode of a characteristic plateau curve for the concentration range of HOCl tested (Figure 2A). The complete inhibition of HOCl-mediated apoptosis induction by the HOCl scavenger taurine, the superoxide anion scavenger SOD, the NADPH oxidase inhibitor AEBSF and the hydroxyl radical scavenger mannitol confirmed that the reaction HOCl + O₂⁻→ •OH + O₂ + Cl⁻ represented the basis for apoptosis induction, with hydroxyl radicals being the ultimate apoptosis inducers. HOCl-mediated apoptosis induction was abrogated by the addition of FeCl₂. This is explained by Fe⁺⁺-triggered Fenton chemistry of HOCl (14), occurring randomly within the assay and therefore mostly at sites distant from the cell membrane. Due to the extremely short free diffusion path-length of the resultant hydroxyl radicals they do not reach the cell membrane and thus have no apoptosis-inducing effect. Unexpectedly, the catalase-mimetic EUK-134 and the singlet oxygen scavenger histidine caused a similar degree of partial inhibition of HOCl-mediated apoptosis induction at higher concentrations of HOCl (Figure 2B). This finding indicates that singlet oxygen, most likely derived from the reaction between HOCl and hydrogen peroxide, seems to positively affect overall apoptotic signaling via the HOCl pathway in tumor cells. When tumor cell catalase was inhibited by 25 mM 3-AT and thus the local concentration of H₂O₂ in the vicinity of the cells was increased, apoptosis induction by lower concentrations of exogenous HOCl was inhibited and then apoptosis induction resumed as a parallel-shifted response curve at higher concentrations of HOCl (Figure 3). The
The inhibitory effect caused by 3-AT was abrogated when the catalase-mimetic EUK-134 (2 μM) was added. This finding confirmed the negative effect of increased H₂O₂ concentration on HOCl-mediated apoptosis induction (21, 22). For a more detailed analysis of modulatory side-effects after HOCl application to tumor cells, MKN-45 tumor cells were treated with HOCl up to 430 μM, after additional inhibitors had been added. The results shown in Figure 4 confirmed the steep increase in apoptosis induction by HOCl up to 107 μM, followed by a plateau. When histidine had been added before HOCl, the increase in apoptosis induction above 50 μM HOCl was blocked. Addition of histidine 10 minutes after HOCl had no effect. Similarly to histidine, addition of 10 μM of the catalase-mimetic EUK-134 blocked the initial increase above 50 μM HOCl. These findings confirm that singlet oxygen generated through the interaction of HOCl and hydrogen peroxide enhances the HOCl-mediated signaling in the concentration range of HOCl used in this experiment. The data presented in Figure 4 confirm that HOCl-mediated apoptosis induction correlated with the HOCl concentration and was dependent on superoxide anions and hydroxyl radicals. It seems to be mediated by the mitochondrial pathway of apoptosis, as caspase-9 inhibitor, as well as caspase-3 inhibitor, blocked apoptosis induction. Caspase-8 inhibitor and the peroxynitrite decomposition catalyst FeTPPS had no inhibitory effect on HOCl-mediated apoptosis induction, giving no indication for the involvement of death receptor-mediated processes in this experimental setting and excluding
peroxynitrite as reaction partner for singlet oxygen generation. Whereas a singlet oxygen-dependent process increased the efficiency of HOCl-mediated apoptosis up to 400 μM HOCl, a likewise singlet oxygen-dependent different process gradually reduced the efficiency of HOCl-mediated apoptosis induction when much higher concentrations of HOCl were applied (Figure 5). This is especially observed from the adverse effect of the singlet oxygen scavenger histidine at low and high concentrations of HOCl. Cells treated with the catalase-mimetic EUK-134 followed the same pattern of inhibition as histidine, indicating that hydrogen peroxide/HOCl interaction was the basis for singlet oxygen generation and action in these two adverse processes (Figure 5). As cell-derived superoxide anions are most likely rate-limiting when HOCl is added as a single bolus, the singlet oxygen-triggered increase in the efficiency of apoptosis induction by exogenous HOCl might be explained by an
increase in available superoxide anions. To clarify whether this was true, MKN-45 tumor cells were pre-treated with 250 μM HOCl to allow for singlet oxygen generation and then the cells were challenged with 250 μM HOCl, in the presence of increasing concentrations of SOD. If the pre-treatment with HOCl indeed causes an increase in superoxide anion concentration, this should be reflected in an increase in the concentration of SOD necessary for a complete blocking of the reaction compared to untreated control cells (46, 47). As inhibition of HOCl-mediated apoptosis induction by CuSOD is characterized as a bell-shaped curve, a relative shift of the inhibition curves should be easily monitored (46, 47). In order to avoid apoptosis-inducing effects of HOCl-derived hydroxyl radicals during pre-treatment of the cells, the hydroxyl radical scavenger mannitol was added during the initial HOCl pre-
treatment which was thus restricted to singlet oxygen-dependent processes. HOCI pre-treatment (in the presence of mannitol) was performed in the absence and presence of the singlet oxygen scavenger histidine or caspase-8 inhibitor. After 10 minutes of such pre-treatment, cells were washed and seeded in fresh medium. SOD was added in serial dilutions and the assays were challenged with 250 μM HOCI for induction of apoptosis. As can be seen in Figure 6, apoptosis induction in cells not pretreated with, but only challenged by HOCI, was blocked by approximately 1 U/ml SOD, in a sharp bell-shaped inhibition curve. Cells pre-treated with HOCI in the presence of mannitol and challenged with a second application of HOCI required eight times more SOD for inhibition of apoptosis. This is indicative of a marked increase in free superoxide anions (46, 47). The presence of caspase-8 inhibitor during HOCI pre-treatment did not affect superoxide anion generation, indicating that enhancement of superoxide anion production by caspase-8 played no role under these conditions. However, pre-treatment of the cells with HOCI in the presence of histidine did not cause increased superoxide anion production, demonstrating that the HOCI-induced increase in superoxide anion production was dependent on a singlet oxygen-mediated reaction that was driven by HOCI. As
Figure 10. Singlet oxygen modulates the apoptotic response of tumor cells to exogenous HOCl. A: NADPH oxidase (NOX1) in the membrane of tumor cells generates extracellular superoxide anions (#1). These dismutate to hydrogen peroxide either spontaneously (#2) or driven by superoxide dismutase (SOD) (#3). Hydrogen peroxide is efficiently decomposed by membrane-associated catalase (CAT) (#4) and therefore HOCl synthesis by cell-derived peroxidase is prevented (not shown). Exogenous HOCl that is added to the system (experimentally or by attacking neutrophils) may either react with superoxide anions (#5) and yield hydroxyl radicals that induce lipid peroxidation (LPO) and thus trigger induction of apoptosis (#6), or may react with hydrogen peroxide and form singlet oxygen (#7). At the lower concentration range of HOCl, the concentration of generated singlet oxygen is sufficient to inactivate SOD (#8), but not catalase. There is no significant apoptosis induction due to direct action of singlet oxygen, as demonstrated by the inhibition profiles in this study. B: As a consequence of SOD inactivation by singlet oxygen, there is now a higher concentration of free superoxide anions and less hydrogen peroxide is present. Therefore, catalase only needs to decompose hydrogen peroxide generated by spontaneous dismutation (#1-3). The reaction between HOCl and superoxide anions (#4) becomes more efficient under these conditions, as HOCl is less consumed by hydrogen peroxide and more superoxide anions are available for interaction with HOCl. Thus, lipid peroxidation (#5) and apoptosis induction are increased. C: When higher concentrations of HOCl are added, there is sufficient singlet oxygen generation to inactivate catalase in addition to SOD. As a result, more HOCl is consumed by hydrogen peroxide (#3) and HOCl/superoxide anion interaction is decreasing (#4), leading to a relative decrease in apoptosis induction. The data summarized in Figure 10 indicate that singlet oxygen generation and action must be extremely fast, as HOCl-dependent singlet oxygen generation and subsequent HOCl-dependent apoptosis induction are triggered by HOCl added in the same bolus.
SOD has been shown to be inactivated by singlet oxygen (48, 49), the finding demonstrated in Figure 6 might be well-explained by singlet oxygen-dependent inactivation of SOD. This would imply that the extracellular superoxide anion concentration of tumor cells was modulated by SOD. If this was the case, the application of neutralizing antibodies directed towards SOD should increase the efficiency of HOCl-mediated apoptosis induction, due to an increase in free superoxide anions. Figure 7A demonstrates that this is indeed so. When the cells had been pre-treated with anti-SOD, the efficiency of subsequent HOCl-mediated apoptosis induction strongly increased, indicative of a higher available concentration of superoxide anions. Pre-treatment with an irrelevant antibody did not cause a shift of the concentration-response curve, whereas an antibody directed against catalase caused a decrease in efficiency of HOCl-mediated apoptosis induction, reflected in a rightward shift of the curve. When anti-SOD treatment was combined with HOCl pre-treatment in the presence of mannitol, no additional effect was seen compared to the control by anti-SOD alone. HOCl pre-treatment alone (in the presence of mannitol) showed a singlet-oxygen-dependent enhancing effect of similar strength as anti-SOD. This finding indicates that anti-SOD and HOCl pre-treatment attack the same target structure, i.e. SOD.
order to test a suspected catalase inactivation mediated by high concentrations of HOCl, MKN-45 cells were pretreated with 2.5 mM or 250 μM HOCl, both in the absence and presence of mannitol. After 5 minutes, the cells were washed, the NADPH inhibitor AEBSF was added, and the cells were challenged with increasing concentrations of peroxynitrite and apoptosis induction was monitored. Exogenously added peroxynitrite can be decomposed by membrane-associated catalase and thus its apoptosis-inducing effect mediated by its interaction with the cell membrane can be inhibited. In contrast, intracellular catalase cannot interfere with the interaction of extracellular peroxynitrite and the cell membrane. Therefore this test was focused on the activity of the protective catalase on the membrane of the tumor cells. When tumor cells were treated with either of the two concentrations of HOCl in the absence of mannitol, they showed strong and direct apoptosis induction after re-seeding (Figure 8). There was only a weak additional effect exerted by exogenous peroxynitrite challenge under these conditions. Cells pre-treated with 2.5 mM HOCl in the presence of the hydroxyl radical scavenger mannitol did not exhibit apoptosis induction after reseeding. This control ensured that the apoptosis-inducing effect of HOCl, based on hydroxyl radical generation, had been efficiently blocked by mannitol. However, the addition of peroxynitrite to these cells caused a strong concentration-dependent increase in apoptosis, indicating that pre-treatment with 2.5 mM HOCl had caused substantial inactivation of catalase that rendered the tumor cells susceptible to exogenous peroxynitrite. As the presence of the singlet oxygen scavenger histidine during HOCl pre-treatment blocked this sensitization completely, the inactivation reaction seemed to be dependent on the action of singlet oxygen. Pre-treatment of the cells with the lower HOCl concentration, i.e. 250 μM HOCl, in the presence of mannitol was not sufficient for inactivation of catalase and therefore did not induce a response to exogenous peroxynitrite, as expected from the preceding data. So far, these data indicate that in addition to induction of apoptosis through hydroxyl radical formation, increasing HOCl concentrations first led to singlet oxygen-dependent inactivation of SOD, followed by inactivation of catalase at higher HOCl concentrations. The inactivation of these enzymes thereby seemed to have adverse effects on the overall HOCl-mediated apoptosis induction – enhancement of HOCl-dependent apoptosis induction after SOD inactivation and inhibition of HOCl-dependent apoptosis induction after catalase inactivation. If this conclusion is correct, pre-treatment of tumor cells with gradually increasing concentrations of the singlet oxygen generator photofrin should initially enhance subsequent HOCl-mediated apoptosis induction, whereas higher concentrations should lead to inhibition of apoptosis induction. Figure 9 shows that 0.12 μg/ml photofrin with light treatment was stimulatory, whereas pre-treatment with higher concentrations of photofrin had a concentration-dependent negative effect on apoptosis induction. This negative effect was prominent as a series of rightward shifts of the HOCl concentration curves. The hypothesis of a differential role of increasing singlet oxygen concentrations was thus experimentally verified.

**Discussion**

This study was based on an conceptional and experimental dissection of the steps ‘HOCl synthesis’ from ‘HOCl action’ and thus allowed focus on the multiple functions of HOCl for the modulation and induction of apoptosis. The application of exogenous HOCl allowed for an explicit study of the interaction of preformed HOCl with cells from distinct stages of multistep carcinogenesis, thereby eliminating the restrictions on HOCl synthesis by tumor cell-specific protective catalase. These data confirm the finding of HOCl-mediated apoptosis induction through hydroxyl radicals specifically in malignant cells (transformed cells as well as *bona fide* tumor cells) (19, 20, 22) and demonstrate a so far unrecognized dual modulatory role of singlet oxygen, generated after the interaction of HOCl with hydrogen peroxide. Abundant extracellular superoxide anion production, a characteristic feature of malignant cells (19, 20-22), is essential to allow the reaction

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\HOCl + \frac{1}{2}O_2^- \rightarrow \cdot\text{OH} + O_2 + Cl^- (13-15).
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Hydroxyl radical production in close vicinity of the cell membrane, thereby represents the critical step that leads to lipid peroxidation and induction of the mitochondrial pathway of apoptosis. Transformed cells (i.e. cells with tumorigenic potential), as well as tumor cells (cells isolated from *bona fide* tumors) that are affected by exogenous HOCl, are both characterized by and dependent on extracellular superoxide anion production (19-24, 28, 40). In contrast, non-transformed cells are lacking continuous extracellular superoxide anion production and therefore neither can react with exogenous HOCl nor drive HOCl generation. The effect of exogenous HOCl on malignant cells mimicks the HOCl-based attack of neutrophils on malignant cells (8-12), a situation in which membrane-associated catalase of tumor cells cannot protect these cells. This is contrasted by intracellular induction of apoptosis (19, 21) or autocrine apoptotic self-destruction, a process in which HOCl is generated by cell-derived signaling molecules (22, 23, 41).

The use of the singlet oxygen scavenger histidine revealed differential singlet oxygen action that was dependent on the concentration of HOCl applied to tumor cells. Singlet oxygen seemed to be generated by the interaction of HOCl with hydrogen peroxide, as seen by its dependence on the HOCl concentration and by an analogous inhibition profile of histidine and the catalase mimetic EUK-124. HOCl/hydrogen peroxide interaction represents one classical way for the formation of singlet oxygen

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\text{H}_2\text{O}_2 + \text{HOCl} \rightarrow \text{H}_2\text{O} + ^1\text{O}_2 + \text{H}^+ + \text{Cl}^- (52, 53).
\]
As tumor cells carry catalase on the outside of their membrane, the concentration of hydrogen peroxide in the vicinity of tumor cells can be expected to be low and therefore rate-limiting in the reaction leading to singlet oxygen. However, as catalase is bound to the cells, dismutation of superoxide anions beyond its range of action will lead to low concentrations of hydrogen peroxide that are not reached by catalase. As the peroxynitrite decomposition catalyst FeTPPS had no effect, the alternative reaction for singlet oxygen formation through HOCl/peroxynitrite interaction (54, 55) did not seem to play any role in the experimental setup studied here. In the concentration range between 50 and 400 μM HOCl applied to MKN-45 tumor cell cultures, singlet oxygen seemed to enhance the apoptosis inducing effect of HOCl. As the overall apoptosis induction was blocked by SOD and mannitol (both inhibitors being indicative for HOCl action), a direct apoptosis-inducing effect of singlet oxygen can be ruled out. In addition, the singlet oxygen-dependent inhibition of HOCl-mediated apoptosis induction at higher HOCl concentrations would not be in line with the assumption of direct apoptosis induction by singlet oxygen. Rather, singlet oxygen seemed to inactivate membrane-associated SOD. Thus the SOD-dependent limitation of superoxide anions for the reaction with HOCl was abrogated. This conclusion is based on the findings that i) anti-SOD caused a strong sensitization to HOCl-mediated apoptosis induction and ii) HOCl in the relevant concentration range caused an increase in available superoxide anions, which was dependent on singlet oxygen, but independent of caspase-8. The indepence of this reaction from caspase-8 differentiates HOCl-dependent singlet oxygen generation from caspase-8-dependent singlet oxygen generation after hydrogen peroxide/peroxynitrite interaction (23). The dependency of superoxide anion enhancement on singlet oxygen is in line with inactivation of SOD, as SOD is known to be effectively inactivated by singlet oxygen (48, 49). The increased superoxide anion availability would be the predicted result of that inactivation. As the combination of HOCl-treatment with addition of anti-SOD did not show an additive effect, the common target of both treatments seems to be SOD. Generation of singlet oxygen by the interaction of hydrogen peroxide and HOCl and subsequent inactivation of SOD must be an extremely fast reaction, as it affected the reaction of the initially added bolus of HOCl, as seen in Figures 2 and 4. A similarly fast reaction rate can be assumed for the formation of singlet oxygen at very high concentrations of HOCl, leading to a decrease in the overall effect of HOCl. This is explained by singlet oxygen-mediated catalase inactivation, followed by consumption of HOCl through excess hydrogen peroxide. This is in agreement with the finding that catalase can be inactivated by singlet oxygen (48, 49). In line with this conclusion, inhibition of tumor cell catalase by 3-AT or antibodies directed against catalase had the same effect on HOCl-mediated apoptosis induction as did pre-treatment with high concentrations of HOCl. Finally, high concentrations of HOCl were directly shown to inactivate catalase through singlet oxygen formation, leading to sensitivity towards exogenous peroxynitrite. The differential inactivation of SOD and catalase by singlet oxygen that is generated by increasing HOCl concentration was confirmed by direct application of increasing concentrations of the singlet oxygen generator photofrin. A detailed scenario for the interaction of exogenous HOCl with superoxide anion-producing tumor cells, focusing on singlet oxygen-dependent modulation of and on hydroxyl radical-dependent induction of apoptosis is summarized in Figure 10. The data presented here significantly extend our picture on the protection of tumor cells against ROS-mediated apoptosis signaling. As a first and dominant line of defense, membrane-associated catalase rigorously prevents HOCl synthesis through decomposition of hydrogen peroxide and completely blocks NO/peroxynitrite signaling through decomposition of peroxynitrite and oxidation of NO (22, 23). In addition, SOD seems to have a co-modulatory role that interferes with, but does not completely block superoxide anion/HOCl interaction, as a basal level of HOCl/superoxide anion interaction is possible without inhibiting SOD. After inhibition or inactivation of SOD, HOCl/superoxide anion interaction is then increased due to an increase in free superoxide anions, but HOCl synthesis may be decreased as hydrogen peroxide generation largely depends on spontaneous dismutation under these conditions. The increase in free superoxide anions and the parallel decrease in hydrogen peroxide will have an overall positive effect on apoptosis signaling through the NO/peroxynitrite pathway, due to a potential positive effect on peroxynitrite formation caused by a higher superoxide anion concentration and a higher NO concentration after abrogation of consumption of NO by hydrogen peroxide (22) and a decreased consumption of peroxynitrite by hydrogen peroxide. Inactivation of SOD by monoclonal antibodies directed towards SOD1, as used in this study, does not allow precisely definition of whether SOD1 or SOD3 are actually involved in protection, as it cannot be excluded that both enzymes share homology at their active sites. These data demonstrate a dual role of tumor cell-specific membrane-associated catalase in the control of HOCl synthesis. Catalase prevents HOCl synthesis within tumor cell populations as it efficiently decomposes hydrogen peroxide derived from the tumor cell’s own ROS generation. It thus protects the tumor cells against their own HOCl-based apoptosis signaling. The situation is completely turned around when an exogenous source for HOCl becomes available. Under these conditions, tumor cell catalase optimizes the reaction between exogenous HOCl and tumor cell superoxide anions, as it abrogates the consumption reaction between HOCl and hydrogen peroxide. Inhibition of catalase or its inactivation through singlet oxygen (that may be generated by the interaction between hydrogen peroxide...
and HOCl) then causes a decrease of apoptosis induction through exogenous HOCl. The protective enzymes of tumor cells, *i.e.* catalase and SOD seem to cooperate in the interference with ROS signaling at several distinct points, as outlined in detail in Figure 11. The knowledge of their interaction and modes to abrogate their protective effect may be useful for strategies that aim at the sensitization of tumor cells for their own apoptotic destruction in novel approaches of tumor therapy.

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**References**


