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Extracellular localization of catalase is associated with the transformed state of malignant cells

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Abstract: Oncogenic transformation is dependent on activated membrane-associated NADPH oxidase (NOX). However, the resultant extracellular superoxide anions are also driving the NO/peroxynitrite and the HOCl pathway, which eliminates NOX-expressing transformed cells through selective apoptosis induction. Tumor progression is dependent on dominant interference with intercellular apoptosis-inducing ROS signaling through membrane-associated catalase, which decomposes H₂O₂ and peroxynitrite and oxidizes NO. Particularly, the decomposition of extracellular peroxynitrite strictly requires membrane-associated catalase. We utilized small interfering RNA (siRNA)-mediated knockdown of catalase and neutralizing antibodies directed against the enzyme in combination with challenging H₂O₂ or peroxynitrite to determine activity and localization of catalase in cells from three distinct steps of multistage oncogenesis. Nontransformed cells did not generate extracellular superoxide anions and only showed intracellular catalase activity. Transformed cells showed superoxide anion-dependent intercellular apoptosis-inducing ROS signaling in the presence of suboptimal catalase activity in their membrane. Tumor cells exhibited tight control of intercellular apoptosis-inducing ROS signaling through a high local concentration of membrane-associated catalase. These data demonstrate that translocation of catalase to the outside of the cell membrane is already

associated with the transformation step. A strong local increase in the concentration of membrane-associated catalase is achieved during tumor progression and is controlled by tumor cell-derived H₂O₂ and by transglutaminase.

Keywords: apoptosis; catalase; multistep oncogenesis; peroxynitrite; reactive oxygen species.

Introduction

Catalase was originally regarded as an efficient monofunctional peroxisomal enzyme for the detoxification of H₂O₂. This picture has substantially changed to a multifunctional enzyme that exhibits classical catalase, peroxidase (Keilin and Hartree, 1955; Kremer, 1970), and oxidase function (Vetrano et al., 2005). Moreover, catalase not only decomposes H₂O₂, it also degrades peroxynitrite in an enzymatic reaction that, like its classical reaction with H₂O₂, involves formation of compound I (CATFe^{IV}=O⁺) (Gebicka and Didil, 2009; Heinzelmann and Bauer, 2010). In addition, compound I of catalase can oxidize NO (CATFe^{IV}=O⁺+2·NO+H₂O→CATFe^{III}+2H⁺+2NO₂⁻) (Wink and Mitchell, 1998; Brunelli et al., 2001), whereas native ferricatalase (CATFe^{III}) is reversibly inhibited by NO (Brown, 1995). Thus, catalase has the potential to execute a central modulatory function at the cross-point between H₂O₂- and NO/peroxynitrite-mediated signaling pathways.

The classical work by Deichman's group has shown that experimental tumor progression *in vivo* requires the establishment of a 'H₂O₂-catabolizing state' of the malignant cells (Deichman and Vendrov, 1986; Deichman et al., 1989, 1998; Deichman, 2000, 2002). Tumor cells isolated from tumors that had been established through inoculation of transformed cells into syngeneic animals were much more resistant to induction of cell death by exogenous H₂O₂ than the original *in vitro* transformed cells. The biological significance of this H₂O₂-resistant phenotype was confirmed by the finding that H₂O₂-resistant tumor cells that had been isolated from experimentally induced tumors were much more efficient to induce a new round of tumor formation than the cell population that had been transformed *in vitro* and that had been initially

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used to induce these tumors. In line with the findings of Deichman's group, Smith et al. (2007) have shown that rat glioma cells have a higher concentration of catalase than normal astrocytes and that inhibition of catalase sensitizes the tumor cells to oxidative stress. Recent work from our group has shown that increased resistance against exogenous ROS and particularly against intercellular ROS signaling through the HOCl and the NO/peroxynitrite pathway is not only found in cell lines from Deichman's experimental tumor progression system, but also in all human tumor cell lines tested so far (Heinzelmann and Bauer, 2010; Bauer, 2014). In contrast, cells transformed to the malignant state *in vitro* (and not passaged in an animal) are regularly sensitive to intercellular apoptosis-inducing ROS signaling (Herdener et al., 2000; Heigold et al., 2002; Bauer, 2012). The 'H₂O₂-catabolizing phenotype' of tumor cells as defined by Deichman (Deichman and Vendrov, 1986; Deichman et al., 1989, 1998; Deichman, 2000, 2002) seems to be identical to resistance against intercellular ROS signaling, as defined by our group (Bechtel and Bauer, 2009; Heinzelmann and Bauer, 2010; Bauer, 2012). It is explained by the localization of functional catalase on the outside of the cell membrane of tumor cells (Bechtel and Bauer, 2009; Heinzelmann and Bauer, 2010). The extracellular localization of catalase on the membrane of tumor cells has been established by (a) specific immunofluorescence staining of living tumor cells, but not of normal cells, with cell-impermeable antibodies directed toward catalase, (b) substantial decrease of catalase-mediated protection of tumor cells through trypsinization, (c) sensitization of tumor cells for H₂O₂- or peroxynitrite-dependent apoptosis induction, as well as for autocrine ROS-mediated apoptosis induction through inhibition of catalase with cell-impermeable antibodies directed against catalase, and (d) efficient protection of tumor cells against apoptosis induction by exogenous peroxynitrite (Heinzelmann and Bauer, 2010). As extracellularly added peroxynitrite would induce lipid peroxidation when it passes the cell membrane, intracellular catalase is ineffective in the protection against extracellular peroxynitrite, whereas catalase located at the outside of the cell membrane detoxifies peroxynitrite before it can attack the membrane (Heinzelmann and Bauer, 2010). Tumor cell-protective catalase efficiently interferes with selective intercellular apoptosis-inducing ROS signaling of malignant cells that is essentially based on and controlled by their expression of NADPH oxidase 1 (NOX1) (Bechtel and Bauer, 2009; Heinzelmann and Bauer, 2010; Kundrát et al., 2012; Bauer, 2012, 2014). Intercellular apoptosis-inducing ROS signaling of malignant cells and catalase-dependent interference with intercellular ROS signaling is

shown in Supplementary Figure 1. Two intercellular apoptosis-inducing signaling pathways of major importance, i.e. the NO/peroxynitrite and the HOCl pathway (Herdener et al., 2000; Heigold et al., 2002; Bauer, 2012) (summarized in Supplementary Figure 1) are driven by extracellular superoxide anions that are characteristic and essential for transformed cells (Irani et al., 1997; Suh et al., 1999; Yang et al., 1999; Herdener et al., 2000; Arnold et al., 2001; Schwieger et al., 2001; Heigold et al., 2002; Mitsushita et al., 2004; Tominaga et al., 2007; Laurent et al., 2008). The HOCl signaling pathway is based on dismutation of superoxide anions to H₂O₂ and the use of the substrate H₂O₂ by peroxidase for the generation of HOCl. Subsequent superoxide anion/HOCl interaction leads to the formation of apoptosis-inducing hydroxyl radicals in close vicinity to the membrane where the superoxide anions are generated (Herdener et al., 2000; Bauer, 2012). The significance of the results of inhibitor studies that have been used to characterize the intercellular apoptosis-inducing ROS-dependent signaling pathways and the nature of the HOCl-synthesizing peroxidase are discussed in detail under Supplementary Material (Supplementary Figures 2–7). The NO/peroxynitrite pathway depends on the interaction between superoxide anions and NO, leading to the formation of peroxynitrite. After protonation of peroxynitrite, the resulting peroxynitrous acid decomposes into NO₂ and hydroxyl radicals (Heinzelmann and Bauer, 2010; Bauer, 2012). Membrane-associated catalase of tumor cells protects the cells against both pathways, as it prevents HOCl synthesis through decomposition of H₂O₂ and as it oxidizes NO and detoxifies peroxynitrite (Heinzelmann and Bauer, 2010) (Supplementary Figure 1).

Recent work has shown that tumor cell required inhibition of membrane-associated protective catalase for the reactivation of intercellular ROS-mediated apoptosis signaling, whereas cells transformed *in vitro* showed intercellular apoptosis-inducing ROS signaling without inhibition of catalase (Bechtel and Bauer, 2009; Heinzelmann and Bauer, 2010). However, the catalase inhibitor 3-amino-1,2,4-triazole (3-AT) caused a distinct enhancement of intercellular apoptosis-inducing ROS signaling of transformed cells (Bechtel and Bauer, 2009), indicating that catalase might have a modulatory role for intercellular apoptosis-inducing ROS signaling of these cells, although its concentration seems not to be sufficient to prevent apoptosis induction. Therefore, in this study, we used cells from three characteristic steps of multistep oncogenesis, i.e. normal nontransformed cells, transformed cells, and *bona vide* tumor cells to experimentally address the question whether translocation of catalase to the outside of the cell membrane is acquired at the early step of oncogenic

transformation of the cells to the malignant state or later, during successful tumor formation and progression. In addition, this study aimed to clarify the nature of the discrepancy between the finding that tumor progression caused increased resistance against H_2O_2 and peroxynitrite (Deichman and Vendrov, 1986; Deichman et al., 1989, 1998; Deichman, 2000, 2002; Heinzelmann and Bauer, 2010; Bauer, 2012) and the frequently published finding of overall lower total concentration of catalase in tumor cells compared to normal tissue (Sato et al., 1992; Coursin et al., 1996; Baker et al., 1997; Bostwick et al., 2000; Chung-Man et al., 2001; Ho et al., 2001; Cullen et al., 2003; Kwei et al., 2004).

Results

Differential effect of siRNA-mediated knockdown of catalase on H_2O_2 - and peroxynitrite-dependent apoptosis induction

Nontransformed 208F rat fibroblasts, src oncogene-transformed 208Fsrc3, and human gastric carcinoma tumor cells MKN-45 were transfected with siRNA directed against catalase (siCAT) or control siRNA (siCo). The dependence of functional catalase knockdown on the concentration of specific siRNA and on time is presented under Materials and Methods (Figure 7). Twenty-four hours after transfection, the cells were challenged either with increasing concentrations of H_2O_2 -generating glucose oxidase (GOX) or exogenously added peroxynitrite. H_2O_2 and peroxynitrite at sufficient concentration induce apoptosis in nonmalignant and malignant cells (Ivanovas and Bauer, 2002). Thereby, *ex vivo* tumor cells are more resistant to apoptosis induction by these agents due to their membrane-associated catalase (Heinzelmann and Bauer, 2010). As H_2O_2 readily passes cell membranes through aquaporins (Bienert et al., 2006, 2007), its concentration can be controlled by intracellular and extracellular catalase. In addition to intracellular catalase, peroxiredoxin contributes essentially to the H_2O_2 -catabolizing activity (Sies, 2014). In contrast, exogenously added extracellular peroxynitrite can only be counteracted by extracellular catalase, as peroxynitrite causes lipid peroxidation as soon as it meets the membrane (Heinzelmann and Bauer, 2010). Low concentrations of GOX were sufficient to induce apoptosis in siCo-transfected nontransformed 208F and transformed 208Fsrc3 cells with similar efficiency (Figure 1A, B). GOX concentrations between 0.6 and 1.25 mU/ml caused half-maximal apoptosis induction after 2 h. SiCo-transfected

MKN-45 tumor cells were more resistant against H_2O_2 -dependent apoptosis induction (Figure 1C) than nontransformed or transformed cells and required 10 mU/ml of GOX for half-maximal apoptosis induction. siCAT-mediated knockdown of catalase activity caused a marked sensitization of all three cell types for apoptosis induction by H_2O_2 . The degree of sensitization was the lowest for nontransformed, intermediate for transformed, and the highest for tumor cells, as the ratio between the GOX concentration required for half-maximal apoptosis induction in siCo- and siCAT-transfected cells was 4 for nontransformed cells, 8 for transformed, and 32 for tumor cells. SiCo-transfected nontransformed and transformed cells responded to the peroxynitrite challenge in a more sensitive way than tumor cells (Figure 1D–F). Half-maximal apoptosis induction in nontransformed and transformed cells was achieved by approximately 30 μ M peroxynitrite, whereas nearly 250 μ M were required for half-maximal apoptosis induction in the tumor cells. Importantly, siRNA-mediated knockdown of catalase activity did not change the sensitivity of nontransformed cells for exogenous peroxynitrite (Figure 1D), but markedly sensitized transformed as well as tumor cells for the peroxynitrite-mediated effect (Figure 1E, F). These data confirm that tumor cells have higher resistance against exogenous H_2O_2 and peroxynitrite than normal and transformed cells. They also indicate that normal cells possess no extracellular catalase, whereas transformed and tumor cells do so, but at different concentrations.

To differentiate between intracellular and extracellular catalase activity in a quantitative way, the effect of siRNA-mediated knockdown of catalase activity (indicative for the effect of total catalase activity) was compared to the effect of neutralizing antibodies directed against catalase (indicative for extracellular catalase activity of the cells, as the antibody is cell-impermeable). This approach was applied to apoptosis induction by H_2O_2 (which is decomposed by intracellular and extracellular catalase) and exogenous peroxynitrite (which can only be detoxified by extracellular catalase). Figure 2A–F confirms that (i) tumor cells were more resistant to apoptosis induction by H_2O_2 and peroxynitrite than normal and transformed cells and that (ii) knockdown of catalase activity markedly sensitized nontransformed, transformed, and tumor cells for apoptosis induction by H_2O_2 , but only sensitized transformed and tumor cells for the action of peroxynitrite. Antibody against catalase did not sensitize nontransformed cells for the effect of H_2O_2 (Figure 2A), and therefore, the remarkable effect knockdown of catalase activity in nontransformed cells seems to be solely due to intracellular catalase. In contrast, antibody directed against

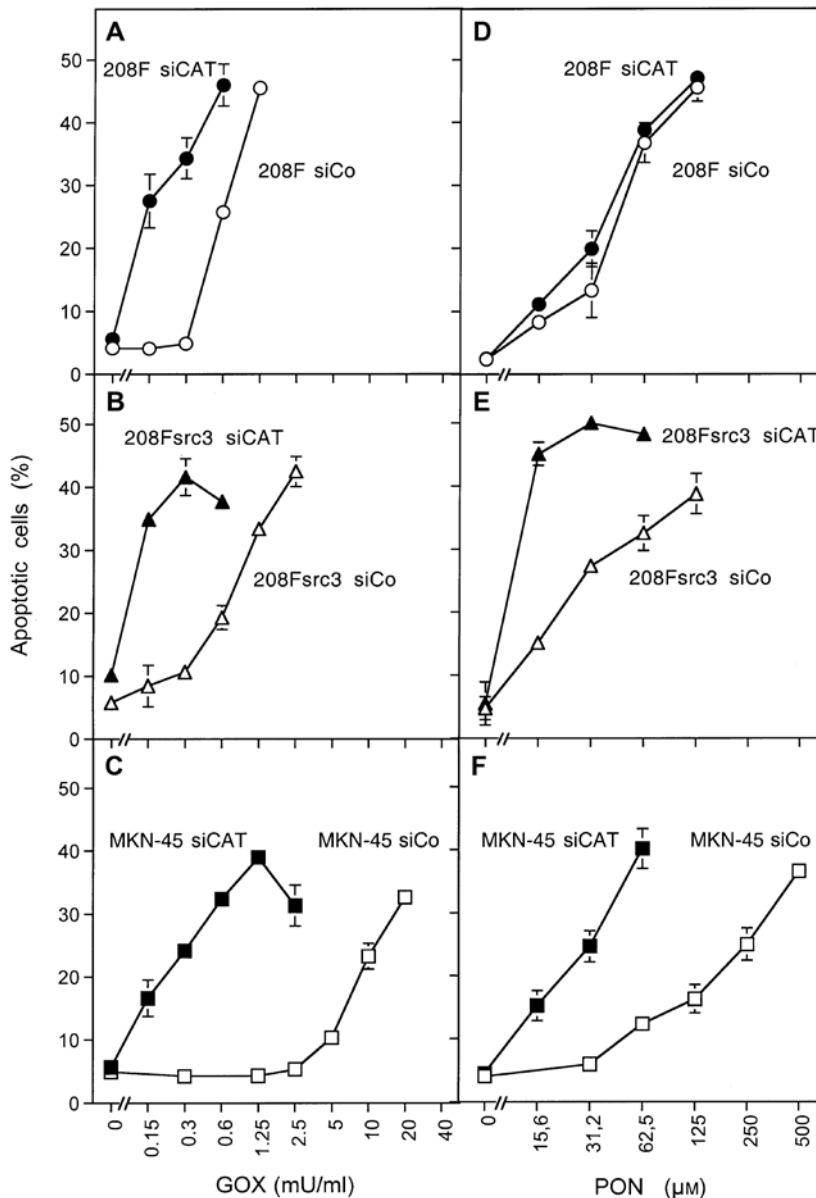


Figure 1: Differential effect of siRNA-mediated knockdown of catalase on the sensitivity of nontransformed, transformed, and tumor cells for apoptosis induction by H₂O₂ and peroxynitrite.

Nontransformed rat fibroblasts 208F (A, D), src oncogene-transformed 208Fsrc3 cells (B, E), and human MKN-45 gastric carcinoma cells (C, F) were transfected with either 10 nM siCo or 10 nM siRNA directed against murine catalase (208F, 208Fsrc3 cells) or against human catalase (MKN-45). Twenty-four hours after transfection, the cells were seeded at a density of 6000 cells/100 μl assay and treated with increasing concentrations of H₂O₂-producing GOX (A–C) or peroxynitrite (D–F). (One unit of GOX is defined as producing 1 μmol H₂O₂/min at 35°C at a pH of 5.1 and optimal saturation of the medium with oxygen). Apoptosis induction was monitored in duplicate assays after 2 h. This experiment has been repeated twice. Statistical analysis: Apoptosis induction by GOX and peroxynitrite was highly significant for all three cell lines treated with siCo or siCAT ($p < 0.001$). The shift between siCo- and siCAT-treated cells shown in A, B, C, E, and F was highly significant ($p < 0.001$), whereas there was no significant shift in D.

catalase (but not irrelevant control antibody) sensitized transformed as well as tumor cells, for the action of H₂O₂ (Figure 2B, C). This sensitizing effect for H₂O₂-dependent apoptosis induction was weaker than the effect of knockdown of catalase activity, indicating that a substantial

concentration of catalase, but not all of it, was located on the outside of transformed and tumor cells. Neither knockdown of catalase activity nor neutralizing antibody directed against catalase caused sensitization of normal cells for peroxynitrite-dependent apoptosis induction

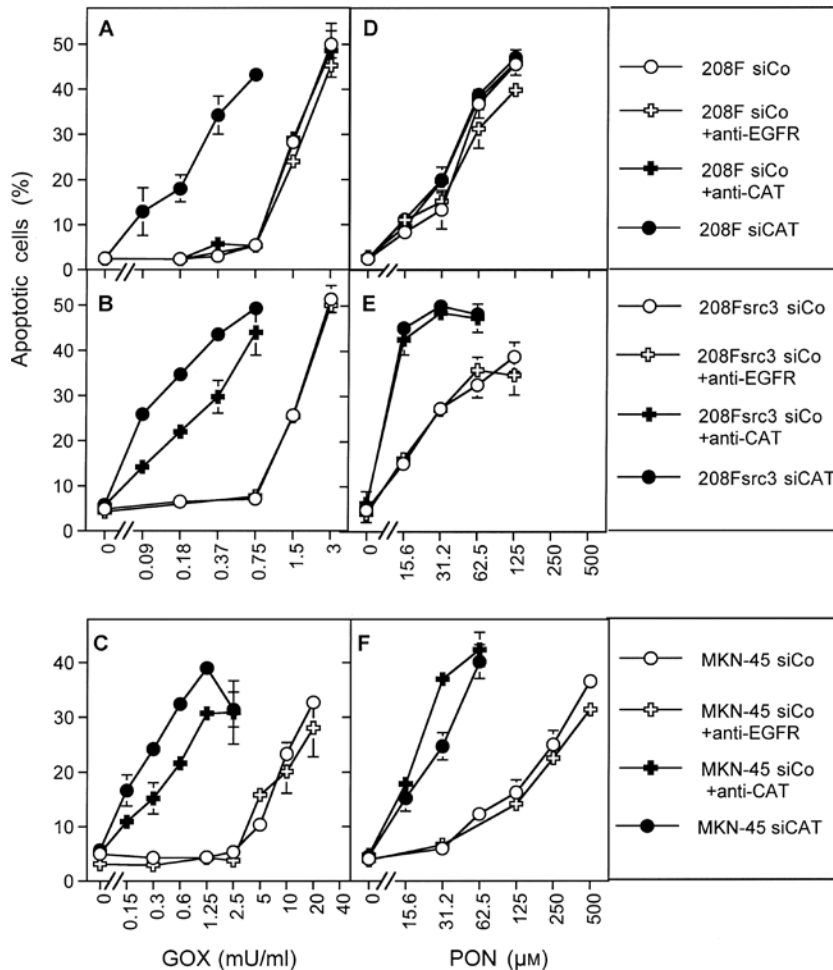


Figure 2: Differentiation between intracellular and extracellular membrane-associated catalase through siRNA-mediated knockdown of catalase and specific inhibitory antibodies directed against catalase.

Nontransformed rat fibroblasts 208F (A, D), src oncogene-transformed 208Fsrc3 cells (B, E), and human MKN-45 gastric carcinoma cells (C, F) were transfected with either 10 nM siCo or siRNA directed against murine catalase (208F, 208Fsrc3 cells) or human catalase. Twenty-four hours after transfection, the cells were seeded at a density of 6000 cells/100 μ l assay. In parallel assays, siCo-treated cells also received either 0.2 μ g/ml neutralizing antibody directed against catalase (aCAT) or control antibody (aEGFR). The cells were incubated for 20 min at 37°C and were then treated with increasing concentrations of H₂O₂-producing GOX (A–C) or with peroxynitrite (D–F). (One unit of GOX is defined as producing 1 μ mol H₂O₂/min at 35°C at a pH of 5.1 and optimal saturation of the medium with oxygen). Apoptosis induction was monitored in duplicate assays 2 h after addition of GOX or peroxynitrite. This experiment has been repeated twice. Statistical analysis: Apoptosis induction by GOX and peroxynitrite was highly significant for all three cell lines treated with siCo or siCAT ($p < 0.001$). (A) The shift between siCo- and siCAT-treated cells was highly significant ($p < 0.001$), whereas there was no significant difference among siCo, siCo+anti-EGFR, and siCo+anti-Cat cells. (B) The shift between siCo and siCAT or siCo+anti-CAT-treated cells and between siCAT and siCo+anti-CAT-treated cells was highly significant ($p < 0.001$), whereas there was no significant difference between siCo- and siCo+anti-EGFR-treated cells. (C) The shift between siCo- and siCAT or siCo+anti-CAT-treated cells was highly significant ($p < 0.001$). The difference between siCAT and siCo+anti-CAT-treated cells was significant ($p < 0.01$), whereas there was no significant difference between siCo and siCo+anti-EGFR-treated cells. (D) There was no significant difference between the curves. (E) The shift between siCo and siCAT or siCo+anti-CAT-treated cells was highly significant ($p < 0.001$). There was no significant difference between siCAT- and siCAT+anti-CAT-treated cells and between siCo and siCo+anti-EGFR-treated cells. (F) The shift between siCo and siCAT or siCo+anti-CAT-treated cells was highly significant ($p < 0.001$). There was no significant difference between siCAT- and siCAT+anti-CAT-treated cells and between siCo and siCo+anti-EGFR-treated cells.

(Figure 2D), thus confirming the absence of protective catalase on their outside. In contrast, the sensitizing effect of antibody directed against catalase for peroxynitrite-mediated apoptosis induction in transformed and tumor cells was more or less identical to the effect of knockdown

(Figure 2E, F), indicating that all protective catalase activity directed against exogenous peroxynitrite must be located on the outside. Tumor cells showed a remarkable higher extracellular catalase activity compared to transformed cells.

Extracellular catalase modulates intercellular apoptosis-inducing ROS signaling of transformed cells and protects tumor cells against intercellular apoptosis-inducing ROS signaling

For a detailed characterization of the modulating effects of extracellular catalase on the efficiency and quality of intercellular ROS-mediated signaling, nontransformed and transformed cells were treated with transforming growth factor β (TGF- β) and increasing concentrations of antibody against catalase in combination with specific inhibitors. Transformed 208Fsrc3 cells showed substantial autocrine apoptosis induction that was enhanced by antibody toward catalase (Figure 3A, B), but not by control antibody directed toward laminin (Figure 3C). Nontransformed 208F cells did not show autocrine apoptosis induction and were not affected by antibody directed to catalase (Figure 3C). The enhancing effect of catalase antibody on transformed 208Fsrc3 cells was abrogated by a very low concentration (0.2 μM) of the catalase mimetic EUK-134 (Figure 3A), indicating that it was based on an increase in H_2O_2 . A tenfold higher concentration of EUK-134 (2 μM), as well as caspase 3 and caspase 9 inhibitor caused complete inhibition of apoptosis (Figure 3A), demonstrating that H_2O_2 was necessary for intercellular apoptosis-inducing ROS signaling, and that apoptosis induction was mediated by caspases and the mitochondrial pathway. In the absence of catalase antibody, apoptosis induction in transformed cells (seeded at a density of 10 000 cells/100 μl) seemed to be dependent on the cooperative interaction between the HOCl and the NO/peroxynitrite pathway, as it was (partially) blocked by the HOCl scavenger taurine and the peroxidase inhibitor aminobenzoyl hydrazide (ABH), as well as by the peroxynitrite decomposition catalyst 5-,10-,15-,20-tetrakis(4-sulfonatophenyl) porphyrinato iron(III) chloride (FeTPPS) (Figure 3B). With increasing concentrations of catalase-specific antibody, inhibition by FeTPPS decreased (indicating a decreasing role of NO/peroxynitrite signaling), and the reaction seemed to depend mostly on HOCl pathway signaling, as it was completely inhibited by ABH and taurine (Figure 3B). Apoptosis induction under all conditions was blocked by the NADPH oxidase (NOX) inhibitor 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF) and the hydroxyl radical scavenger mannitol, in line with the established central role of superoxide anions and hydroxyl radicals in both signaling pathways (Heinzelmann and Bauer, 2010; Bauer, 2012). Extracellular catalase activity with modulatory potential, at a concentration that was too low for complete

protection against intercellular apoptosis-inducing ROS signaling, was further confirmed for 208Fsrc3 using additional approaches, such as inactivation of catalase by extracellular singlet oxygen (Escobar et al., 1996) generated by the photosensitizer photofrin (data not shown) or by the reactive aldehyde 4-hydroxynonenal (D'Souza et al., 2008; Bauer and Zarkovic, 2015). Extracellular catalase was demonstrated after induction of RAS expression in IR-1 cells (208F rat fibroblasts with an inducible RAS oncogene) (Schwieger et al., 2001), accompanied by NOX1 activation and transformation and in spontaneously transformed hamster embryonal (STHE) fibroblasts (Deichman et al., 1989) (data not shown). Tumor cells isolated from tumors that had been established by inoculation of STHE cells into syngeneic hamsters expressed higher concentrations of extracellular catalase than STHE cells and were fully protected against intercellular apoptosis-inducing ROS-mediated signaling (data not shown).

Our data thus demonstrate that expression of membrane-associated catalase is a typical phenotypic feature of transformed cells. Extracellular catalase seems to modulate their intercellular apoptosis-inducing ROS signaling but is not sufficient to protect the cells completely against apoptosis induction. In contrast, membrane-associated catalase of *bona fide* tumor cells seems to be expressed at sufficiently high concentration to strictly prevent ROS-dependent apoptosis signaling (Bechtel and Bauer, 2009; Heinzelmann and Bauer, 2010), as illustrated exemplarily in Figure 4A. MKN-45 cells did not show autocrine apoptosis unless their catalase was specifically inhibited by neutralizing antibodies directed against catalase. Control antibody directed toward laminin had no sensitizing effect. Apoptosis was induced by anti-CAT in the mode of an optimum curve, with NO/peroxynitrite signaling at low concentrations of antibody [indicated by the inhibition through the NOS inhibitor *N*- ω -nitro-L-arginine methyl ester hydrochloride (L-NAME)] and HOCl signaling at medium and high concentrations of antibody (indicated by the inhibitory effects of the peroxidase inhibitor ABH and the HOCl scavenger taurine). Catalase-mediated protection of tumor cells against intercellular apoptosis-inducing ROS signaling has been found in all human and rodent tumor cell lines tested so far (data not shown). A more complete inhibition profile at low and high concentrations of antibody directed toward catalase is presented in the supplementary material (Supplementary Figure 3). It fully supports the conclusion that NO/peroxynitrite signaling prevails at low concentrations and HOCl signaling at high concentrations of antibody directed against catalase. This is also in line with recently published work (Heinzelmann and Bauer, 2010) and in analogy to the effects of low and

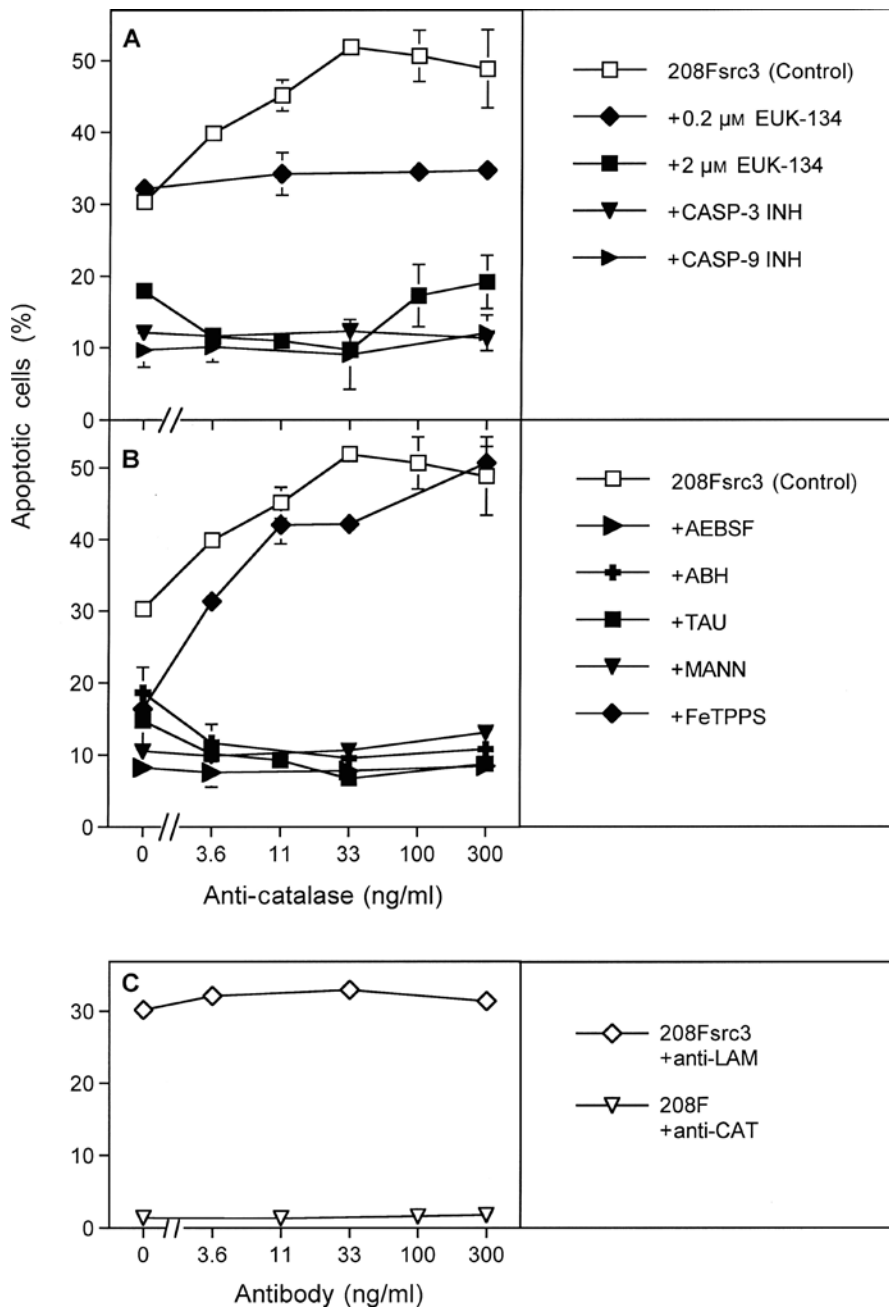


Figure 3: Neutralizing antibody against catalase enhances autocrine apoptosis-inducing ROS signaling of transformed cells and has an impact on the signaling quality.

A total of 10 000 transformed 208Fsrc3 cells/100 μ l in the presence of 20 ng/ml TGF- β 1 were treated with the indicated concentrations of neutralizing antibody directed against catalase (A, B) or with irrelevant control antibody (anti-laminin) (C). For additional control, nontransformed 208F cells were treated with neutralizing antibodies against catalase and TGF- β 1 (C). After 20 min of incubation, assays received either no further addition (control) or 0.2 or 2 μ M of the catalase mimetic EUK-134, 50 μ M caspase 3 inhibitor, 25 μ M caspase 9 inhibitor (A), 100 μ M of the NOX inhibitor AEBSF, 50 mM of the HOCl scavenger taurine, 25 μ M of the peroxynitrite decomposition catalyst FeTPPS, 150 μ M of the peroxidase inhibitor ABH, or 10 mM of the hydroxyl radical scavenger mannitol (B), as indicated. The percentage of apoptotic cells was determined in duplicate assays after 14 h. This experiment has been repeated twice and has also been performed with STHE fibroblasts and with IR-1 cells with an inducible RAS oncogene, confirming the extracellular location of catalase of transformed cells. Statistical analysis: The enhancing effect of 3.6 ng/ml anti-CAT on apoptosis induction in 208Fsrc3 cells was significant ($p < 0.01$) and the effect of higher concentrations highly significant ($p < 0.001$), while there was no significant effect of anti-laminin. Nontransformed cells did not show significant apoptosis induction. The effects of the inhibitors were highly significant ($p < 0.001$). The site of action of inhibitors and scavengers and the details of intercellular apoptosis-inducing ROS signaling through the NO/peroxynitrite and the HOCl signaling pathway are demonstrated in the supplementary material (see Supplementary Figure 2).

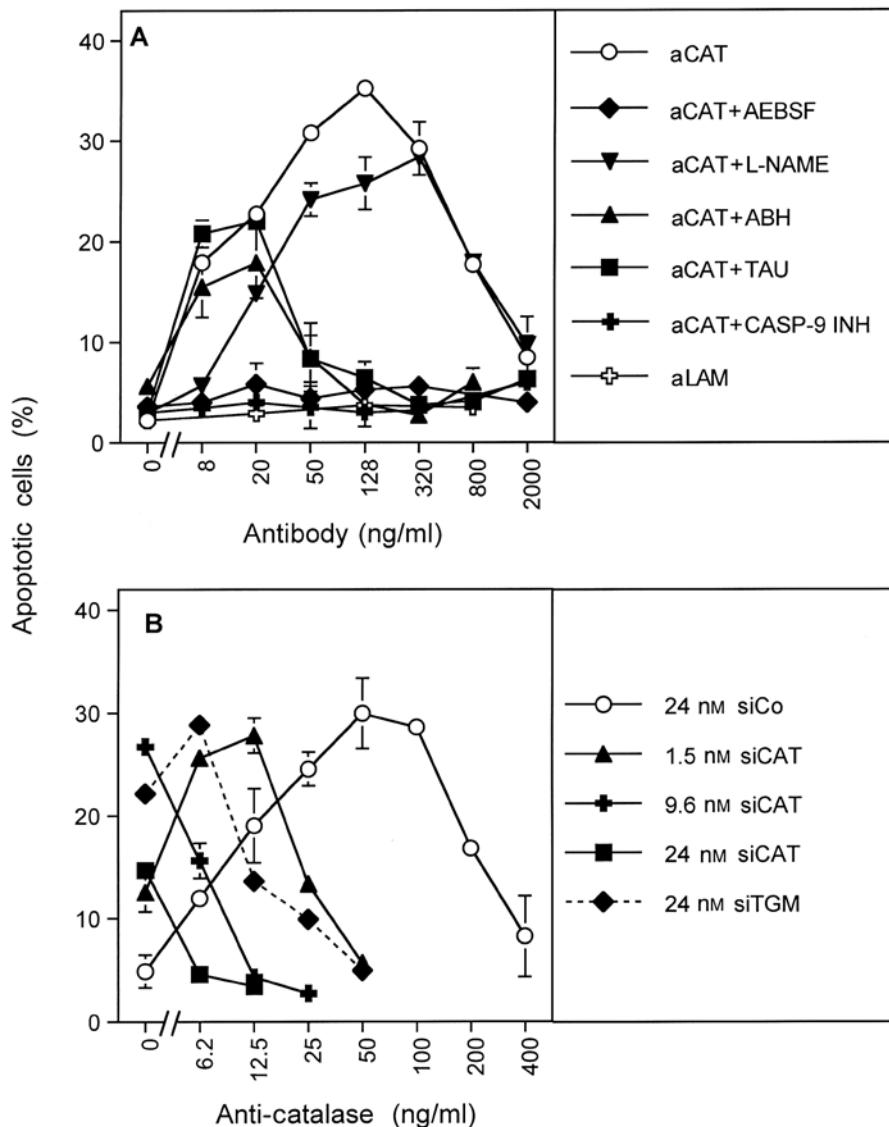


Figure 4: Neutralizing antibody against catalase sensitizes tumor cells for ROS-mediated apoptosis induction and cooperates additively with siRNA-mediated knockdown of catalase.

(A) A total of 12 500/100 μ l human gastric carcinoma cells MKN-45 received either no further addition or 100 μ M of the NOX inhibitor AEBSF, 2.4 mM of the NOS inhibitor L-NAME, 150 μ M of the mechanism-based peroxidase inhibitor 4-ABH, 50 mM of the HOCl scavenger taurine, or 25 μ M of caspase 9 inhibitor. Increasing concentrations of neutralizing antibody against catalase (aCAT) or irrelevant antibody (anti-laminin, 'aLAM') were added. Apoptosis induction was monitored in duplicated assays after 5.5 h. This experiment has been repeated more than 20 times; in addition, analogous experiments with more than 50 different human tumor cell lines have been performed, leading to the consistent conclusion of protection of tumor cells against intercellular apoptosis-inducing ROS signaling by membrane-associated catalase. Neutralizing anti-CAT caused apoptosis induction in the mode of an optimum curve, defined by NO/peroxynitrite signaling in the lower concentration of antibodies and HOCl signaling in the higher concentration range. The supraoptimal decline of apoptosis induction at very high concentrations of aCAT is due to the consumption reaction (6) between HOCl and H_2O_2 shown in Supplementary Figure 2. Statistical analysis: Apoptosis induction mediated by anti-CAT, inhibition of apoptosis by AEBSF, and caspase 9 inhibitor were highly significant ($p < 0.001$). Inhibition of apoptosis induction by L-NAME at low concentrations of antibody and by ABH and taurine at high concentrations were highly significant ($p < 0.001$). (B) MKN-45 cells were transfected with 24 nM siCo or 1.5, 9.6, and 24 nM siRNA against human catalase. siCAT transfections were supplemented with siCo to reach a total concentration of 24 nM siRNA in all assays. In addition, cells were transfected with 24 nM siTGM. After 24-h incubation, the cells were reseeded at a density of 12 500 cells/assay, and increasing concentrations of neutralizing antibodies against catalase were added. Apoptosis induction was monitored after 5.5 h in duplicate assays. This experiment has been repeated twice. Statistical analysis: The shifts of the curves shown in panel (B) are highly significant ($p < 0.001$). The mechanism of protection against intercellular apoptosis-inducing ROS signaling and details of intercellular apoptosis-inducing ROS signaling through the NO/peroxynitrite and the HOCl signaling pathway, including the use of specific inhibitors for the elucidation of signaling pathways, are demonstrated in the supplementary material (see Supplementary Figures 1–4).

high concentrations of the catalase inhibitor 3-AT (Supplementary Figure 4). Inhibition of catalase by specific antibody and siRNA-mediated knockdown of catalase interacted additively (Figure 4B). Cells with suboptimal catalase knockdown mediated by 1.5 nM siRNA were brought to the optimum of apoptosis induction by low concentrations of antibodies, before higher concentrations of the antibody then caused the typical supraoptimal reaction. This caused a leftward shift of the optimum curve seen for antibody action alone. Cells transfected with 9.6 and 24 nM siCAT were immediately inhibited by low concentrations of antibody, and therefore, only the supraoptimal part of the optimum curve remained to be seen. As inhibition of catalase by specific antibody and siRNA-mediated knockdown of catalase interacted additively (Figure 4B) and also show the same inhibitor profile (Supplementary Figure 5), it was confirmed that both treatments affect the same target.

Interestingly, siRNA-mediated knockdown of transglutaminase (siTGM) had an analogous effect on apoptosis induction as siRNA-mediated knockdown of catalase, as it sensitized the tumor cells for apoptosis induction that cooperated additively with neutralizing antibody directed toward catalase (Figure 4B). In line with this finding, inhibition of transglutaminase- or siRNA-mediated knockdown of its activity caused an increase in free catalase in the supernatant of the tumor cells and a decrease of resistance of the cells against exogenous H_2O_2 and peroxynitrite (Supplementary Figures 13–16).

The concentration of protective catalase activity of tumor cells is modulated by their own NOX-dependent H_2O_2 generation

The strong catalase-dependent resistance of tumor cells against intercellular apoptosis-inducing ROS signaling might be the result of selection of cells with constitutively high expression of membrane-associated catalase or, alternatively, selection of cells that respond very strongly to their own NOX1-dependent ROS production by an appropriate dynamic catalase expression response. To differentiate between these two phenotypical alternatives, MKN-45 cells were pretreated for 14 h in the presence of increasing concentrations of the NOX inhibitor AEBSF, SOD, catalase, or the catalase mimetic EUK-134. After pretreatment, the cells were washed, seeded at low density and challenged with increasing concentrations of H_2O_2 -generating GOX. Under these conditions,

autocrine apoptosis induction is not effective due to low cell density and apoptosis is directly induced by H_2O_2 . This allows to monitor the relative changes of catalase in the system that counteracts the effect of H_2O_2 . As shown in Figure 5A, B, control cells required around 2 mU/ml GOX for half-maximal apoptosis induction. Pretreatment with AEBSF caused a marked sensitization of the cells for H_2O_2 . When the cells had been pretreated with 100 μM AEBSF, <0.1 mU/ml GOX was necessary for half-maximal apoptosis induction, i.e. compared to untreated control cells, 1/20 of the GOX concentration was sufficient to establish the same biological effect. Thus, pretreatment with 100 μM AEBSF seems to have sensitized the cells by reduction of their catalase activity to <5% of control cells. The degree of sensitization was directly dependent on the concentration of AEBSF applied during the preincubation period (Figure 5A). Pretreatment with catalase or the catalase mimetic EUK-134 also showed a similarly strong sensitization for subsequent H_2O_2 -dependent apoptosis induction (Figure 5B), whereas pretreatment with SOD caused an increase in resistance against H_2O_2 by a factor of ~2 (Figure 5A). A similar effect was demonstrated for pretreatment with TGF- β (Supplementary Figure 8B), which has been recently shown to enhance NOX1 activity and thus contributes to an increased concentration of H_2O_2 (Temme and Bauer, 2013). Taken together, these data demonstrate that tumor cell catalase expression is controlled by tumor cell-derived H_2O_2 . Superoxide anions, generated by NOX are thereby not involved directly in the control of catalase expression but rather function as a source for H_2O_2 generation through their dismutation, as seen from the differential effects of the NOX inhibitor AEBSF and SOD. When AEBSF-pretreated tumor cells that had lost the majority of its protective catalase activity were washed and incubated in AEBSF-free medium, catalase activity was restored with time and nearly reached the concentration of untreated control cells after 7-h incubation (Figure 5C), indicating that the modulation of catalase activity was a process of high plasticity. Further controls related to the modulation of catalase expression of tumor cells by their own H_2O_2 are presented in Supplementary Figures 8 and 9.

To determine the significance of downmodulation of protective catalase of tumor cells for their resistance against autocrine apoptosis-inducing ROS signaling, MKN-45 cells were pretreated with increasing concentrations of AEBSF, catalase, SOD, and EUK-134 and/or remained without addition. After 14 h of pretreatment, the cells were washed and resuspended in fresh medium under conditions that were suitable for the induction of autocrine ROS-mediated apoptosis induction. As can be seen in Figure 6, tumor cells pretreated without additions

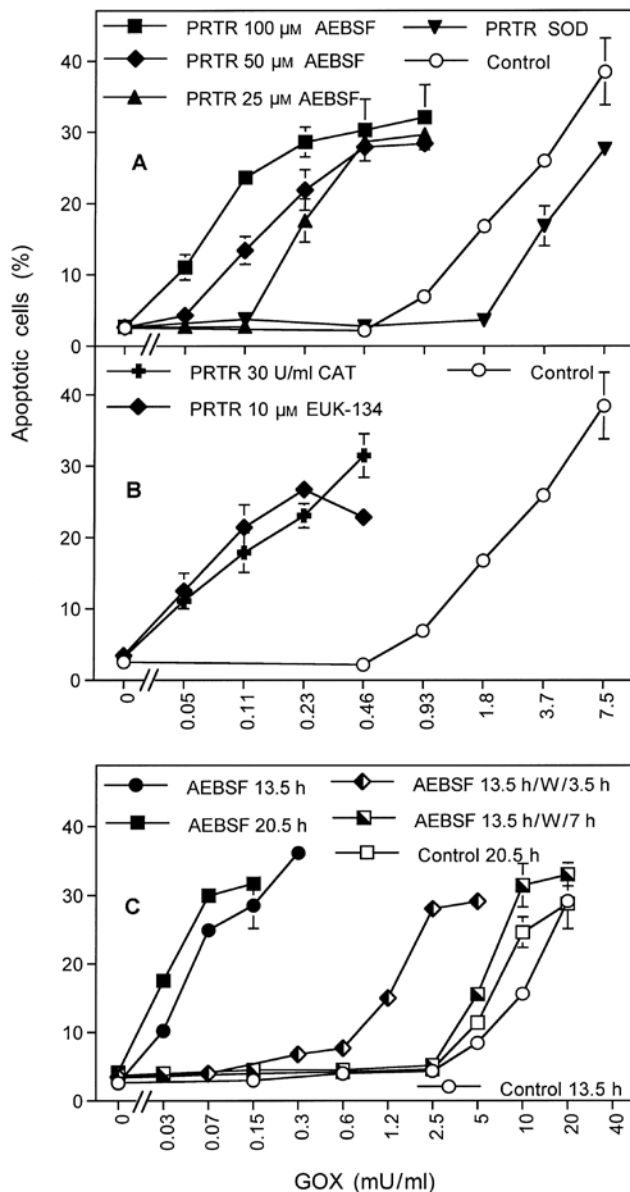


Figure 5: Catalase concentration of tumor cells is modulated by their extracellular H_2O_2 concentration.

(A, B) MKN-45 cells (200 000/ml) were pretreated with 25, 50, or 100 μM AEBSF, 100 U/ml MnSOD, 30 U/ml catalase, or 10 μM EUK-134 for 14 h at 37°C. Control cells were incubated without addition. Cells were centrifuged, washed in three cycles and reseeded at a density of 4000 cells/100 μl . GOX was added at the indicated concentrations, and apoptosis induction in duplicate assays was monitored after 2 h. This experiment has been repeated twice. Statistical analysis: All shifts of curves described are highly significant ($p < 0.001$). The differences between the effects of varying concentrations of AEBSF are highly significant ($p < 0.001$). (C) MKN-45 cells (200 000/ml) were pretreated with 100 μM AEBSF ('AEBSF') or without AEBSF ('control') for 13.5 or 20.5 h at 37°C. In addition, cells pretreated with AEBSF for 13.5 h were centrifuged, washed in three cycles, and incubated in the absence of AEBSF for 3.5 or 7.5 h ('AEBSF 13.5 h/W/3.5 h', 'AEBSF 13.5 h/W/7 h'). Before being challenged by H_2O_2 -producing GOX, cells were centrifuged, washed in three cycles, and reseeded at a density of 5000 cells/100 μl . GOX was added at the indicated concentrations and apoptosis induction was monitored after 2 h in duplicate assays. (One unit of GOX is defined as producing 1 μmol H_2O_2 /min at 35°C at a pH of 5.1 and optimal saturation of the medium with oxygen.) This experiment has been repeated twice. Statistical analysis: The effects of AEBSF pretreatment as well as of removing AEBSF from pretreated cells and cultivating the cells in its absence are highly significant ($p < 0.001$). Further controls are shown in the supplementary materials (Supplementary Figures 8 and 9).

by the result that pretreatment with 25 μM AEBSF (which induced the lowest degree of sensitization, as shown in Figure 5) allowed NO/peroxynitrite signaling, but not HOCl signaling, whereas pretreatment with higher concentrations of AEBSF allowed subsequent HOCl signaling. As shown in Figure 4A, sensitization for NO/peroxynitrite signaling can be achieved already by low inhibition of protective catalase, whereas establishment of HOCl signaling requires a higher degree of catalase inactivation.

Discussion

Our data confirm that tumor cells are much more resistant against apoptosis induction by exogenous H_2O_2 and peroxynitrite than transformed and normal cells. The resistance of tumor cells correlates with the H_2O_2 -catabolizing phenotype that has been described by Deichman et al. as a necessary requirement for tumor progression (Deichman and Vendrov, 1986; Deichman et al., 1989, 1998; Deichman, 2000, 2002) and that has been shown due to the activity of membrane-associated catalase (Heinzelmann and Bauer, 2010). Our data also confirm that most of the protective catalase activity of tumor cells is located at

or in the presence of SOD did not show apoptosis induction, whereas cells pretreated with AEBSF, catalase, or EUK-134 showed remarkable apoptosis induction. Thereby, apoptosis induction in cells pretreated with 25 μM AEBSF was exclusively dependent on NO/peroxynitrite signaling, as it was inhibited by FeTPPS and not by taurine, whereas apoptosis induction in cells pretreated with higher concentrations of AEBSF or with catalase or EUK-8 was solely dependent on HOCl signaling. These findings indicate that removal of H_2O_2 during pretreatment had strongly reduced the concentration of extracellular catalase that is protective against intercellular apoptosis-inducing ROS signaling. The strict quantitative nature of this process is seen

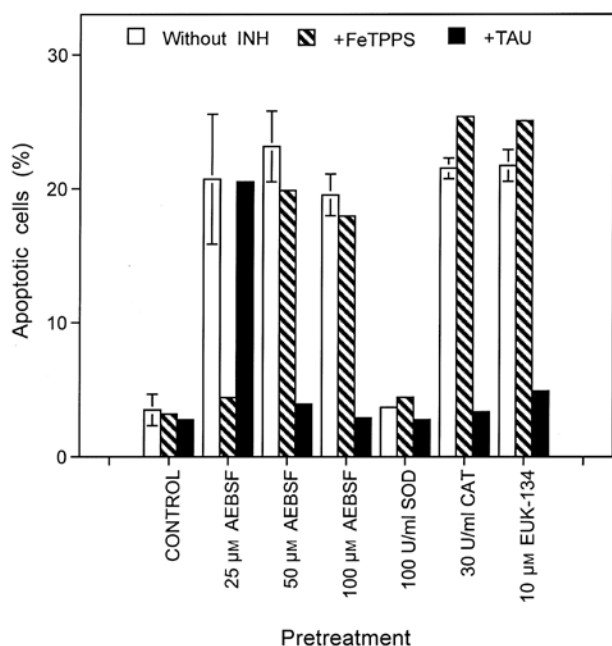


Figure 6: Impact of downmodulation of tumor cell-associated catalase for reactivation of intercellular apoptosis-inducing ROS signaling.

Cells pretreated under the conditions described in Figure 5 were washed and reseeded under conditions of autocrine apoptosis induction (12 500 cells/100 µl). The cells remained either free of further additions or received 25 µM FeTPPS or 50 mM taurine. After 6 h, apoptosis induction was monitored in duplicate assays. The figure shows that conditions that downmodulated catalase (i.e. AEBSF treatment, treatment with catalase, or the catalase mimetic EUK-134), but not control cells or SOD-pretreated cells, went into autocrine apoptosis induction after pretreatment. Pretreatment with 25 µM AEBSF that had caused the least degree of sensitization in the parallel experiment shown in Figure 5 caused apoptosis induction mainly through the NO/peroxynitrite pathway, whereas all other conditions caused apoptosis induction through HOCl signaling. This experiment has been repeated once. Statistical analysis: Apoptosis induction after pretreatment with AEBSF, catalase, and EUK-134 was highly significant ($p < 0.001$), whereas pretreatment with SOD did not cause significant apoptosis induction. In cells that had been pretreated with 25 µM AEBSF, inhibition of apoptosis by FeTPPS was highly significant ($p < 0.001$), whereas there was no significant inhibition by taurine. In cells that had been pretreated with 50 or 100 µM AEBSF, catalase or EUK-134 inhibition by taurine was highly significant ($p < 0.001$), whereas there was no significant inhibition by FeTPPS.

their outside, thus allowing effective interference with exogenous peroxynitrite and with intercellular apoptosis-inducing ROS signaling pathways, i.e. the HOCl and the NO/peroxynitrite signaling pathways. When the protective catalase on the outside of tumor cells is blocked by antibodies, the residual protection by intracellular catalase of the tumor cells against exogenous H_2O_2 is even less efficient than the protection of normal cells that only have

intracellular catalase. Antibodies directed against catalase have no effect on the intracellular catalase of normal cells, but siRNA-based knockdown of catalase activity sensitizes normal cells toward the apoptosis-inducing activity of exogenous H_2O_2 that has the ability to enter the cells through aquaporins. In contrast, siRNA-mediated knockdown of intracellular catalase of normal cells has no effect on protection against exogenous peroxynitrite that requires extracellular catalase for detoxification, as the attack of peroxynitrite on the membrane cannot be prevented from inside the cell.

Unexpectedly, transformed cells that show similar total resistance against ROS as normal cells and that are much more sensitive than tumor cells showed a distribution of catalase activity with the major activity located on the outside and the minor part inside, as deduced from the challenge experiments in the presence of antibody directed toward catalase and from the protection of the cells against exogenous peroxynitrite. This pattern of catalase distribution resembles that of tumor cells, whereas the total concentration of extracellular catalase of transformed cells is not sufficiently high to protect the transformed cells against intercellular apoptosis-inducing ROS signaling at optimal cell density. In contrast, tumor cells achieve tight control of intercellular apoptosis-inducing ROS signaling through the activity of their membrane-associated extracellular catalase and thus are completely protected. This finding allows to conclude that (i) the change in the distribution pattern of catalase is related to the step of transformation and not to that of tumor progression and that (ii) tumor progression is characterized by an increase in total extracellular catalase activity up to a fully protective level. This conclusion seems to define rather general phenotypic features of transformed and tumor cells, as it has been substantiated by the study of additional transformed and tumor cells and using additional methods (Bauer and Zarkovic, 2015).

The interplay between catalase at the distinct locations with ROS is clearly different and characteristic for each step during tumor development. Nontransformed cells lack constitutively active NOX1 in their membrane (Schwieger et al., 2001; Heigold et al., 2002; Bauer, 2012) and therefore cannot establish autocrine ROS-mediated apoptosis induction (Bechtel and Bauer, 2009) or an apoptotic response to exogenous NO or HOCl (Engelmann et al., 2000; Heigold et al., 2002). Nontransformed cells do not express membrane-associated catalase and possess intracellular catalase only. Even after knockdown of intracellular catalase activity, nontransformed cells seem to have sufficient alternative antioxidant capacity (such as glutathione, glutathione peroxidase, and peroxiredoxin)

(Sies, 2014) to cope with H_2O_2 generated by mitochondria, as knockdown of catalase activity did not cause cell death. However, when challenged with exogenous H_2O_2 , normal cells with knockdown of their intracellular catalase activity react in a very sensitive way and die by apoptosis or necrosis.

Knockdown of catalase activity or inhibition of extracellular catalase of transformed cells has a remarkable positive modulatory effect on intercellular apoptosis-inducing ROS signaling that is driven by membrane-associated NOX1 of transformed cells. This effect is especially prominent when transformed cells are seeded at suboptimal concentration for autocrine ROS-mediated apoptosis induction. Although most of their catalase is on the outside of transformed cells, it is not sufficient to completely inhibit intercellular apoptosis-inducing ROS signaling that causes elimination of these cells. Inhibition of extracellular catalase of transformed cells may not only enhance otherwise suboptimal intercellular apoptosis-inducing ROS signaling, but also change the quality of signaling, as inhibition of catalase causes an increase in H_2O_2 . As a consequence, HOCl synthesis and HOCl signaling are enhanced and NO/peroxynitrite signaling is decreasing due to the consumption of NO by H_2O_2 (Heinzelmann and Bauer, 2010). The parallel relative decrease in intracellular catalase activity of transformed cells might enhance the proliferation-stimulating effect of intracellular H_2O_2 and also potentially increase the chance of induction of genomic instability by intracellular ROS.

In contrast to the situation seen for transformed cells, the high concentration of catalase activity located on the outside of tumor cells is sufficient to protect them completely against intercellular apoptosis-inducing ROS signaling that is driven by superoxide anions generated by NOX1. The localization of catalase on the outside of the membrane, in close vicinity to superoxide anion generating NOX1, seems to be a strategic advantage for the enzyme. It thus can efficiently interfere with H_2O_2 generation, as dismutation of superoxide anions can be expected to be the highest in the area of their highest local concentration, i.e. the site of their generation. In addition, membrane-associated SOD seems to contribute to the dismutation of superoxide anions and thus prevents superoxide anion-dependent inhibition of catalase through reduction of compound I to the inactive compounds II and III (Bauer, 2014). The interaction between H_2O_2 and catalase prevents the HOCl signaling pathway. Nevertheless, due to the immobilization of catalase, a minor part of H_2O_2 can be expected to escape into the intracellular department. The high local concentration of extracellular catalase in the membrane of tumor cells also is sufficient

to decompose peroxynitrite that is generated in the direct vicinity of NOX1 before peroxynitrite has a chance to become protonated and to decompose spontaneously into NO_2 and apoptosis-inducing hydroxyl radicals. This critical protective step against peroxynitrite is flanked by catalase-dependent oxidation of NO, thus limiting the chance of peroxynitrite formation. For kinetic reasons, the tight association of catalase with the membrane seems to be especially essential for protection against peroxynitrite generated by the tumor cells. When tumor cell catalase activity was knocked down by siRNA and protection against cell-generated peroxynitrite was achieved by exogenous soluble catalase, 500–1000 U/ml had to be applied to achieve protection, whereas protection against H_2O_2 -dependent HOCl signaling required 100-fold less of the enzyme (Bauer, unpublished result). In contrast to normal cells, active intracellular catalase is much lower in concentration in the tumor cells. This may allow for ROS-dependent mutagenesis in the intracellular compartment as driving force for induction of genomic instability as well as for protection of proliferation-stimulating H_2O_2 in the inside of the tumor cell, two characteristic hallmarks of tumorigenesis (Hanahan and Weinberg, 2011). In line with this conclusion, overexpression of intracellular catalase in tumor cells has been shown to decrease proliferation of the cells as well as tumor growth *in vivo* (Arnold et al., 2001).

The high local concentration of catalase at a restricted site, i.e. the membrane of the cell, and the relatively low concentration of the enzyme within the cell meet three ROS-related requirements of tumorigenesis in a perfect way. The membrane-associated extracellular catalase protects the tumor cells against the apoptosis-inducing effects of their own apoptosis-inducing ROS signaling and the low intracellular catalase facilitates the procarcinogenic effects of ROS, i.e. induction of proliferation and of genomic instability. As the compartment with relatively high local concentration of catalase in close vicinity of the cell membrane represents a very minor part of the total mass of the tumor cell, the finding of high concentration of catalase on the outside of the membrane is still consistent with the established finding that the average concentration of catalase in tumor cells is usually lower than in normal cells (Sato et al., 1992; Coursin et al., 1996; Baker et al., 1997; Bostwick et al., 2000; Chung-Man et al., 2001; Cullen et al., 2003; Kwei et al., 2004). Therefore, the findings by Deichman's group (Deichman and Vendrov, 1986; Deichman et al., 1989, 1998; Deichman, 2000, 2002) and our own findings (Bechtel and Bauer, 2009; Heinzelmann and Bauer, 2010) are not discrepant to the findings by Sato et al. (1992),

Coursin et al. (1996), Baker et al. (1997), Bostwick et al. (2000), Chung-Man et al. (2001), Cullen et al. (2003), and Kwei et al. (2004), but rather are focused on a specific site on tumor cells with biologically relevant high catalase expression that is dramatically different from the much lower catalase expression inside the cell.

To our knowledge, the demonstration of membrane-associated catalase activity on tumor cells (Bechtel and Bauer, 2009; Heinzelmänn and Bauer, 2010), which is in contrast to the established site of catalase action, i.e. the peroxisomes, represents a novel finding. However, the release of catalase from tumor cells has already been shown 20 years ago by Sandström and Buttke (1993) and confirmed by Moran et al. (2002). These authors also demonstrated the functional relevance of extracellular catalase for the survival of the tumor cells, in analogy to our findings. As their studies were specifically focused on soluble catalase in the supernatant of the tumor cells, they possibly may have missed extracellular membrane-associated catalase. Based on our data, membrane-associated catalase, due to its high local concentration is optimal for protection of tumor cells against the HOCl signaling pathway and especially the NO/peroxynitrite pathway (Heinzelmänn and Bauer, 2010). Despite the long time since the release of catalase from tumor cells has been first described, the mechanism of transport of catalase through the membrane has not yet been clarified. In line with the findings that (a) catalase represents a potential substrate for transglutaminase (Valdivia et al., 2006) and that (b) transglutaminase can be found extracellularly (Akimov and Belkin, 2001), transglutaminase 2 was shown to be responsible for the attachment of catalase to the cell membrane of tumor cells (Supplementary Figures 13–16).

The change in localization of catalase seems to be related to the transformation step. The high amount of catalase at the later stage of *bona vide* tumor cells, however, does not indicate a phenotype with constitutively high expression of catalase in the membrane. It rather indicates the potential to react with high expression of catalase after sensing cell-derived H_2O_2 . Removal of H_2O_2 during culture of tumor cells causes a downregulation of protective catalase to a degree that may be fatal for the cell after the antioxidants are removed and the cell is challenged by its usual concentration of ROS and can re-establish intercellular apoptosis-inducing signaling. This finding shows that the interplay between cell-derived ROS and catalase in tumor cells is a rather dynamic process and not at all static. Based on the findings by Zhou et al. (2001), the control of catalase expression by H_2O_2 might be controlled by redox-sensitive transcription factors like

activating protein-1 (AP-1) or nuclear factor κ light-chain-enhancer of activated B cells (NF- κ B). Our findings also allow the speculation that tumor cells in a high antioxidative environment will be protected by this environment against their own apoptosis-inducing ROS signaling pathways, but most likely, they will not express much catalase on their surface due to the missing inducing signal by H_2O_2 . This may rise problems for detection of such cells when it is based on the marker ‘membrane-associated catalase’. Speculatively, this situation might correlate to the ‘dormant state’ of tumor cells. This would be a state in which the tumor cell does not proliferate despite active NOX, as the proliferation signals are scavenged by antioxidants, but also does not show the characteristic marker extracellular catalase. In analogy to the experiment shown in Figure 6, such dormant tumor cells might be threatened when an extracellular ROS stimulus is applied faster than they can respond in re-expression of protective catalase.

Materials and methods

Materials

The NOX inhibitor AEBSF, catalase from bovine liver and from human erythrocytes, the broad-spectrum matrix metalloproteinase inhibitor (R)-N4-hydroxy-N1-[(S)-2-(1H-indol-3-yl)-1-methylcarbamoyl-ethyl]-2-isobutyl-succinamide (Galardin; GM6001) (Santiskulvong and Rozengurt, 2003), the H_2O_2 generator GOX, the NOS inhibitor N- ω -nitro-L-arginine methylester hydrochloride (L-NAME), the transglutaminase inhibitor LDN-27219, the hydroxyl radical scavenger mannitol, Mn-containing SOD from *Escherichia coli*, the HOCl scavenger taurine, neutralizing monoclonal antibodies against human catalase (clone CAT-505, mouse, IgG1), and control antibody directed against EGF receptor, laminin, or GAPDH were obtained from Sigma-Aldrich (Schnelldorf, Germany). The mechanism-based peroxidase inhibitor 4-ABH was purchased from Acros Organics (Geel, Belgium). Peroxynitrite, the peroxynitrite decomposition catalyst FeTPPS and EUK-8 [manganese N,N'-bis(salicylidene)ethylenediamine chloride] were obtained from Calbiochem (Merck Biosciences GmbH, Schwalbach/Ts, Germany). Inhibitors for caspase 3 (Z-DEVD-FMK) and caspase 9 (Z-LEHD-FMK) were obtained from R&D Systems (Wiesbaden-Nordenstadt, Germany). The catalase mimetic EUK-134 {chloro[[2,2'-(1,2-ethanediyl)bis[(nitrilo- κ N)methylidene]]bis[6-methoxyphenolato- κ O]]]-manganese was a product of Cayman and was obtained from Biomol (Hamburg, Germany). TGF- β 1 was purified from human platelets (Bauer et al., 1982) and kept as a stock solution of 1.5 μ g/ml in Eagle's minimal essential medium (EMEM) plus 5% fetal bovine serum (FBS) at -20°C. Detailed information on inhibitors has been previously published (Bechtel and Bauer, 2009; Heinzelmänn and Bauer, 2010).

Cells and media for cell culture: Nontransformed rat fibroblasts 208F and their derivatives transformed through constitutive expression of v-src ('208Fsrc3') were a generous gift by Drs C. Sers and R. Schäfer,

Berlin, Germany (Iten et al., 1989). 208F and 208Fsrc3 cells were cultured in EMEM, containing 5% FBS. FBS (Biochrom, Berlin, Germany) had been heated for 30 min at 56°C prior to use. Medium was supplemented with penicillin (40 U/ml), streptomycin (50 µg/ml), neomycin (10 µg/ml), moronal (10 U/ml), and glutamine (280 µg/ml).

The human gastric adenocarcinoma cell line MKN-45 (ACC 409) was purchased from DSMZ (Braunschweig, Germany). MKN-45 cells were cultured in RPMI 1640 medium, containing 10% FBS and supplements as described above.

Characterization of the cell lines used: 208F, 208Fsrc3, and MKN-45 cells represent cells from three distinct steps of experimental tumor progression. Nontransformed 208F cells do not show criss-cross morphology, colony formation in soft agar, and are not tumorigenic. They do generate sufficient extracellular superoxide anions to establish intercellular apoptosis-inducing ROS signaling (Herdener et al., 2000; Schwieger et al., 2001; Heigold et al., 2002). 208Fsrc3 cells have been transformed *in vitro* and have the potential to induce tumorigenesis in immunocompromised mice, but have not yet been confronted to natural antitumor mechanisms of an organism. 208Fsrc3 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 apoptosis-inducing ROS signaling (Herdener et al., 2000; Schwieger et al., 2001; Heigold et al., 2002).

The human gastric carcinoma cell line MKN-45 has been established from an adenocarcinoma of the stomach and thus is representative for the late stage of tumor progression. MKN-45 cells form colonies in soft agar. Despite remarkable extracellular superoxide anion generation, they are resistant toward intercellular apoptosis-inducing ROS signaling due to expression of membrane-associated catalase (Heinzelmann and Bauer, 2010). Inhibition of catalase by neutralizing antibodies or 3-AT reactivate intercellular apoptosis-inducing ROS signaling through the NO/peroxynitrite and the HOCl signaling pathway (Heinzelmann and Bauer, 2010).

Methods

Apoptosis induction by exogenous H₂O₂ and peroxynitrite: Cells were seeded at low cell density (4000 cells/100 µl medium or 6000 cells/100 µl medium in 96-well tissue culture plates, as described in the figure legends) and GOX or peroxynitrite were added at concentrations indicated in the figures. GOX utilizes glucose from the medium for the continuous generation of H₂O₂.

Assays were performed in duplicate and the percentages of apoptotic cells were determined after the indicated incubation time at 37°C and 5% CO₂ through inverted phase contrast microscopy as described below.

Autocrine apoptosis induction by intercellular apoptosis-inducing ROS signaling: Cells were seeded in 96-well tissue culture clusters at a density of 10 000 cells/100 µl or 12 500 cells/100 µl of complete medium, as indicated in the respective figures. Assays that contained 208Fsrc3 cells (or nonreactive 208F cells for control) received 20 ng/ml TGF-β1 in addition. MKN-45 tumor cells required the addition of the indicated concentrations of neutralizing antibody directed against catalase to allow intercellular apoptosis-inducing ROS signaling. Assays were performed in duplicate. After the indicated time of incubation at 37°C and 5% CO₂, the percentages of apoptotic cells were determined by inverted phase contrast microscopy as described below.

Quantitation of the percentage of apoptotic cells: The percentage of apoptotic cells was determined by inverted phase contrast microscopy based on the classical criteria for apoptosis, i.e. nuclear condensation/fragmentation or membrane blebbing (Kerr et al., 1972). The characteristic morphological features of intact and apoptotic cells, as determined by inverted phase contrast microscopy have been recently published (Beck et al., 1997; Heigold et al., 2002; Heinzelmann and Bauer, 2010; Bauer et al., 2014). At least 200 neighboring cells from randomly selected areas were scored for the percentage of apoptotic cells at each point of measurement. A systematic examination of our method (Bauer et al., 2014) has shown a remarkable coherence between the pattern of cells with condensed/fragmented nuclei (stained with bis-benzimide) and TUNEL-positive cells in assays with substantial apoptosis induction, whereas there was no significant nuclear condensation/fragmentation in control assays. The significance of this spatial coherence was confirmed through quantitation of cells with condensed/fragmented nuclei vs. TUNEL-positive cells. As the TUNEL reaction leads to selective loss of apoptotic cells during the staining procedure, quantitation of apoptosis induction was not based on the TUNEL reaction, but rather on the determination of cells with condensed or fragmented nuclei and membrane blebbing. As recently shown (Bauer et al., 2014), this method is strictly quantitative when it is adequately performed. For further control of the significance of this method, a comparison of the quantitation of annexin V staining by fluorescence microscopy and by FACS analysis, in conjunction with phase contrast microscopy confirmed that each of these methods reached the same degree of significance in determining the percentage of apoptotic cells (Bauer et al., 2014).

siRNA-mediated knockdown of catalase, NOX, peroxidase, and transglutaminase activity: Control siRNA (siCo) and siRNA for the knockdown of specific enzymes were obtained from Qiagen (Hilden, Germany).

Sequences of siRNAs used:

1. siCo:
Sense: r(UUC UCC GAA CGU GUC ACG U)dTdT
Antisense: r(ACG UGA CAC GUU CGG AGA A)dTdT
SiCo was determined by the manufacturer as not affecting the expression of any known gene.
2. siCAT for the knockdown of human catalase (used for MKN-45 cells):
Product number: Hs_Cat_4_HP siRNA
Target sequence: CCG GAT CTC ACT TGG CGG CAA
Sense: r(GGA UCU CAC UUG GCG GCA A)dTdT
Antisense: r(UUG CCG CCA AGU GAG AUC C)dGdG
3. siCAT for the knockdown of murine catalase (used for 208F and 208Fsrc3 cells):
Product number: Mm_Cat_4_HP siRNA
Target sequence: CCC AAT AGG AGA TAA ACT TAA
Sense: r(CAA UAG GAG AUA AAC UUA A)dTdT
Antisense: r(UUA AGU UUA UCU CCU AUU G)dGdG
4. HP custom siRNA directed against human NOX1 ('siNOX1'):
Sequences:
Sense: r(GAC AAA UAC UAC ACA A)dTdT
Antisense: r(UUG UGU AGU AGU AUU UGU C)dGdG
5. HP custom siRNA directed against human DUOX1 ('siDUOX1'):
Sense: r(AGU CUA ACA CCA CAA CUA A)dTdT
Antisense: r(UUA GUU GUG GUG UUA GAC U)dGdG

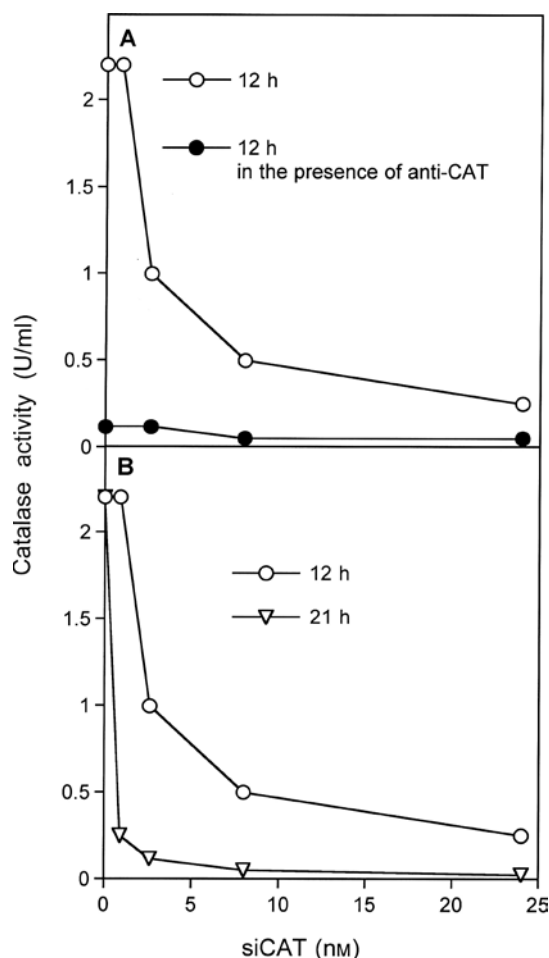


Figure 7: Quantitation of the specific functional knockdown of tumor cell catalase activity by siCAT.

A total 300 000 MKN-45 cells/ml were transfected with the indicated concentrations of human siCAT supplemented with siCo (not directed against a target) to reach a final total concentration of siRNA of 24 nM. After 12 and 21 h, cells were centrifuged, washed with fresh medium, and reseeded at a density of 5000 cells/100 μ l. Control IgG (monoclonal antibody directed toward laminin) or neutralizing monoclonal antibody directed against human catalase were added at a concentration of 0.2 μ g/ml. After 15 min at 37°C, the cells were challenged with increasing concentrations of H_2O_2 -generating GOX and apoptosis induction was monitored after 2 h in duplicate assays. (One unit of GOX is defined as producing 1 μ mol H_2O_2 /min at 35°C at a pH of 5.1 and optimal saturation of the medium with oxygen.) Apoptosis induction in response to the GOX concentration was plotted for the cells treated without siCAT and with increasing concentrations of siCAT (shown in Supplementary Figures 10 and 11). These plots allowed to determine the concentration of GOX whose apoptosis-mediating effect was neutralized by a given cell sample. A calibration curve that determined the concentrations of purified human catalase needed for neutralization of the effects of defined concentrations of GOX showed a strict linear response curve and was used to calculate the decrease in catalase activity in response to siCAT pretreatment. As shown in the figure, substantial functional knockdown, dependent on the concentration of siCAT, was already seen after 12 h for siCAT concentrations of 2.6 nM and higher and for all siCAT concentrations used (0.9–24 nM) at 21 h. As neutralizing antibody-directed human catalase completely abrogated catalase activity, whereas control antibody had no effect, the specific concentration- and time-dependent functional knockdown of human catalase is assured. Further controls related to this experiment are shown in the supplementary material (Supplementary Figures 10 and 11).

6. HP GenomeWide siRNA directed against human transglutaminase 2:

Product number: Hs_TGM2_6_HP siRNA

Cat. Nr: SI03055465

Target sequence: CAC AAG GGC GAA CCA CCT GAA

Transfection: SiRNAs were dissolved in suspension buffer supplied by Qiagen at a concentration of 20 μ M. Suspensions were heated at 90°C for 1 min, followed by incubation at 37°C for 60 min. Aliquots were stored at -20°C.

Before transfection, 88 μ l of medium without serum and without antibiotics were mixed with 12 μ l Hyperfect solution (Qiagen) and the required volume of siRNA to obtain the wanted final concentration. The mixture was treated by a Vortex mixer for a few seconds and then allowed to sit for 10 min. It was then gently and slowly added to 300 000 MKN-45 cells in 1 ml RPMI 1640 medium containing 10% FBS and antibiotics or to 200 000 208F or 208Fsrc3 cells in 6-well tissue culture clusters and 2.3 ml medium. The cells were incubated at 37°C, 5% CO_2 for 24 h. The cells were collected, centrifuged, and resuspended in fresh medium at the desired concentration.

Transfection efficiency: Control experiments showed that the transfection efficiency was much >90% when Hyperfect transfection reagent (Qiagen), the protocol summarized above were used.

Functional determination of the siRNA-mediated knockdown of catalase:

As functional catalase, and its localization, was in the focus of this study, knockdown of catalase activity rather than structural knockdown were required. The quantitative analysis of functional siRNA-mediated knockdown of catalase activity in MKN-45 cells was more than 97% for 10 nM siCAT after 24 h (Figure 7). The use of specific catalase-neutralizing monoclonal antibody and control antibody ensured that catalase was specifically quantified in the experiment described in Figure 7. Further details of the quantitation are presented in Supplementary Figures 10 and 11. The validity of quantitative determination of catalase activity through inhibition of GOX-dependent, H_2O_2 -mediated apoptosis induction has been recently published (Bauer and Zarkovic, 2015). The specificity of the catalase knockdown was further confirmed by abrogation of its effect through addition of exogenous catalase (Supplementary Figure 7C). Besides Hs_CAT_4_HP and Mm_Cat_4_HP siRNA, five other different siRNA preparations against human catalase and four siRNA preparations against murine catalase caused sufficient knockdown of functional catalase to establish ROS-mediated autocrine apoptosis induction, whereas negative siCo and siRNAs against more than 20 apoptosis- or ROS-related molecular targets but different from catalase did not (data not shown).

SiRNA experiments showed that the half-life of catalase activity is much shorter than the half-life of immunoreactive (but obviously

inactive) catalase protein. The reason for this discrepancy is explained in the supplementary material (Supplementary Figure 12). It is obvious that the method used in our study is restricted to the analysis of the regulation of catalase activity and is not suitable for structural studies related to catalase.

Functional knockdown of NOX1 was determined through direct quantitation of superoxide anion production by siCo- and siNOX1-transfected cells 24 h after transfection, following the protocol described by Temme and Bauer (2013). Functional knockdown was 94%.

Functional knockdown of DUOX1 was determined by direct quantitation of peroxidase release from siCo- and siDUOX1-transfected cells (Supplementary Figure 7B), using a recently described competition test for the quantitation of peroxidase (Bauer, 2011) and was 88%.

Functional knockdown of transglutaminase was demonstrated as pretreatment of MKN-45 tumor cells with 24 nM siRNA directed against transglutaminase had the same strong effect as 10 μ M of transglutaminase inhibitor LDN-27219 (see Supplementary Figure 14 for details).

Statistical analysis

In all experiments, assays were performed in duplicate and empirical standard deviations were calculated to demonstrate reproducibility in parallel assays, but not for statistical analysis of variance, which would require more than two parallel assays. The experiments have been repeated at least twice (with duplicate assays). The Yates continuity corrected χ^2 -test was used for the statistical determination of significances ($p < 0.01$, significant; $p < 0.001$, highly significant). As rather broad concentration ranges are used in our studies, the presentation of the data requires semilogarithmic presentation in most figures.

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