Lactobacilli enhance reactive oxygen species-dependent apoptosis-inducing signaling

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1. Introduction

Infectious agents such as viruses, bacteria and parasites are linked to slightly more than 20% of the global cancer burden [1]. Distinct members of several virus groups, such as herpes viruses, papilloma viruses, retroviruses, hepatitis B and C viruses and others may contribute to oncogenesis directly and/or indirectly. They utilize mechanisms such as introduction of viral oncogenes, modification of proto oncogenes, induction of immunosuppression, prevention of apoptosis, induction of chromosomal instability or induction of reactive oxygen species (ROS) generation through establishment of chronic inflammation [1,2]. Helicobacter pylori (H. pylori), an outstanding bacterial carcinogen, is involved in the induction of gastric cancer and MALT lymphoma mainly through its induction of distinct ROS/RNS-related steps during tumor initiation and promotion/progression [3,4] reviewed in reference [5]. H. pylori induces an indirect prooxidative mechanism through attraction of neutrophils and by direction of their NOX2 assembly to the cell membrane (reviewed in reference [5]). In addition, H. pylori induces NOX1 expression in mucosal cells [6,7]. These H. pylori-mediated effects lead to a high concentrations of superoxide anions and their dismutation product H$_2$O$_2$, thus causing mutagenic effects that initiate malignant transformation. As H. pylori is protecting himself against high local concentrations of ROS through expression of SOD and catalase, it also has the potential to prevent elimination of transformed cells through ROS/RNS-dependent intracellular apoptosis-inducing signaling [8] and thus to contribute to tumor progression.

Whereas the mechanisms of prooncogenic effects of viruses and of H. pylori have been studied in detail and are well characterized, much less is known about the antioncogenic potential of microbes. The study of the role of probiotic bacteria for the prevention of colon cancer has led to the conclusion that the tumorpreventive effects of probiotic bacteria might be due to their control of the microbial flora, establishment of beneficial metabolic effects and stimulation of the immune system [9,10].

Lactobacilli are among the best studied microbes with probiotic effects. Lactobacilli are part of the normal oral and intestinal flora and represent the predominant microorganisms in the vaginal flora of healthy premenopausal women [11,12]. Lactobacilli adhere to epithelial cells and thus cause steric prevention of infection with undesirable
microorganisms. Lactobacilli cause low pH through production of lactate and also release bactericidal compounds [11,12]. The major antimicrobial activity of lactobacilli seems to depend on their production of H₂O₂ [13–17]. H₂O₂-producing strains of lactobacilli use a NADH oxidase that directly generates H₂O₂ in a two-electron reduction of O₂ (NADH + H⁺ + O₂ → NAD⁺ + H₂O₂) [18–20]. In the urogenital tract, the presence of lactobacilli seems to be essential for the suppression of other microbes [11–17,21,22]. The relevance of lactobacillus-derived H₂O₂ for the protective effect against other microbes has been demonstrated in vitro [23–26] and correlates well with the finding that 96% of healthy women, but only 6% of women with vaginosis, carry H₂O₂-producing lactobacilli [13,14]. Klebanoff et al. have demonstrated that the antimicrobial effect of H₂O₂-generating lactobacilli is efficiently enhanced in the presence of peroxides (such as myeloperoxidase and eosinophilic peroxidase) and halides [14]. This points to a role of HOCl as superior antimicrobial compound [27–30]. Importantly, as demonstrated by Klebanoff et al., the vaginal fluid of the majority of tested women contains sufficiently high concentration of peroxideid to allow biologically significant HOCI synthesis in the presence of H₂O₂-generating lactobacilli [14].

As the H₂O₂/peroxide/halide system had been shown to efficiently kill tumor cells [31–35], Klebanoff et al. suggested that H₂O₂ generating lactobacilli and peroxideid might not only control microbes, but also prevent tumorigenesis [14]. This idea is in line with the experimental demonstration of a potential antitumorigenic effect of lactobacilli [36–45]. It is also strengthened by epidemiological findings, showing that vaginal tumors, i.e. tumors at the site of massive colonization with lactobacilli, are extremely rare, whereas tumors in the neighbouring (bacillus-free) cervix occur frequently [46,47]. These findings are particularly intriguing, as both types of tumors are connected to infection with papilloma viruses.

Despite the long history of the knowledge of a potential tumor-preventive role of lactobacilli, the pioneering work of Klebanoff and the impact of lactobacillus-mediated control for the female human population, the exact mechanism of lactobacillus-mediated control of oncogenesis has not been unravelled in the past.

Our present knowledge on the role of NOX1 expression and extracellular superoxide anion generation by malignant cells [48–52], reviewed in references [53–55], on HOCI synthesis and on HOCI/superoxide anion interaction in biological systems, and on the role of intercellular ROS-mediated apoptosis-inducing signaling during the control of oncogenesis (reviewed in references [53–55] and presented more detailed under Supplementary Materials) allowed to readdress the important questions and suggestions originally raised by Klebanoff et al. [14].

Our experimental findings presented here demonstrate that lactobacilli-derived H₂O₂ is necessary, but not sufficient for selective elimination of malignant cells. Rather, the interaction between i) lactobacillus-derived H₂O₂, ii) peroxidaseid present in the vaginal fluid and iii) NOX1 expression by malignant cells seem to warrant selective elimination of malignant cells without harming normal tissue.

2. Materials and methods

2.1. Materials

The NOX1 inhibitor 4-(2-Aminothiophenyl)benzenesulfonyl fluoride (AEBSF), the catalase inhibitor 3-amino triazole (3-AT), catalase from bovine liver, the hydroxyl radical scavenger dimethylthiourea, glucose oxidase (GOX), the singlet oxygen scavenger histidine, the hydroxyl radical scavenger mannitol, myeloperoxidase (MPO), the NOS inhibitor L-arginine (L-NAME), the HOCI scavenger taurine, Mn-SOD from E. coli, were obtained from Sigma-Aldrich (Schnelldorf, Germany). The peroxidase inhibitor 4-Aminobenzoyl hydrazide (ABH) was obtained from Acros Organics (Geel, Belgium). Inhibitors for caspase-3 (Z-DEVD-FMK), caspase-8 (Z-IETD-FMK) and caspase-9 (Z-LEHD-FMK) were obtained from R&D Systems (Wiesbaden-Nordenstadt, Germany).

The peroxynitrite decomposition catalyst 5-, 10-, 15-, 20-Tetrakis(4-sulfonatophenyl)porphyrinato iron(III) chloride (FeTPPS) and the cell-permeable SOD mimetic Mn(III) 5,10,15,20-tetrakis(N-methylpyridinium-2-yl)porphyrin (MsTM-2PyP) were obtained from Calbiochem (Merck Biosciences GmbH, Schwalbach/Ts, Germany).

Transforming growth factor β1 (TGFβ1) was purified from human platelets [56] and kept as a stock solution of 1.5 µg/ml in Eagle’s Minimum Essential Medium (EMEM) plus 5% fetal bovine serum (FBS) at −20 °C.

Detailed information on inhibitors and scavengers has been presented in detail in the supplementary material of references 8 and 58.

2.2. Cells and media for cell culture

The human gastric adenocarcinoma cell line MKN-45 (ACC 409) (established from the poorly differentiated adenocarcinoma of the stomach (medullary type) of a 62 year-old woman) and the human HPV-18-positive cervix adenocarcinoma cell line SISO (ACC-327) were purchased from DSMZ, Braunschweig, Germany. Nontransformed 208 F rat fibroblasts and 208 F rat fibroblasts transformed through constitutive expression of v-src (“208 F src3”), have been established by and were a generous and valuable gift by Drs C. Sers and R. Schäfer, Berlin, Germany. 208Fsrc3 cells have been recently characterized with respect to intercellular ROS signaling [59]. MKN-45 were cultured in RPMI 1640 medium, containing 10% fetal bovine serum (FBS). Fetal bovine serum (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). Care was taken to avoid cell densities below 300,000/ml and above 10⁶/ml. SISO, 208 F and 208Fsrc3 cells cultivated as adherent cultures in Eagle’s Minimum Essential Medium (EMEM), supplemented with 5% heat-treated FBS, penicillin (40 U/ml), streptomycin (50 µg/ml), neomycin (10 µg/ml), moronal (10 U/ml) and glutamine (280 µg/ml).

2.2.1. Lactobacilli

The H₂O₂-producing strains L. gasseri, L. jensenii and L. acidophilus were obtained from Dr. A. Serr, Institute of Medical Microbiology, University Medical Centre, Freiburg. Lactobacilli were cultivated on yeast extract, cysteine, blood agar and were suspended in medium prior to use in experiments. Cell culture experiments in the presence of lactobacilli were performed in the absence of antibiotics.

3. Methods

3.1. Apoptosis induction

3.1.1. Autocrine apoptosis induction by intercellular ROS signaling

Cells in complete medium were seeded in 96-well tissue culture clusters at a standard density of 12 500 cells/100 µl or at densities stated in the respective figure legends. All assays were performed in duplicate. Assays were cultivated at 37 °C in the presence of 5% CO₂. Optimal autocrine apoptosis induction in transformed 208Fsrc3 cells required the addition of purified TGFβ1–1 (20 ng/ml). Without addition of TGFβ1–1 the kinetics of apoptosis induction was delayed. Nontransformed cells do not show autocrine ROS-mediated apoptosis induction.

Reactivation of intercellular apoptosis-inducing ROS signaling of bona fide tumor cells like MKN-45 required the inhibition of membrane-associated catalase by 3-amino triazole (3-AT). The concentrations used are indicated in the respective figures. Autocrine apoptosis...
induction in tumor cells did not require the addition of exogenous TGFβ-1, but was inhibited by siRNA-mediated knockdown of TGFβ-1 or TGFβ receptor (Bauer, submitted for publication).

3.2. Apoptosis induction mediated by H$_2$O$_2$-generating lactobacilli

Cells were seeded at the concentrations indicated in the respective figures in FBS-containing medium, containing glutamine but no antibiotics. Lactobacilli were added at the indicated concentrations and the duplicate assays were cultivated at 37 °C, 5% CO$_2$ for the indicated times. Where indicated, 200 nM MPO/ml or inhibitors had been added. Due to the contribution of H$_2$O$_2$ by the lactobacilli, the kinetics of apoptosis induction was enhanced compared to autocrine apoptosis induction.

3.3. Determination of the percentage of apoptotic cells

After the indicated time of incubation at 37 °C and 5% CO$_2$, 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 [8,60–62]. The characteristic morphological features of intact and apoptotic cells, as determined by inverted phase contrast microscopy have been published [8,59,60,63,64]. At least 200 neighbouring cells from randomly selected areas were scored for the percentage of apoptotic cells at each point of measurement. Control assays ensured that the morphological features 'nuclear condensation/fragmentation' as determined by inverse phase contrast microscopy were correlated to intense staining with bisbenzimide and to DNA strand breaks, detectable by the TUNEL reaction [51,59,63,64]. A recent systematic comparison of methods for the quantitation of apoptotic cells has shown that there is a perfect coherence between the pattern of cells with condensed/fragmented nuclei (stained with bisbenzimide) and TUNEL-positive cells in assays with substantial apoptosis induction, whereas there was no significant nuclear condensation/fragmentation in control assays [8,64]. Further controls ensured that ROS-mediated apoptosis induction was mediated by the mitochondrial pathway of apoptosis, involving caspase-9 and caspase-3 (64, Bauer, submitted for publication).

3.4. Knockdown by treatment with specific small interfering ribonucleic acids (siRNAs)

3.4.1. SiRNAs:

SiRNAs were obtained from Qiagen (Hilden, Germany)

The following siRNAs were used:

Control siRNA which does not affect any known target in human and murine cells (siCo):

- sense: r(UUCUCCGAACGUGUCAGCUCdTdT,
- antisense: r(ACGUGACACGUUCGGAGAA)dTdT.

SiRNAs directed towards NADPH oxidase-1 (NOX1):

- custom-made siRNA directed towards NADPH oxidase-1 variant a (siNOX1-a): target sequence: CCG ACA AAT ACT ACA CAA.
- sense: r(GAC AAA UAC UAC ACA A)dTdT,
- antisense: r(UUG UGU AGU AGU AUU UGU C)dGdG.

SiRNAs were dissolved in suspension buffer supplied by Qiagen at a concentration of 20 μM. 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 μl of medium without serum and without antibiotics were mixed with 12 μl of Hyperfect solution (Qiagen) and the required volume of specific siRNA or control siRNA to reach the desired concentration of siRNA during transfection (the standard concentration of siRNA was 10 nM for 208Fsrc3 cells). 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 200,000 208Fsrc3 cells/well in 2.3 ml medium supplemented with 5% FBS and antibiotics (6-well plates). The cells were incubated at 37 °C in 5% CO$_2$ for 24 h. Transfected cells were centrifuged and resuspended in fresh medium at the required density before use.

3.4.2. Determination of the efficiency of siRNA-mediated knockdown

The siRNA transfection system as described above had been optimized to allow a reproducible transfection efficiency of more than 95% of the cells and to avoid toxic effects (Bauer, unpublished data).

The efficiency of knockdown by siNOX1 was based on functional SOD-dependent quantitative assay [65,66] and was more than 90%.

3.5. Statistics

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

4. Results

4.1. Differential response of cells from distinct stages of tumor progression to lactobacilli, in the absence and presence of MPO

When nontransformed 208 F, src-oncogene-transformed 208Fsrc3 and MKN-45 human gastric carcinoma cells were incubated with increasing concentrations of H$_2$O$_2$-generating lactobacilli, apoptosis was induced very efficiently and nonselectively in transformed as well as nontransformed cells, whereas tumor cells were much less sensitive for lactobacillus-mediated apoptosis induction (Fig. 1). 2 500 bacilli/ml were sufficient for half-maximal apoptosis induction in nontransformed and transformed cells, whereas the tumor cells required a more than 30 fold higher concentration of lactobacilli to achieve the same effect. When myeloperoxidase (MPO) was applied together with lactobacilli, nontransformed cells were nearly completely protected against lactobacillus-mediated apoptosis induction, whereas at concentrations up to 10,000 lactobacilli /ml, transformed cells showed the same degree of apoptosis induction in the presence of MPO as in its absence. At higher concentrations of lactobacilli, the plateau type of response that was obtained in the absence of MPO, shifted to supraoptimal inhibition in its presence. Addition of MPO to the gastric carcinoma cells MKN-45 markedly shifted the concentration range of lactobacilli required for apoptosis induction to markedly lower concentrations.

The same striking effect of lactobacilli and MPO was seen for the human HPV-18 positive cervical carcinoma cell line SISO (Fig. 2). Similar to MKN-45 cells, SISO cells required relatively high concentrations of lactobacilli to achieve apoptosis induction. Three different H$_2$O$_2$-producing strains of lactobacilli, i.e., L. acidophilus, gasseri and jensenii caused a similar degree of apoptosis induction. In the presence of MPO, apoptosis induction in SISO cells by all three strains of lactobacilli seemed to become much more efficient and therefore required much lower concentrations of bacteria. Apoptosis induction by lactobacilli both in the absence and presence of MPO seemed to depend on HOCl, as seen by the inhibitory effect of taurine.
4.2. Elucidation of ROS signaling in the presence of lactobacilli

In order to understand the complex and divergent picture of response of nontransformed, transformed and tumor cells to lactobacilli in the absence or presence of additional MPO, inhibitor studies were performed for the three types of cells. As shown in Fig. 3, in the absence of MPO, lactobacillus-mediated apoptosis induction in nontransformed cells was inhibited by catalase, but not by taurine (a HOCl scavenger), MnSOD (an extracellular superoxide anion scavenger), mannitol (an extracellular hydroxyl radical scavenger) or FeTPPS (a cell-permeable peroxynitrite scavenger). These findings indicate that apoptosis induction by lactobacilli in nontransformed cells was dependent on H₂O₂, but not on ROS signaling via the HOCl or the NO/peroxynitrite pathway. Inhibition by the cell-permeable SOD mimetic MnTM-2-PyP and the cell-permeable hydroxyl radical scavenger DMTU indicates that H₂O₂-dependent intracellular Fenton chemistry (H₂O₂ + Fe²⁺ → OH⁻ + ·OH + Fe³⁺) might represent the ultimate apoptosis-inducing step in nontransformed cells, where DMTU scavenges intracellular hydroxyl radicals and MnTM-2-PyP scavenges intracellular superoxide anions. These may be necessary for reducing transformed cells was inhibited by catalase, but not by taurine (a HOCl scavenger), MnSOD (an extracellular superoxide anion scavenger), mannitol (an extracellular hydroxyl radical scavenger) or FeTPPS (a cell-permeable peroxynitrite scavenger). These findings indicate that apoptosis induction by lactobacilli in nontransformed cells was dependent on H₂O₂, but not on ROS signaling via the HOCl or the NO/peroxynitrite pathway. Inhibition by the cell-permeable SOD mimetic MnTM-2-PyP and the cell-permeable hydroxyl radical scavenger DMTU indicates that H₂O₂-dependent intracellular Fenton chemistry (H₂O₂ + Fe²⁺ → OH⁻ + ·OH + Fe³⁺) might represent the ultimate apoptosis-inducing step in nontransformed cells, where DMTU scavenges intracellular hydroxyl radicals and MnTM-2-PyP scavenges intracellular superoxide anions. These may be necessary for reducing
A. Apoptosis induction in nontransformed 208F cells (5,000 cells/100 µl complete medium) mediated by 10^4 L. gasseri/ml is inhibited by 60 U/ml catalase (CAT), 10 mM of the cell-permeable SOD mimetic MnTM-2-PyP, and 10 mM the cell permeable hydroxyl radical scavenger dimethylthiourea (DMTU), but not by 50 mM of the HOCl scavenger taurine (TAU), 100 U/ml of cell-impermeable MnSOD, 10 mM of the cell impermeable hydroxyl radical scavenger mannitol (MANN) or 25 µM FeTPPS. In the presence of lactobacilli and exogenous MPO (MANN) or 25 µM FeTPPS, apoptosis induction in 208F cells is inhibited by catalase, taurine (TAU), MnSOD and mannitol (MANN), indicating the HOCl signaling is responsible for cell death. Control: assays without lactobacilli and without inhibitors. B. Apoptosis induction in transformed 208Fsrc3 cells (5,000 cells/100 µl complete medium) mediated by 10^4 L. gasseri/ml is inhibited by 60 U/ml catalase, but not by 50 mM taurine (TAU), 100 U/ml MnSOD, 10 mM mannitol (MANN) or 25 µM FeTPPS. In the presence of lactobacilli and exogenous MPO, apoptosis induction in 208Fsrc3 cells is inhibited by catalase, taurine (TAU), MnSOD and mannitol (MANN), indicating the HOCl signaling is responsible for cell death. Control: assays without lactobacilli and without inhibitors. In both experiments, the percentages of apoptotic cells most likely exhibit the same mechanism of nonselective induction of apoptosis as H_2O_2 as nontransformed cells. However, in the presence of MPO, transformed cells most likely exhibit the same mechanism of nonselective induction of apoptosis by H_2O_2 as nontransformed cells. However, in the presence of MPO, apoptosis induction in transformed cells by lactobacilli seemed to depend on H_2O_2, as it was blocked by catalase. However, it did not depend on HOCl or peroxynitrite signaling, as taurine, MnSOD, mannitol and FeTPPS had no inhibitory effect. Therefore, in the absence of MPO, transformed cells most likely exhibit the same mechanism of nonselective induction of apoptosis by H_2O_2 as nontransformed cells. However, in the presence of MPO, lactobacilli-mediated apoptosis induction in transformed cells followed a completely different reaction mode as in its absence. It was also completely different from the reactions found for nontransformed cells. The complete inhibition by catalase, taurine, MnSOD and mannitol indicates that lactobacilli and MPO establish apoptosis induction in transformed cells through HOCl signaling. Extracellular superoxide anions as well as hydroxyl radicals seem to be crucial for apoptosis induction. MPO added without lactobacilli had no apoptosis-inducing effect, pointing to the role of MPO-dependent HOCl synthesis with H_2O_2 as substrate.

A higher degree of complexity of ROS signaling mediated by lactobacilli in the absence and presence of MPO was seen for tumor cells (Fig. 4). At lower cell density (40,000 cells/ml) and in the absence of MPO, relatively high concentrations of lactobacilli caused H_2O_2-dependent apoptosis induction in the tumor cells. This apoptosis induction was independent of HOCl or NO/peroxynitrite signaling, as neither taurine, SOD, FeTPPS or the singlet oxygen scavenger histidine caused an inhibitory effect. Apoptosis induction seemed to be mediated by H_2O_2, as exogenous catalase prevented apoptosis induction. However, in the presence of MPO, 40,000 MKN-45 cells/ml responded to increasing concentrations of lactobacilli through specific HOCl signaling, as seen by the complete inhibition by catalase, taurine, SOD and mannitol, i.e., scavengers that are indicative for the HOCl signaling pathway. Peroxynitrite as well as singlet oxygen played no role under these conditions.

When higher concentrations of MKN-45 cells (125,000 cells/ml instead of 40,000 cells/ml) were challenged with lactobacilli, their response required markedly higher concentrations of lactobacilli and seemed to follow a completely different pattern compared to the lower cell density. Apoptosis induction in 125,000 MKN-45 cells per ml by lactobacilli in the absence of MPO was dependent on singlet oxygen (as it was inhibited by histidine), peroxynitrite (seen by the complete inhibition mediated by FeTPPS) as well as the signaling components of the HOCl signaling pathway, i.e., hydrogen peroxide, HOCl, superoxide anions and hydroxyl radicals, as seen by the typical inhibitor profile. In the presence of MPO, apoptosis induction mediated by lactobacilli in 125,000 MKN-45 cells/ml was independent of the action of singlet oxygen and peroxynitrite, but was directly dependent on signaling via the HOCl signaling pathway, as indicated by the inhibitory effect of catalase, taurine, SOD and mannitol.

4.3. H_2O_2-generating glucose oxidase (GOX) mimics the effect of lactobacilli

In order to determine whether H_2O_2 was sufficient to explain the so far described effects of lactobacilli, 208Fsrc3 cells were treated with increasing concentrations of glucose oxidase (GOX), an enzyme that steadily generates H_2O_2. To test for the crucial role of target cell-derived extracellular superoxide anions in the interaction with HOCl, 208Fsrc3 control cells and cells with siRNA-mediated knockdown of NOX-1 were used. With respect to extracellular superoxide anion generation, 208Fsrc3 cells with knockdown of NOX1 thus showed the phenotype of non-malignant cells. Where indicated, assays received MPO in addition. As can be seen in Fig. 5, GOX mimicked the activity...
of lactobacilli in its ability to induce apoptosis in 208Fsrc3 cells. Functional NOX-1 was not necessary for the direct apoptosis inducing effect when GOX was applied alone. In the presence of MPO, transformed control cells showed apoptosis induction following an optimum curve. Apoptosis induction in the range of the optimum curve was dependent on cell-derived superoxide anions as it was absent in cells with siRNA-mediated knockdown of NOX-1. These data show that the effect of lactobacilli on transformed cells, both in the absence and presence of MPO can be completely mimicked by H$_2$O$_2$ steadily produced by GOX. It is also seen from this experiment that active NOX-1 is crucial for HOCl-mediated apoptosis induction in transformed cells. 208Fsrc3 cells with knockdown of NOX1 were equivalent to the nontransformed phenotype and therefore responded to H$_2$O$_2$, but not to HOCl that was generated through the interaction between H$_2$O$_2$ and MPO.

The potential of GOX to completely substitute for the lactobacillus effect was also confirmed for tumor cells (Supplementary Fig. 3). Further details of the interaction between GOX and tumor cells at high cell density are shown and discussed in Supplementary Figs. 4 and 5.

Taken together, the response of the tumor cells to GOX was indistinguishable to that to lactobacilli, indicating that lactobacilli-derived hydrogen peroxide was necessary and sufficient to explain the effects of lactobacilli on apoptosis induction. The crucial role of NOX1 for HOCl-mediated apoptosis induction in tumor cells was also demonstrated.

5. Discussion

Our data show that H$_2$O$_2$-generating lactobacilli establish and modulate complex and differential ROS-mediated apoptosis inducing
signaling of cells from distinct stages of multistep oncogenesis. All contributions of lactobacilli to the apoptosis-related signaling effects analyzed in this study can be completely attributed to H2O2 generated by the bacteria, as 1) catalase efficiently blocked the lactobacilli-mediated effects and 2) H2O2-generating GOX completely mimicked the bacterial effects in model experiments.

Importantly, lactobacillus-derived H2O2 is shown to be necessary, but not to be sufficient for selective apoptosis induction in malignant cells. Rather, lactobacillus-derived H2O2 caused apoptosis nonselectively in nontransformed and transformed cells, most likely through intracellular Fenton chemistry (Figs. 6A, 7A). Membrane-associated catalase of tumor cells counteracted the apoptosis-inducing effect (Fig. 8A), therefore apoptosis induction in tumor cells by H2O2 required higher concentrations of lactobacilli than being effective on non-malignant and transformed cells. It is obvious therefore, that H2O2 generation by lactobacilli per se would have no beneficial effect for the vaginal epithelium and also would not explain the tumor preventive effect of lactobacilli. This conclusion is in line with findings of nonselective induction of apoptosis in nontransformed and transformed cells by direct application of H2O2 in vitro [67,68].

However, the physiological situation described by Klebanoff et al. [14], i.e. the combination of H2O2-generating lactobacilli in the presence of substantial concentrations of peroxidase, allows for selective antioncogenic action. As outlined in Figs. 6–8B, in the presence of peroxidase H2O2 is converted into HOCl (4). As nontransformed cells lack extracellular superoxide anion generation, HOCl (in the concentration range relevant for these studies) has no effect on non-malignant cells, as seen by the protective effect of MPO in the presence of lactobacilli (Fig. 1A).
**H2O2 and peroxidase are available at su**

MPO can be predicted to act also on individual transformed cells, as decomposed by membrane-associated catalase (#9). The ratio between tumor cells and cells [66,70], the e

signaling pathway requires high local density of transformed target 2). Moreover, whereas autocrine apoptosis signaling through the HOCl DUOX-coded peroxidase themselves (see Supplementary Figures 1, in the vaginal

signaling of transformed cells which need to generate H2O2 and catalase is used a substrate for the synthesis of HOCl. The interaction between HOCl and hydroxyl radicals (#11) which cause lipid peroxidation (#12) and apoptosis induction

is thus confined to the membrane of the superoxide anion-generating malignant cells. This warrants the selectively of apoptosis induction for malignant cells.

isotoxin is generated in the vaginal fluid, derived from lactobacilli. This antimicrobial effect is established by lactobacilli in the presence of MPO. This may be predicted to act also on individual transformed cells, as H2O2 and peroxidase are available at sufficient concentration in the vaginal fluid. Also, though bona fide tumor cells are efficiently protected against the establishment of HOCl signaling in an autocrine mode [57,60], they are susceptible to HOCl-mediated apoptosis induction in the vaginal milieu, as they cannot efficiently counteract HOCl synthesis by lactobacillus-derived H2O2 and peroxidase, and as catalase cannot protect them against distantly generated HOCl. Only high concentrations of tumor cells may have a modulatory counter-action towards apoptosis induction. Thus, it may be concluded that the control system consisting of H2O2 - generating lactobacillus and peroxidase in the vaginal fluid may eliminate malignant cells from early and late stages of oncogenesis, independent of their establishment of a protective system through membrane-associated catalase and SOD. The potential ROS-based antitumor effect of H2O2-producing lactobacilli might be complemented by immunostimulatory effects of lactobacterial components [73,74], leading to further efficiency of this biological tumor-preventive system. These mechanisms might explain why vaginal tumors are rare, despite the driving oncogenic potential of papilloma viruses [46].

One might expect that continuous generation of H2O2 by lactoba-
cillus might finally lead to concentrations of H2O2 that are damaging to the vaginal epithelium. One might also assume that the conversion of H2O2 to HOCl through the action of peroxidase might finally lead to toxic concentrations of HOCl. This is obviously not the case under physiological conditions. This finding is explained by the consumption reaction between H2O2 and HOCl (H2O2 + HOCl → O2 + H2O + H++ Cl−) [75,76]. This reaction thus seems to balance the concentrations of H2O2 and HOCl. As the reaction between HOCl and superoxide anions (k=10^7 M−1 s−1) is two orders of magnitude faster than the interaction between HOCl and H2O2 (k=10^4 M−1 s−1), this balancing consumption reaction would, however, not interfere with selective apoptosis induction of superoxide anion-producing malignant cells. The reaction product singlet oxygen (1O2) that is derived from the interaction between H2O2 and HOCl has recently been shown not to be damaging to non-malignant cells (provided it is supplied extracellulary) [77], but to efficiently inactivate membrane-associated catalase of tumor cells. Therefore, singlet oxygen generated in the vaginal fluid through H2O2/HOCl interaction might contribute to sensitization of tumor cells for ROS-dependent apoptosis induction and thus establish an additional antitumor effect.

Klebanoff’s suggestion that H2O2 generating lactobacilli and peroxidase might not only control microbes, but also prevent tumorigenesis [14] was based on the finding that HOCl (generated through the interaction between lactobacilli and peroxidase) seemed to be a superior antimicrobial compound [27–30] and that the H2O2/peroxidase/halide system efficiently kills tumor cells [31–35].

It may be concluded that the antimicrobial and the antitumoral effect of the lactobacillus/peroxidase system both depend on HOCl synthesis through the utilization of lactobacillus-derived H2O2 as substrate for HOCl synthesis by peroxidase. However, it seems likely that the final controlling steps are different. Whereas selective apoptosis induction of malignant cells is based on hydroxyl radical generation through the interaction between HOCl and NOX1-derived superoxide anions, an analogous reaction for intruding bacteria is not known, as there is no indication for the generation of superoxide anions on the outside of bacteria. But it seems to be likely that singlet oxygen, derived from the continuously ongoing consumption reaction between H2O2 and HOCl (H2O2 + HOCl → O2 + H2O + H++ Cl−) [75,76] may be acting effectively against bacteria, as singlet oxygen has been shown to be 10 000 fold more active against bacteria compared to H2O2 [78–80]. This strong antibacterial activity of singlet oxygen might be explained by the inactivating effect of singlet oxygen on the bacterial respiratory chain [81].

Based on the available experimental and epidemiological data, the lactobacillus/peroxidase system seems to represent an effective redox-related control system directed towards tumorigenesis. NOX1-derived superoxide anions, the hallmark of the malignant state of cells, thereby plays the determining role. These findings are also encouraging for therapeutic approaches that try to instrumentalize the specific ROS chemical biology of tumor cells [55,82–84].
J. Jürgensmeier, C.P. Schmitt, E. Viesel, P. Hö
S. Elmore, Apoptosis: a review of programmed cell death, Toxicol. Pathol. 35 (2007) 495–4557.

G. Bauer, N. Zarkovic, Revealing mechanisms of selective, concentration-dependent potentials of 4-hydroxy-2-nonenal to induce apoptosis in cancer cells through inactivation of membrane-associated catalase, Free Radic. Biol. Med. 81 (2015) 128–144.

E. Beck, R. Schäfer, G. Bauer, Sensitivity of transformed fibroblasts for intercellular induction of apoptosis is determined by their transformed phenotype, Exp. Cell Res. 234 (1997) 47–56.

S. Heinzelmann, G. Bauer, Multiple protective functions of catalase against intercellular apoptosis-inducing ROS signaling of human tumor cells, Biol. Chem. 391 (2010) 675–693.

J.F.R. Kerr, A.H. Wyllie, A.R. Currie, Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics, Br. J. Cancer 26 (1972) 239–257.

S. Elmore, Apoptosis: a review of programmed cell death, Toxicol. Pathol. 35 (2007) 495–515.

J. Jürgensmeier, C.P. Schmitt, E. Viesel, P. Höfner, G. Bauer, TGF-β-treated normal fibroblasts eliminate transformed fibroblasts by induction of apoptosis, Cancer Res. 54 (1994) 393–398.

K. Schett, G. Bauer, Direct and indirect inactivation of tumor cell protective catalase by salicylic acid and anthocyanidins reactivates intercellular ROS signaling and allows for synergistic effects, Carcinogenesis 36 (2015) 400–411.

J. Temne, G. Bauer, Low-dose gamma irradiation enhances superoxide anion production by nonirradiated cells through TGF-β1-dependent bystander signaling, Rad. Res. 179 (2013) 422–432.

S. Potgiesser, S. Heinzelmann, G. Bauer, Intercellular HOCl-mediated apoptosis induction in malignant cells: interplay between NOX1-dependent superoxide anion generation and DUOX-related HOCl-generating peroxidase activity, Anticancer Res. 35 (2015) 5927–5943.

B. Ivanovas, G. Bauer, Selective and nonselective apoptosis induction in transformed and nontransformed fibroblasts by cyanogen reactive oxygen and nitrogen species, Anticancer Res. 22 (2002) 841–856.

B. Böhm, S. Heinzelmann, M. Motz, G. Bauer, Extracellular localization of catalase is associated with the transformed state of malignant cells, Biol. Chem. 396 (2015) 1339–1356.

I. Engelmann, S. Dormann, M. Saran, G. Bauer, Transformed target cell-derived superoxide anions drive apoptosis induction by myeloperoxidase, Redox Rep. 5 (2000) 207–214.

M. Hesdener, S. Heigold, M. Saran, G. Bauer, Target cell-derived superoxide anions cause efficiency and selectivity of intercellular induction of apoptosis, Free Radical Biology & Medicine 29 (2000), 2000, pp. 1260–1271.

L.P. Candeias, K.B. Patel, M.R.L. Stratford, P. Wardman, Free hydroxyl radicals are formed on reaction between the neutrophil-derived species superoxide anion and hypochlorous acid, FEBS 333 (1993) 151–153.

J.K. Felder, L.P. Candeias, P. Wardman, Kinetics and mechanisms of hypochlorous acid reactions, Arch. Biochem. Biophys. 323 (1995) 120–126.

R. Shin, Y. Itoh, M. Kataoka, S. Ino-Miura, R. Miura, T. Miuntani, T. Fujisawa, Anti-tumor activity of heat-killed lactobacillus plantarum BF-LP284 on Meth-A tumor cells in BALB/c mice, Int. J. Food Sci. Nutr. 67 (2016) 641–649.

A. Abedin-Do, Z. Taherian-Esfahani, S. Ghasfouri-Fard, E. Motuevasi, Immunomodulatory effects of lactobacillus strains: emphasis on their effects on cancer cells, Immunotherapy 7 (2015) 1307–1329.

R.E. Connick, The interaction of hydrogen peroxide and hypochlorous acid in acidic solutions containing chloride ion, J. Am. Chem. Soc. 69 (1947) 1509–1514.

A.M. Held, D.J. Hallo, J.K. Hurst, Mechanisms of chlorine oxidation of hydrogen peroxide, J. Am. Chem. Soc. 100 (1978) 5732–5740.

M. Riehmuller, N. Burger, G. Bauer, Singlet oxygen treatment of tumor cells triggers extracellular singlet oxygen generation, catalase inactivation and reactivation of intercellular apoptosis-inducing signaling, Redox Biol. 5 (2015) 157–168.

T.A. Dahl, W.R. Middenazd, P.E. Hartman, Pure singlet oxygen cytotoxicity for bacteria, Photochemistry and Photobiology 46 (1987), 1987, pp. 345–352.

T. Maisch, J. Baier, B. Franz, M. Maier, M. Landthaler, R.-M. Szemies, W. Baumler, The role of singlet oxygen and oxygen concentration in photodynamic inactivation of bacteria, Proc. Natl. Acad. Sci. USA 104 (2007) 7223–7228.

M. Schafer, C. Schmitz, G. Horneck, High sensitivity of Deinococcus radiodurans to photodynamically-produced singlet oxygen, Int. J. Rad. Biol. 74 (1998) 249–253.

H. Tatsuzawa, T. Marsyama, N. Misawa, K. Fujimori, K. Hori, Y. Sano, Y. Kambayashi, M. Nakano, Inactivation of bacterial respiratory chain enzymes by singlet oxygen, FEBS Lett. 439 (1998) 329–333.

G. Bauer, The antitumor effect of singlet oxygen, Anticancer Res. 36 (2016) 5649–5664.