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Research Paper

Lactobacilli enhance reactive oxygen species-dependent apoptosis-inducing signaling

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ABSTRACT

H_2O_2-producing lactobacilli in the vaginal fluid have been suggested to play a potential tumor-preventive role in addition to the control of undesirable microorganisms. As the vaginal fluid also contains a significant concentration of peroxidase that might utilize lactobacilli-derived H_2O_2 as substrate for HOCl synthesis, a dominant biological role of HOCl in both natural defence systems has been postulated.

Our study shows that lactobacillus-derived H_2O_2 per se is not likely to be beneficial for the vaginal epithelium, as it causes apoptosis nonselectively in nontransformed as well as transformed cells. However, the combination of lactobacilli and peroxidase, i.e. the situation that is actually found in vivo, leads to the conversion of H_2O_2 to HOCl which does not affect non-malignant cells, as these do not generate extracellular superoxide anions. In contrast, malignant cells, due to their abundant extracellular superoxide anion generation allow the generation of apoptosis-inducing hydroxyl radicals through HOCl/superoxide anion interaction. In total, our data show that the combination of H_2O_2 -generating lactobacilli and peroxidase causes the selective elimination of malignant cells and thus might contribute to the tumorpreventive potential of lactobacilli. These findings are in good agreement with epidemiological data. The contribution of lactobacilli in this system can be completely mimicked by H_2O_2-generating glucose oxidase, indicating that it is fully explained by bacterial generation of H_2O_2.

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_2O_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 intercellular 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

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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 H_2O_2 [13–17]. H_2O_2-producing strains of lactobacilli use a NADH oxidase that directly generates H_2O_2 in a two-electron reduction of O_2 ($NADH + H^+ + O_2 \rightarrow NAD^+ + H_2O_2$) [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 H_2O_2 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 H_2O_2-producing lactobacilli [13,14]. Klebanoff et al. have demonstrated that the antimicrobial effect of H_2O_2-generating lactobacilli is efficiently enhanced in the presence of peroxidases (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 peroxidase to allow biologically significant HOCl synthesis in the presence of H_2O_2-generating lactobacilli [14].

As the H_2O_2/peroxidase/halide system had been shown to efficiently kill tumor cells [31–35], Klebanoff et al. suggested that H_2O_2 generating lactobacilli and peroxidase 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 tumorpreventive 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 HOCl synthesis and on HOCl/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 H_2O_2 is necessary, but not sufficient for selective elimination of malignant cells. Rather, the interaction between i) lactobacillus-derived H_2O_2, i) peroxidase present in the vaginal fluid and i) 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-Aminoethyl)benzenesulfonyl fluoride (AEBSF), the catalase inhibitor 3-aminotriazole (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 N-omega-nitro-L-arginine methylester hydrochloride (L-NAME), the HOCl 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 (MnTM-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 has been previously published [8,51,57,58]. The site of action of 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 10^6/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 H_2O_2-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% CO_2. Optimal autocrine apoptosis induction in transformed 208Fsrc3 cells required the addition of purified TGFß−1 (20 ng/ml). Without addition of TGFß−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-aminotriazole (3-AT). The concentrations used are indicated in the respective figures. Autocrine apoptosis

induction in tumor cells did not require the addition of exogenous TGFß−1, but was inhibited by siRNA-mediated knockdown of TGFß−1 or TGFß receptor (Bauer, submitted for publication).

3.2. Apoptosis induction mediated by H_2O_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 mU MPO/ml or inhibitors had been added. Due to the contribution of H_2O_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(UUCUCCGAACGUGUCACGU)dTdT,

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 ACT ACA CAA.

sense: r(GAC AAA UAC 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 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_2O_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_2O_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.

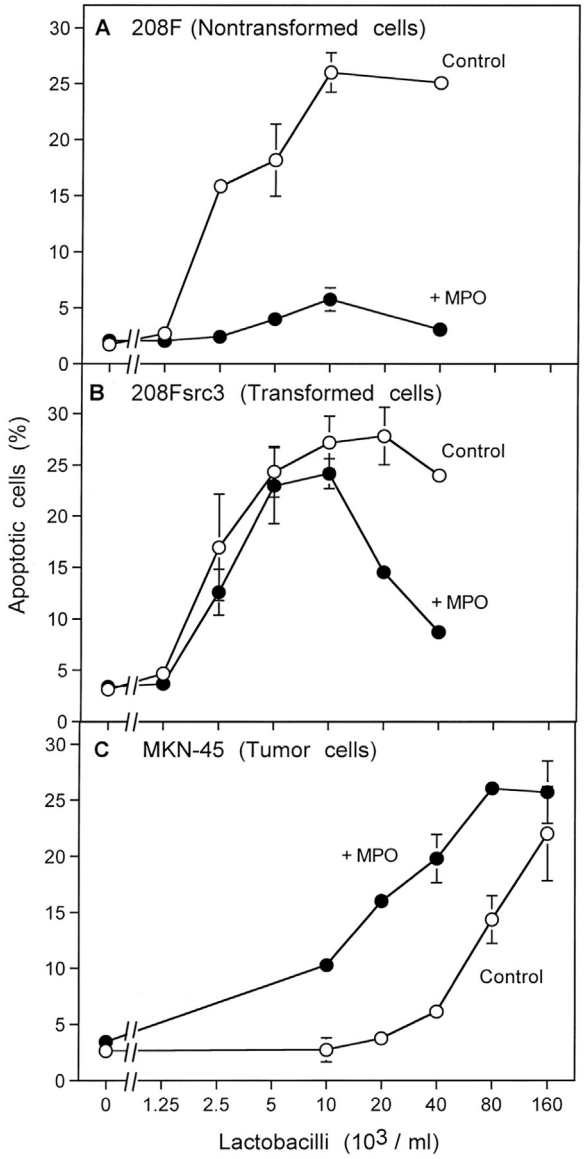

Fig. 1. Differential apoptotic response of nontransformed, transformed and tumor cells to increasing concentrations of lactobacilli in the absence or presence of additional MPO. Nontransformed 208 F cells and transformed 208Fsrc3 cells were seeded as monolayers in 96 well plates (6 000 cells/100 µl complete medium), and MKN-45 tumor cells were seeded in suspension (12 500 cells/100 µl). Assays received increasing concentrations of L. gasseri, in the absence or presence of 200 mU/ml MPO. Apoptosis induction by lactobacilli in the absence of MPO was much more efficient in nontransformed and transformed cells compared to tumor cells. The addition of MPO abrogated the apoptosis-inducing effect of lactobacilli on nontransformed cells (A), changed the characteristics of the response curve for transformed cells (B) and enhanced apoptosis induction in tumor cells (C).The percentages of apoptotic cells were determined at 3 h. Statistical analysis: A: Apoptosis induction in the presence of lactobacilli ($2.5 \times 10^3 - 40 \times 10^3$) and the inhibitory effect of MPO on apoptosis induction were highly significant ($p < 0.001$). B: Apoptosis induction in the presence of lactobacilli ($2.5 \times 10^3 - 40 \times 10^3$) and the inhibitory effect of MPO (in the concentration range of $20 \times 10^3 - 40 \times 10^3$ lactobacilli/ml) were highly significant ($p < 0.001$). C: Apoptosis induction in the presence of lactobacilli ($80 \times 10^3 - 160 \times 10^3$) and the enhancing effect of MPO on lactobacillus-mediated apoptosis induction were highly significant ($p < 0.001$).

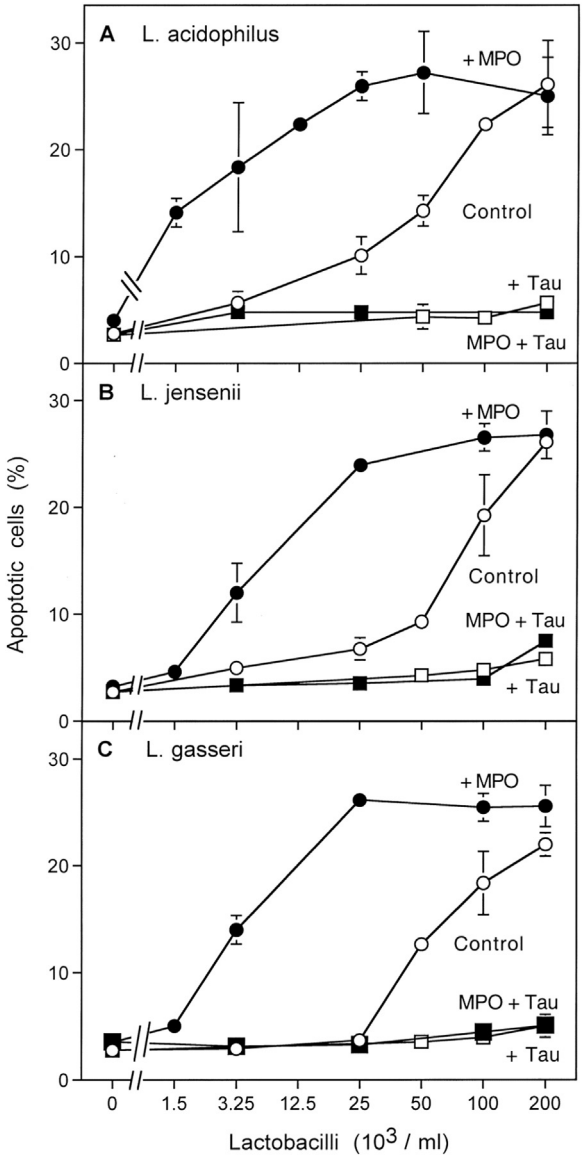

Fig. 2. Apoptosis induction in human SISO tumor cells by increasing concentrations of L. acidophilus (A), L. jensenii (B) and L. gasseri (C), in the absence or presence of MPO. Human SISO cervical carcinoma cells (10 000 cells/100 µl complete medium in 96 well plates) received the indicated concentrations of lactobacilli, in the absence or presence of 200 mU/ml MPO. Where indicated, 50 mM of the HOCl scavenger taurine (TAU) had been added in addition. The characteristics of apoptosis induction by all three strains of lactobacilli shows similar characteristics with regard to dose response, enhancement by MPO and inhibition by the HOCl scavenger taurine (TAU). The percentages of apoptotic cells had been determined after 4 h. Statistical analysis: A-C: Apoptosis induction by all three strains of lactobacilli ($100 \times 10^3 - 200 \times 10^3$), the enhancing effect of MPO and the inhibitory effect of taurine were highly significant ($p < 0.001$).

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 and presence of 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 non-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 H_2O_2, 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 H_2O_2-dependent intracellular Fenton chemistry ($H_2O_2 + Fe^{++} \rightarrow OH^- + \cdot OH + Fe^{+++}$) 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

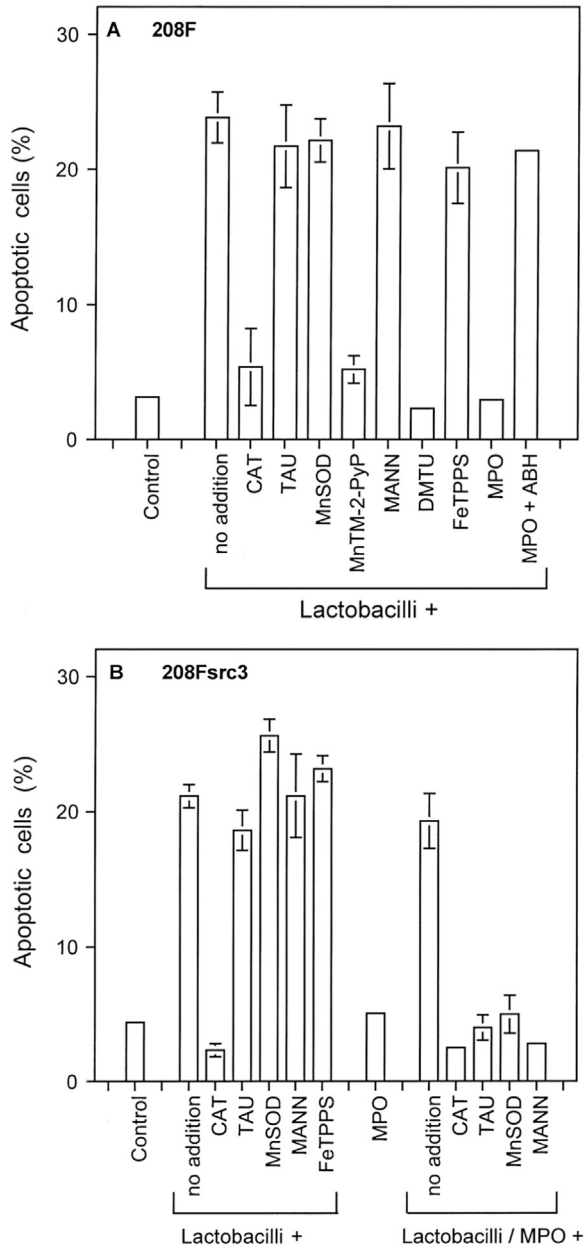

Fig. 3. ROS signaling mediated by lactobacilli. A. Apoptosis induction in nontransformed 208F cells (5 000 cells/100 µl complete medium) mediated by 10^4 L. gasseri/ml is inhibited by 60 U/ml catalase (CAT), 10 µM of the cell-permeable SOD mimetic MnTM-2-PyP, and 10 mM the cell permeable hydroxyl radical scavenger dimethylthio urea (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) and 25 µM of the peroxynitrite decomposition catalyst FeTPPS. MPO (200 mU/ml) abrogates apoptosis induction by lactobacilli in an enzymatic reaction that is blocked by 150 µM of the peroxidase inhibitor ABH. 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 had been determined at 2.5 h. Statistical analysis: A: Apoptosis induction by lactobacilli, inhibition of apoptosis induction by catalase, MnTM-2-PyP, DMTU, MPO, as well as abrogation of MPO-dependent inhibiton through ABH were highly significant ($p < 0.001$). B: Apoptosis induction by lactobacilli, in the absence and presence of MPO was highly significant ($p < 0.001$). Inhibition of lactobacilli-mediated apoptosis induction by catalase, as well as inhibition of apoptosis induction by lactobacilli in the presence of MPO by catalase, taurine, SOD and mannitol were highly significant ($p < 0.001$).

metal 3+ ions back to the metal 2+ form ($Fe^{+++} + O_2^{\cdot -} \rightarrow Fe^{++} + O_2$) that is required for the Fenton reaction. Addition of MPO completely blocked lactobacillus-mediated apoptosis induction in nontransformed cells. This inhibitory effect was due to the specific enzymatic activity of MPO, as it was abrogated by the mechanism-based peroxidase inhibitor ABH. In the absence 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 dependent 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, lactobacillus-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 lactobacillus 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, 208Fscr3 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

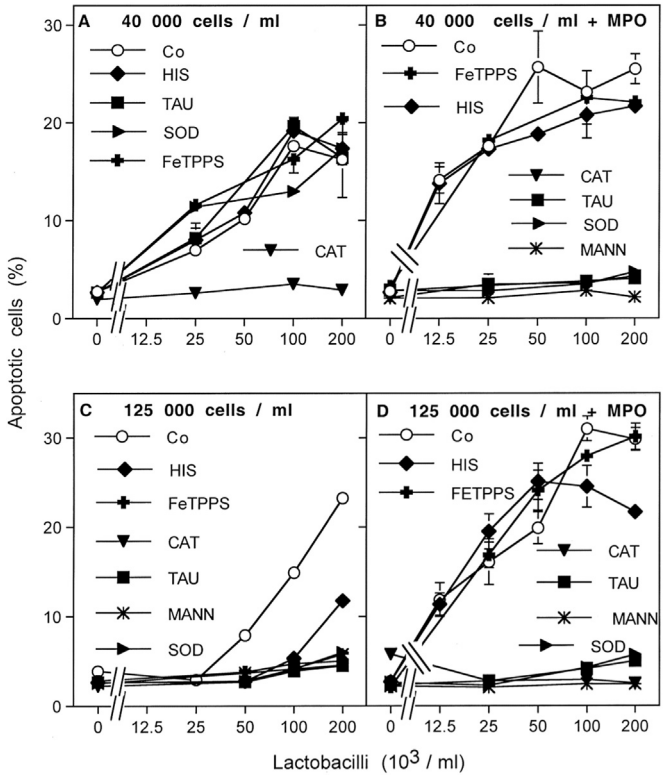

Fig. 4. Differential apoptosis induction mediated by L. gasseri in MKN-45 tumor cells at low (A,B) and high (C,D) cell density, in the absence (A,C) or presence (B,D) of MPO. MKN-45 tumor cells at the indicated densities (96 well plate, 100 µl complete medium) received the indicated concentrations of L. gasseri in the absence of presence of 200 mU/ml MPO. Where indicated, assays received 2 mM histidine (HIS), 50 mM taurine (TAU), 100 U/ml MnSOD (SOD), 25 µM FeTPPS, 60 U/ml catalase (CAT), 10 mM mannitol (MANN). Control assays (Co) were free of inhibitors. The percentages of apoptotic cells were determined after 4 h. A. Lactobacillus-mediated apoptosis induction is due to the effect of H_2O_2 without involvement of singlet oxygen, HOCl or NO/peroxynitrite signaling. B. In the presence of MPO and tumor cells at low cell density, apoptosis induction is mediated by HOCl signaling without requirement for the action of singlet oxygen or peroxynitrite. C. At high cell density and the absence of MPO, apoptosis induction requires singlet oxygen, peroxynitrite, H_2O_2, HOCl, hydroxyl radicals and superoxide anions. D. At high cell density and the presence of MPO, apoptosis induction does not require singlet oxygen and peroxynitrite, but seems to be due to HOCl signaling that involves H_2O_2, HOCl, hydroxyl radicals and superoxide anions as it is inhibited by catalase, taurine, mannitol and SOD. Statistical analysis: A: Apoptosis induction by lactobacilli (50×10^3 – 200×10^3) and inhibition by catalase were highly significant ($p < 0.001$). B: Apoptosis induction by lactobacilli (12.5×10^3 – 200×10^3) and inhibition by catalase, taurine, SOD and mannitol were highly significant ($p < 0.001$). Apoptosis induction by lactobacilli (100×10^3 – 200×10^3) and inhibition by all inhibitors were highly significant ($p < 0.001$). D: Apoptosis induction by lactobacilli (12.5×10^3 – 200×10^3) and inhibition by catalase, taurine, mannitol and SOD were highly significant ($p < 0.001$).

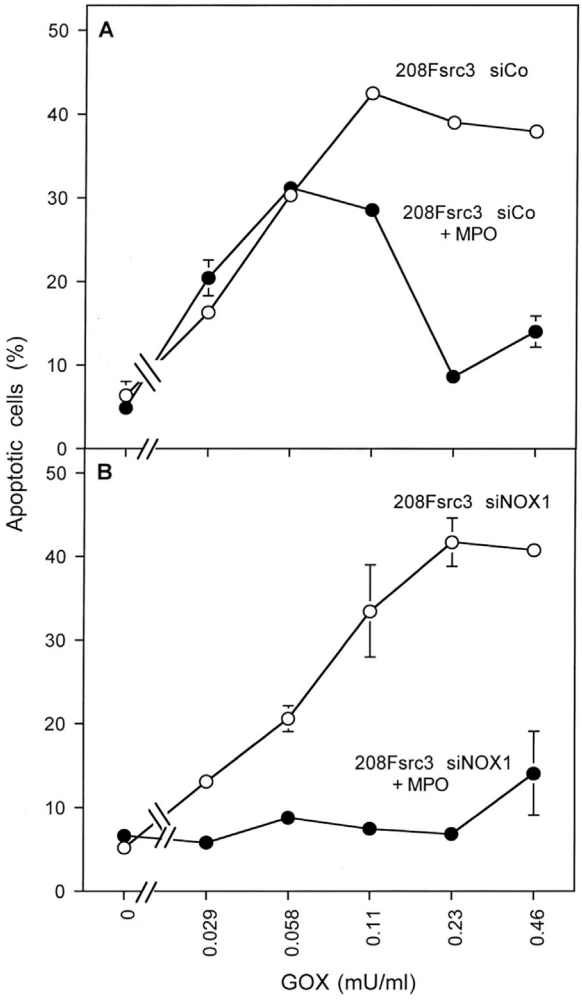

Fig. 5. Apoptosis induction mediated by glucose oxidase (GOX) in transformed 208Fsrc3 cells treated with control siRNA (siCo) or after siRNA-mediated knockdown of NOX1 (siNOX1). 208Fsrc3 cells treated with 24 nM control siRNA (siCO) or siRNA directed towards human NOX1 (siNOX1) for 24 h were seeded at a density of 6000 cells/100 µl. The assays received the indicated concentrations of GOX, both in the absence or presence of 200 mU/ml MPO. The percentages of apoptotic cells were determined after 1.5 h. A. H_2O_2-generating GOX mimicks the effect of lactobacilli on transformed cells (in analogy to the result shown in Fig. 3B), both in the absence and presence of MPO. B. After knockdown of NOX1 the response of 208Fsrc3 cells to GOX is analogous to the effect of lactobacilli to nontransformed cells (both in the absence and presence of MPO, in analogy to the result shown in Fig. 3A). Statistical analysis: A: Apoptosis induction by GOX (0.029 – 0.46 mU/ml) and the inhibitory effect of MPO (at 0.11 – 0.46 mU/ml GOX) were highly significant ($p < 0.001$).B: Apoptosis induction by GOX (0.058 – 0.46 mU/ml) and the inhibitory effect of MPO were highly significant ($p < 0.001$).

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_2O_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_2O_2, but not to HOCl that was generated through the interaction between H_2O_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_2O_2-generating lactobacilli establish and modulate complex and differential ROS-mediated apoptosis inducing

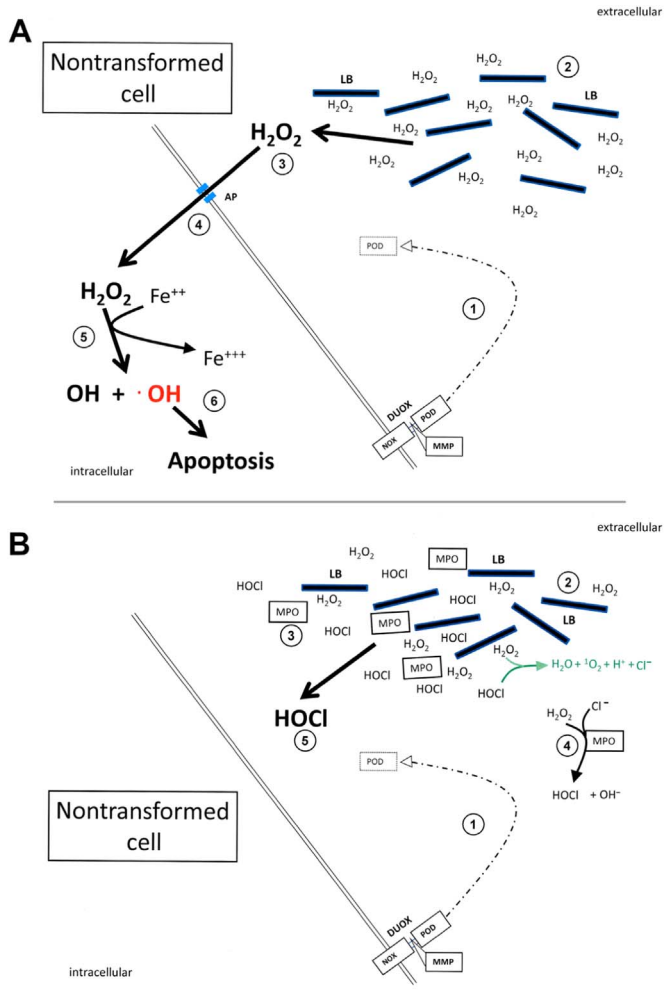

Fig. 6. Interaction of lactobacilli with nontransformed cells. A. The cells release the peroxidase domain of DUOX (POD) (references 66, 85) at concentrations too low to affect this system (#1). Lactobacilli (LB) (#2) generate abundant H_2O_2 (#3) which enters the cells through aquaporins (AP) (#4) (references 86, 87). Intracellular Fenton chemistry (#5) leads to the generation of apoptosis-inducing hydroxyl radicals (#6). The resultant ferric iron can be recycled to ferrous irion through intracellular superoxide anions (not shown in the Figure).B. In the presence of MPO (#3), lactobacillus-derived H_2O_2 (#2) 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).

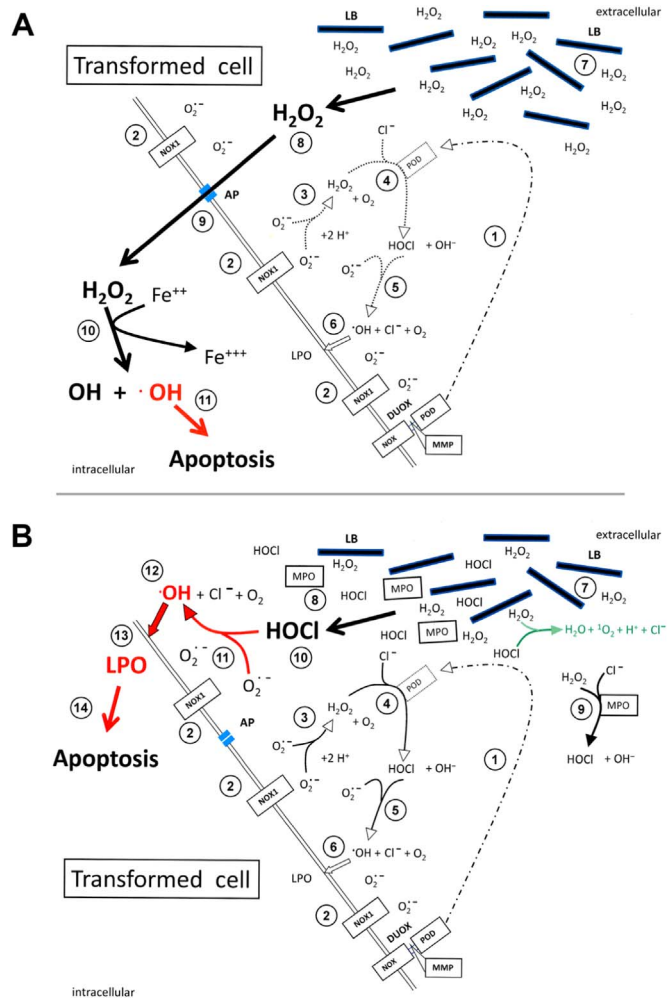

Fig. 7. Interaction of lactobacilli with transformed cells. A. Peroxidase (POD) released from DUOX (#1) and NOX1 (#2) cooperate in HOCl signalling. NOX1-derived extracellular superoxide anions dismutate to H_2O_2 (#3), which is used by POD as substrate for HOCl synthesis (#4). HOCl interacts with superoxide anions (#5), generating hydroxyl radicals (#6) that cause lipid peroxidation, followed by apoptosis. However, this process requires a sufficient density of transformed cells and is relatively slow (see Supplementary Fig. 1 for details). Lactobacilli (LB) (#7) generate H_2O_2 in abundance (#8) which penetrates into the cells through aquaporins (#9) and causes intracellular Fenton chemistry (#10) and subsequent apoptosis induction (#11). B. In the presence of MPO (#8), lactobacillus-derived H_2O_2 (#7) is used as substrate for HOCl synthesis by MPO (#9). HOCl (#10) then interacts with NOX1-derived superoxide anions (#11), leading to the formation of hydroxyl radicals (#12) that cause lipid peroxidation (#13) and apoptosis induction through the mitochondrial pathway (#14).

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 H_2O_2 generated by the bacteria, as i) catalase efficiently blocked the lactobacilli-mediated effects and 2) H_2O_2-generating GOX completely mimicked the bacterial effects in model experiments.

Importantly, lactobacillus-derived H_2O_2 is shown to be necessary, but not to be sufficient for selective apoptosis induction in malignant cells. Rather, lactobacillus -derived H_2O_2 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 H_2O_2 required higher concentrations of lactobacilli than being effective on non-malignant and transformed cells. It is obvious therefore, that H_2O_2 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 H_2O_2 in vitro [67,68].

However, the physiological situation described by Klebanoff et al. [14], i. e. the combination of H_2O_2-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 H_2O_2 is converted into HOCl. As can be deduced from the experimental findings, the concentrations of HOCl reached are not affecting non-malignant cells. This is due to the lack of sustained extracellular superoxide anion generation by nontransformed cells (Fig. 6B) and in line with previous work [69,70]. However, NOX1-derived extracellular superoxide anions of malignant cells (Figs. 7 and 8B) readily interact with HOCl and generate apoptosis-inducing hydroxyl radicals [70–72]. Due to the relatively short free diffusion path length of superoxide anions and the extreme short diffusion path length of hydroxyl radicals, generation and action of hydroxyl radicals

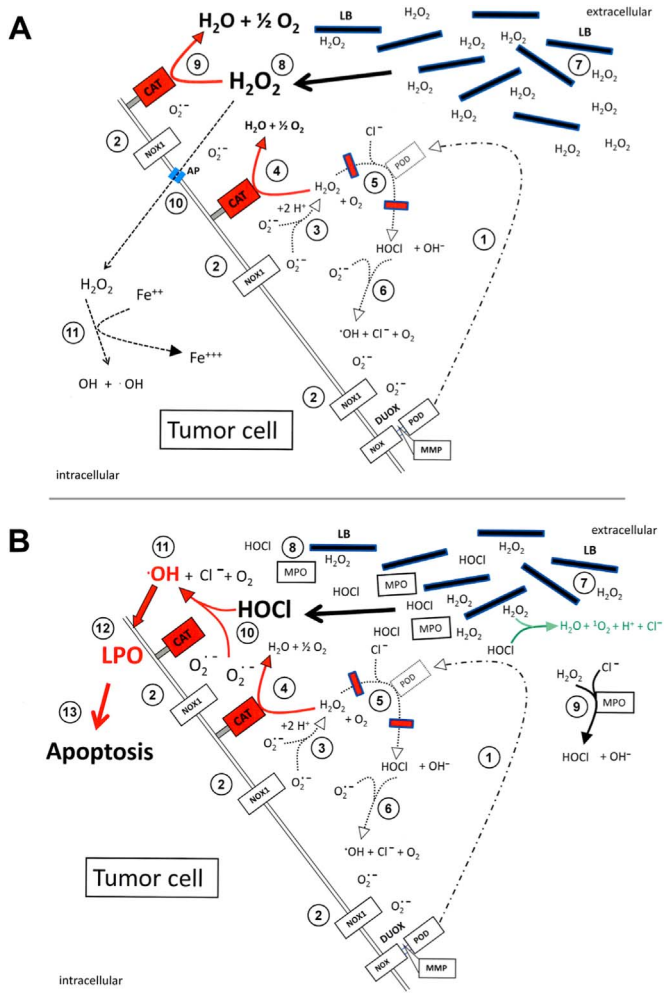

Fig. 8. Interaction of lactobacilli with bona fide tumor cells. A. Membrane-associated catalase decomposes H_2O_2 (#4) and thus prevents apoptosis-inducing HOCl signalling through reaction steps #1-3, 5-6). Lactobacillus (LB) (#7)-derived H_2O_2 (#8) is decomposed by membrane-associated catalase (#9). The ratio between tumor cells and LB determines whether protection by membrane-associated catalase is overrun and H_2O_2 penetrates the cells (#10) and causes the generation of hydroxyl radicals through Fenton chemistry (#11). At very low concentration of tumor cells, a more complex process is inititated by LB, which is analysed in Supplementary Fig. 5. B. In the presence of MPO (#8), lactobacillus-generated H_2O_2 (#9, 10) that is out of reach of tumor cell protective catalase is used a substrate for the synthesis of HOCl. The interaction between HOCl and NOX1-derived extracellular superoxide anions (#10) then leads to the generation of hydroxyl radicals (#11) which cause lipid peroxidation (#12) and apoptosis induction (#13).

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

The biochemical mechanism is analogous to elimination of transformed cells through intercellular HOCl signaling [55,70]. However, due to abundance of lactobacillus-derived H_2O_2 and MPO, the process in the vaginal fluid is faster than autocrine HOCl-dependent apoptosis signaling of transformed cells which need to generate H_2O_2 and DUOX-coded peroxidase themselves (see Supplementary Figures 1, 2). Moreover, whereas autocrine apoptosis signaling through the HOCl signaling pathway requires high local density of transformed target cells [66,70], the effect established by lactobacilli in the presence of MPO can be predicted to act also on individual transformed cells, as H_2O_2 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 H_2O_2 and peroxidase, and as catalase cannot protect them against distantly generated HOCl. Only high concentrations of tumor cells may have a modulatory counteraction towards apoptosis induction. Thus, it may be concluded that the control system consisting of H_2O_2- 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 H_2O_2-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 H_2O_2 by lactobacillus might finally lead to concentrations of H_2O_2 that are damaging to the vaginal epithelium. One might also assume that the conversion of H_2O_2 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 H_2O_2 and HOCl ($H_2O_2 + HOCl \rightarrow {}^1O_2 + H_2O + H^+ + Cl^-$) [75,76]. This reaction thus seems to balance the concentrations of H_2O_2 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 H_2O_2 ($k=10^5\ M^{-1}s^{-1}$), this balancing consumption reaction would, however, not interfere with selective apoptosis induction of superoxide anion-producing malignant cells. The reaction product singlet oxygen ($^1O_2$) that is derived from the interaction between H_2O_2 and HOCl has recently been shown not to be damaging to non-malignant cells (provided it is supplied extracellularly) [77], but to efficiently inactivate membrane-associated catalase of tumor cells. Therefore, singlet oxygen generated in the vaginal fluid through H_2O_2/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 H_2O_2- 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 H_2O_2/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 H_2O_2 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 H_2O_2 and HOCl ($H_2O_2 + HOCl \rightarrow {}^1O_2 + H_2O + H^+ + Cl^-$) [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 H_2O_2 [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].

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.redox.2017.01.015.

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