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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