L-アスコルビン酸はMycoplasma hyorhinisに感染したヒト由来胃がん培養細胞株AZ-521のアポトーシスを促進する

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<tr>
<td>誌名</td>
<td>The journal of veterinary medical science</td>
</tr>
<tr>
<td>ISSN</td>
<td>09167250</td>
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<td>著者</td>
<td>小原, 寿人 原澤, 亮</td>
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<td>掲載ページ</td>
<td>p. 11-15</td>
</tr>
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<td>発行年月</td>
<td>2008年1月</td>
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農林水産省 農林水産技術会議事務局筑波産学連携支援センター
Tsukuba Business-Academia Cooperation Support Center, Agriculture, Forestry and Fisheries Research Council Secretariat
L-Ascorbic Acid Enhances Apoptosis in Human Gastric Carcinoma Cell Line AZ-521 Cells Infected with Mycoplasma hyorhinis

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(Received 1 May 2007/Accepted 18 September 2007)

ABSTRACT. Mycoplasma hyorhinis (M. hyorhinis) exerts multiple effects on cell metabolisms including apoptosis mediated by their endonucleases and nitric oxide production in vitro. Although AsA is preferable to health in general because of its reactive oxygen species scavenging activity, we found that in a human carcinoma cell line AZ-521 infected with M. hyorhinis, apoptosis was enhanced by addition of L-ascorbic acid (AsA) to the cell cultures. No significant differences were evident between the AZ-521 cells with and without AsA (AsA−) after 24 hr of incubation in the mitochondrial fluorescence. M. hyorhinis-infected AZ-521 cells treated with AsA (AsA+) have developed distinct DNA ladders as compared to the control cells AsA− after 24 hr of incubation. Marked cytopathic effects were rather apparent in AsA+ treated cells than in control cells AsA− after 24 hr. Our data demonstrate that AsA addition to cell cultures enhances apoptosis induced by M. hyorhinis infection. We suggest that the presence of another external apoptotic pathway by M. hyorhinis infection.

KEY WORDS: apoptosis, L-ascorbic acid, Mycoplasma hyorhinis.

While most mycoplasmas are not primarily pathogenic to humans and animals, a few specific mycoplasma species are responsible for chronic and degenerative diseases [26]. Mycoplasma hyorhinis (M. hyorhinis) is causative of polyserositis [3], otitis media [16], arthritis [6], and mycoplasmal pneumonia [11] in pigs, and may lead the swine production to serious situation [30].

M. hyorhinis is also a common contaminant in cell cultures [1]. Infection with M. hyorhinis leads the culture cells to apoptosis, which is mediated by their endonucleases [20, 21]. We examined the effect of L-ascorbic acid (AsA) to the apoptosis in cultured cells infected by M. hyorhinis, since it has been known that AsA has reactive oxygen species (ROS) scavenging activity by protecting the membrane phospholipids against the peroxidative damage by ROS, and by suppressing ROS production [2, 8]. However, dietary taken AsA exhibits a pro-oxidant, which attributes to DNA damage, in healthy humans so it is possible that AsA may play a role in diseases such as cancer, rheumatoid arthritis and atherosclerosis [24].

In contrast to our initial assumption that AsA may prevent apoptosis of the cells infected with M. hyorhinis, in the process of the experiments, we found excess AsA addition enhanced apoptosis of the cells infected with M. hyorhinis rather than preventative.

In the present study, we demonstrated that a human carcinoma cell line AZ-521 cells infected with M. hyorhinis lead to apoptosis enhanced by addition of AsA, revealed by fluorescence DNA staining and DNA ladder formation, but no significant differences in the mitochondrial fluorescence as compared to the controls. These suggest the presence of another external apoptotic pathway by M. hyorhinis infection.

MATERIALS AND METHODS

Cell cultures: Human gastric cancer cell line AZ-521 was obtained from the Health Science Research Resources Bank (Osaka). The cell culture was maintained as previously described [31]. To minimize the pH decrease by AsA addition to the cell culture, HEPES buffer (pH 7.5) was added to the DMEM at the final concentration of 50 mM. The cell culture was tested for the absence of mycoplasma contamination by using the PCR Mycoplasma Detection Set (TaKaRa) according to the manufacturer's instruction. The cells were seeded into chamber flasks at 8.5 x 10^5 cells per chamber.

Mycoplasma strain and its growth condition: The type strain of M. hyorhinis BTS7 was used in this study. The propagation of M. hyorhinis and its colony-forming-units (CFU) was determined as described previously except for adding 0.5 % glucose instead of bacteriological mucin [10]. M. hyorhinis was inoculated to the AZ-521 cell culture at a multiplicity of infection (MOI) of 10^3 after seeding the cells into the chamber flasks.

Chemicals and reagents: Analytical grade AsA (Nacalai Tesque Inc.) was used to prepare 0.5 M in a phosphate-buffered saline (PBS) solution and stored at 4°C in the dark. Hoechst 33342 (Dojin Chemicals) and MitRed (Dojin Chemicals) were used for staining DNA and mitochondria of the cells according to the manufacturer's instruction.

Influence of the AsA on the M. hyorhinis propagation and cell viability: AsA was added to the AZ-521 cell culture (AsA +) at the final concentration of 2.0 mM. The same cell culture without AsA (AsA−) was used as a control. Numbers of living and dead cells were counted by staining with...
trypan blue after 32 hr of incubation, for monitoring cytotoxic effect of AsA [23]. CFU of *M. hyorhinis* cultivated in PPLO broth containing AsA and HEPES (pH 7.5) at the final concentrations of 2.0 mM and 50 mM, respectively, was estimated after 24 hr of incubation at 37°C.

**DNA ladder analysis:** AZ-521 cells grown in the DMEM containing 2.0 mM AsA were collected by centrifugation at 4,000 × g for 30 min after 18, 24, and 30 hr of inoculation with *M. hyorhinis*. The cells were washed three times with PBS, and chromosomal DNA was extracted with ApopLadder Ex™ (TaKaRa) according to the manufacturer’s instruction. The DNA eluted in Tris-EDTA buffer (10 mM Tris-HCl pH 8.0 and 1.0 mM EDTA) was used for the analysis. DNA ladder analysis was performed as previously described [29].

**Fluorescent microscopic analysis:** The AZ-521 cells grown in the chamber flasks were examined for apoptotic changes by using fluorescent dyes, MitoRed and Hoechst 33258. Mitochondrial activity was examined by microscopic observation of the AZ-521 cells stained with MitoRed [17]. Hoechst 33258 was used for examination of chromatin DNA. We observed the fluorescent nuclei showing apoptotic changes, mainly due to chromatin condensation at 200 × and 1,000 × magnification [17]. Further, at 18, 24, 30 hr after inoculation, we counted 10 microscopic fields at 400 × magnification the number fluorescent cells infected with *M. hyorhinis* showing apoptotic changes, AsA+ or AsA−. Apoptosis was expressed as the percentage of cells with apoptotic nuclear morphology in relation to the total cell number. Student’s *t* test was used for date analysis.

**RESULTS**

**Influence of the AsA on the *M. hyorhinis* propagation and cell viability:** Growth of *M. hyorhinis* in the PPLO medium containing 2.0 mM AsA was examined. Titers of *M. hyorhinis* in the PPLO medium with and without 2.0 mM AsA were 5.1 × 10⁸ CFU and 3.1 × 10⁸ CFU, respectively. Thus the AsA itself did not affect propagation of *M. hyorhinis*. Viability of the AZ-521 cells was not affected by incubation with 2.0 mM AsA after 32 hr, indicating that the concentration used was not toxic to the cells.

**DNA ladder analysis:** *M. hyorhinis*-infected AZ-521 cells were collected by centrifugation at 18, 24 and 30 hr after incubation AsA+ and AsA−, and the DNA ladder was examined (Fig. 1). Although the DNA ladders of the *M. hyorhinis*-infected AZ-521 cells AsA+ and AsA− were equally faint at 18 hr, those of the AsA-treated cells were more apparent than the control cells at 24 hr. Similarly, DNA ladders of the AsA-treated cells were distinct from those of the control cells at 36 hr post inoculation. These data indicated that AsA enhanced apoptosis that *M. hyorhinis* induced in AZ-521 cells.

**Fluorescent microscopic analysis:** No significant differences were evident between the AZ-521 cells AsA+ and AsA− after 24 hr of incubation in MitoRed assay (Fig. 2). This suggests that the molecular processes of apoptosis induced by *M. hyorhinis* infection is not via an intrinsic pathway, which is regulated by the cytochrome c from mitochondria and the subsequent ATP-dependent cascade [14]. Hoechst 33258 assay revealed that apoptosis in the AsA− treatment AZ-521 cells was enhanced as compared to the control AZ-521 cells at 24 hrs (Fig. 3). Marked chromatin condensation was observed in the AZ-521 cells infected with *M. hyorhinis* at 1,000 × magnification (Fig. 4). All the cells in the AZ-521 cell culture AsA+ and AsA− were both found detached at 30 hr due to cytopathic effect by *M. hyorhinis* infection (data not shown). At 18, 24, 30 hr after inoculation respectively, apoptotic changes were more significantly (*p<0.05*) observed in AsA−treated AZ-521 cells infected with *M. hyorhinis* than in non-treated AZ-521 cells (Fig. 5). This indicates that AsA enhanced apoptosis AZ-521 cells infected by *M. hyorhinis*.

**DISCUSSION**

To the best of our knowledge, the present study is first to report that AsA augments apoptosis in a human gastric carcinoma cell line infected with *M. hyorhinis*. AsA is a vitamin in primates including humans and guinea pigs, because these animals cannot synthesize AsA due to the lack of L-gulono-1,4-lactone oxidase (GULO), the terminal enzyme in the AsA synthesize pathway [5, 19]. AsA is also essential for cellular functions including collagen biosynthesis and is important antioxidant providing primary defense against oxidative insult [22], preventing endothelial dysfunction causing atherogenesis [12]. AsA has ROS scavenging activity by suppressing ROS production as well as by protecting the membrane phospholipids against the peroxidative damage of ROS [2, 8]. AsA enhances inducible nitric oxide synthase (iNOS) activity [18] as well as endothelial nitric oxide synthase (eNOS) activity in various cells [5, 9], and results in increase of nitric oxide (NO) production.
Nitric oxide, a gaseous free radical, has been identified as a ubiquitous signaling molecule in biological systems [15], and is known to induce apoptosis [13, 25] in various cells. Human cell lines infected with *M. hyorhinis* have been reported to enhance NO synthesis [7]. Thus, addition of much amount of AsA to *M. hyorhinis*-infected cells may lead to high concentration NO, resulting in the increase of apoptotic cells.

Roughly at most 0.5 mM AsA is found in human gastric juice [28]. So, the concentration used in the present study was higher than the physiological concentration. There is a report that AsA itself induced cell death in various cell sys-
cleases of M. hyorhinis have been responsible for induction of apoptosis [27]. However, the concentration used was not toxic to the AZ-521 cells at least 32 hr after treatment by AsA.

In the present study, the MitoRed assay did not show differences between the AsA-treated AZ-521 cells and the control AZ-521 cells (Fig. 2), suggesting that apoptosis induced by M. hyorhinis is not via mitochondria. Endonucleases of M. hyorhinis have been responsible for induction of apoptosis [20, 21]. These endonucleases, 47 to 54 kDa, are Ca\(^{2+}\) and Mg\(^{2+}\)-dependent. It has been proposed that M. hyorhinis use their endonucleases to obtain precursors for DNA synthesis, since M. hyorhinis lack the ability for de novo synthesis of nucleotide precursors. Only the endonuclease of M. hyorhinis may not be responsible for apoptosis in the AZ-521 cells, because addition of AsA to the cell cultures enhanced the apoptosis, suggesting presence of another external pathway such as NO. When the apoptosis and NO experiments were performed, it is important to certify that cell cultures are free from mycoplasma infection [4] to avoid erroneous data by contaminating mycoplasmas.

Similarly, Paddenberg et al. reported that cycloheximide treatment leaded to DNA fragmentation of the human pancreatic adenocarcinoma cell line PaTu 8902 cells infected with M. hyorhinis [20], but not of non-treated cells. Interestingly, in the present study, we showed that non-treated AZ-521 cells infected with M. hyorhinis caused DNA fragmentation of the cells. The possible explanation for this phenomenon could be the difference of the cell used and strain of M. hyorhinis.

In conclusion, our data demonstrate that AsA addition to cell cultures enhances apoptosis induced by M. hyorhinis infection. Although molecular function of AsA in enhancement of apoptosis induced by M. hyorhinis infection is currently unknown, our data suggest that the presence of another external apoptotic pathway by M. hyorhinis infection.
REFERENCES


