Abstract. Background: The effects of bacterial eradication therapy cannot be fully explained simply by elimination of the target bacteria, if one considers the effects of eradication therapy in H. pylori-negative cases of low-grade malignancy MALTomas of the rectum. The present study was undertaken to examine the possibility and mechanism of direct induction of apoptosis of the tumor cells by the antibiotics used for bacterial eradication therapy. Materials and Methods: A B cell lymphoma cell line (300-19) derived from BALB/c mice was co-cultured with amoxicillin or clarithromycin, and amoxicillin and clarithromycin at concentrations equal to or 1/10 x MIC of either drug. Cells co-cultured with 1/100 x MIC of the standard anti-tumor agents, adriamycin, vincristine and cyclophosphamide, served as positive controls. Cells cultured without any antibiotic or anti-tumor agent served as controls. In each group, the following analyses were performed: (i) the time-course of changes in the cellular morphology, (ii) the time-course of occurrence of DNA fragmentation, (iii) the appearance of apoptotic changes as evaluated by trypan blue staining, (iv) Bcl-2 expression as examined by immunoblotting, and (v) the expression of TNFRI, Fas, FasL and caspase-3, -8 and -9, as evaluated by immunoblotting. Results: Cells treated with amoxicillin and clarithromycin showed the formation of apoptotic bodies, and degeneration and detachment of the cells in a dose-dependent manner. DNA fragmentation was induced in these cells to a degree similar to that seen in cells treated with the anti-tumor agents. Trypan blue staining also demonstrated apoptosis of the cells and loss of cell viability. Bcl-2 expression was seen only in the control group and FasL was never seen, while the expression of TNFRI, Fas and caspase-3, -8 and -9 was seen in the amoxicillin-treated group, clarithromycin-treated group, amoxicillin and clarithromycin-treated group and the positive control group. Conclusion: Antibiotics used for the eradication of H. pylori can also directly induce apoptosis in mouse B cell lymphoma cells, an action which involves the TNF system.

H. pylori eradication therapy exerts diverse beneficial effects, not only in H. pylori-positive cases of intractable peptic ulcer and H. pylori-positive cases of low-grade malignancy tumors arising from MALT (mucosa-associated lymphoid tissue) in the stomach, but also in H. pylori-negative cases of low-grade malignancy MALTomas of organs other than the stomach (rectum, etc.) (1-3). These effects cannot always be simply explained by H. pylori elimination alone. Furthermore, it has recently been reported that H. pylori eradication can also exert therapeutically beneficial effects in idiopathic thrombocytopenic purpura (ITP) (4-13) and atopic dermatitis (31-35). The question of how the eradication of H. pylori in the stomach affects MALTomas of distant organs or non-gastrointestinal diseases such as ITP and atopic dermatitis has recently received attention. Although a number of reports (14-35) have been published concerning the effects of bacterial eradication therapy, no attempt has yet been made to study the background factors and unsolved mechanisms.

We previously reported that the antibiotics used for bacterial eradication therapy can directly induce apoptosis in inflammatory or immunocompetent cells of the gastric mucosa (36). However, it has remained unknown whether or not these antibiotic effects would also be seen in tumor cells. The present in vitro study was undertaken to examine the possibility and mechanism of direct induction of apoptosis in tumor cells by the antibiotics used for H. pylori eradication therapy.
Figure 1. A. Changes of cells three days after culture (control). B. Changes of the cells one day after co-culture with amoxicillin and clarithromycin (MICx1/10). C. Changes of the cells three days after co-culture with amoxicillin and clarithromycin (MICx1/10).
**Materials and Methods**

A B cell lymphoma (300-19) cell line derived from BALB/c mice was used for this study. RPMI1640 supplemented with 10% FCS served as the culture medium, and the cells were incubated in a CO₂ incubator. When the cells became confluent, their density was adjusted to 2x10^8 cells/mL for use in the experiments described below. The cultured cells were additionally co-cultured with amoxicillin or clarithromycin, amoxicillin and clarithromycin added at concentrations equal to 1/10 x MIC of each drug. Cells co-cultured with 1/100 x MIC of one of the standard anti-tumor agents (vincristine, adriamycin, or cyclophosphamide) served as positive controls. Cells cultured without any antibiotic or anti-tumor agent served as controls. In each group, the following analyses were performed: (i) the time-course of changes in the cellular morphology, (ii) the time-course of occurrence of DNA fragmentation, (iii) the appearance of apoptotic changes as evaluated by trypan blue staining after 60 minutes of incubation, and (iv) the expression of Bcl-2 as examined by immunoblotting; furthermore, (v) the expression of TNFR1, Fas, FasL, Bcl-2 and caspase-3,-8 and -9 in the cells from each group after 24 hours of incubation, by immunoblotting.

**Detection of DNA fragmentation.** Samples were collected from the cell cultures at various time-points. The isolated samples were fractioned into culture supernatant (SN) and cell lysate (CL) fractions, and nuclear DNA fragmentation in these two fractions was separately identified by ELISA (Cellular DNA fragmentation ELISA kit, Roche Diagnostics Japan), conducted essentially according to the manufacturer’s manual. The cells were incubated overnight at 37°C in the presence of BrdU, to label DNA. Then, the labelled cells were collected by centrifugation at 250 x g for 10 minutes and resuspended in culture medium (RPMI 1640 containing 10% FCS) to a cell density of 1x10^5 cells/mL. The resultant cell suspension was transferred to a 96-well microtiter plate in aliquots of 200 µL/well and incubated at 37°C for 1 hour in a CO₂ incubator. At the end of the incubation period, the microtiter plate was centrifuged at 250 x g for 10 minutes and 100 µL/well of the resultant culture supernatant was withdrawn to obtain the SN fraction. To the cells remaining at the bottom of the well of the microtiter plate, 100 µl of incubation buffer (containing EDTA and Tween-20, provided with the ELISA kit) was added and the plate was incubated at room temperature for 30 minutes, to disrupt cell membranes. After the cell lysis step, the microtiter plate was centrifuged again at 250 x g for 10 minutes and 100 µL/well of the resultant supernatant was collected to obtain the cell lysate fraction. To conduct a sandwich ELISA, samples thus obtained were transferred without further dilution to each well of a new microtiter plate coated with a mouse anti-DNA monoclonal antibody (provided with the ELISA kit), and incubated at room temperature for 30 minutes. After being washed with wash buffer, the microtiter plate was subjected to microwave irradiation (AGE Micromat 275Z, 650W) for 5 minutes for denaturation and fixation of the immunocomplexed BrdU-labelled DNA fragments on its surface. Then the microtiter plate was incubated at -20°C for 10 minutes and further allowed to react with anti-BrdU peroxidase conjugates (provided with the ELISA kit) at room temperature for 90 minutes. After the microtiter plate had been washed to remove any unreacted peroxidase, peroxidase bound in the immune complexes formed on the microtiter plate surface was detected photometrically using TMS as the chromogenic substrate.

**Determination of apoptosis by trypan blue staining.** The cells were harvested at different time-points (1, 3, 7, 10 and 14 days) after the start of incubation. The number of cells per mL culture was counted. After being washed with wash buffer, the microtiter plate was subjected to microwave irradiation (AGE Micromat 275Z, 650W) for 5 minutes for denaturation and fixation of the immunocomplexed BrdU-labelled DNA fragments on its surface. Then the microtiter plate was incubated at -20°C for 10 minutes and further allowed to react with anti-BrdU peroxidase conjugates (provided with the ELISA kit) at room temperature for 90 minutes. After the microtiter plate had been washed to remove any unreacted peroxidase, peroxidase bound in the immune complexes formed on the microtiter plate surface was detected photometrically using TMS as the chromogenic substrate.
Figure 2. Changes of the cells 60 minutes after co-culture with antibiotics: judgment of cell death by trypan blue staining.

Arrowhead shows the formation of apoptotic body.

Figure 3. Expression of Bcl-2 by immunoblotting.

1. Control
2. Amoxicillin (MICx1/10)
3. Clarithromycin (MICx1/10)
4. Amoxicillin + Clarithromycin (MICx1/10)
5. Anti-tumor agents (MICx1/100)

Figure 4. Expression of TNFR1 by immunoblotting.

1. Control
2. Amoxicillin (MICx1/10)
3. Clarithromycin (MICx1/10)
4. Amoxicillin + Clarithromycin (MICx1/10)
5. Anti-tumor agents (MICx1/100)
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Figure 5. Expression of Fas/Fas ligand by immunoblotting.

1. Control
2. Amoxicillin (MICx1/10)
3. Clarithromycin (MICx1/10)
4. Amoxicillin + Clarithromycin (MICx1/10)
5. Anti-tumor agents (MICx1/100)

Figure 6. Expression of caspase -3, -8, -9 by immunoblotting.

1. Control
2. Amoxicillin (MICx1/10)
3. Clarithromycin (MICx1/10)
4. Amoxicillin + Clarithromycin (MICx1/10)
5. Anti-tumor agents (MICx1/100)
conducted, followed by blotting onto a polyvinylidene difluoride (PVDF) membrane (Schleicher and Schuell, Inc., Knee, NH, USA). The membrane was blocked with 1% skim milk for 2 hours, incubated with rabbit monoclonal anti-TNFRI, Fas, FasL, Bcl-2, or caspase-3, -8, or -9 antibody (diluted 1:500 in 1% bovine serum albumin (BSA)/ phosphate-buffered saline (PBS); Accurate Chemical and Scientific Corp., West-bury, NY, USA) overnight at 4°C, and with horseradish peroxidase-conjugated goat anti-rabbit antibody (diluted 1:500 in 1% bovine serum albumin (BSA)/ phosphate-buffered saline (PBS); Accurate Chemical and Scientific Corp., West-bury, NY, USA) for 2 hours at room temperature. Signals were detected using a chemiluminescence reagent (Dupont NEN Company, Wilmington, DE, USA). The rabbit monoclonal anti-TNF, CD95, Bcl-2 and caspase-3, -8 and -9 antibodies were purchased from Santa Cruz Biotechnology, Inc. (USA).

**Statistical analysis.** All data were expressed as mean±SD (standard deviation). The significance of changes was evaluated by using the Student's t-test. A p value less than 0.05 was considered to be significant.

**Results**

The cells showed progressive degeneration and detachment after the start of co-culture with antibiotics (Figure 1-A, B, C). At each concentration of the antibiotics and anti-tumor agents, a progressive increase in DNA fragmentation was also seen depending on the time and the concentration of the drugs (Table I). Partial staining of the cells with trypan blue revealed the formation of apoptotic bodies and loss of cell viability one hour after the start of co-culture with amoxicillin and clarithromycin (1/10 x MIC), and the apoptotic changes increased over time (Figure 2). Immunoblotting revealed expression of Bcl-2 only in the control group (Figure 3). Immunoblotting revealed expression of TNFRI in all the groups, including the control group. The level of TNFRI expression increased in the following, ascending, order: control group < 1/10 x MIC antibiotic treatment group (amoxicillin, clarithromycin) < 1/10 x MIC antibiotic treatment group (amoxicillin and clarithromycin) < 1/100 x MIC anti-tumor agent treatment group (Figure 4). FasL was not expressed in any of the groups, although Fas was expressed in all groups (Figure 5). The expression of caspase-3, -8 and -9 was seen in all the groups, except in the control group. The level of caspase -3, -8 and -9 expression increased in the following ascending order: 1/10 x MIC antibiotic treatment group (amoxicillin, clarithromycin) < 1 x MIC antibiotic treatment group (amoxicillin and clarithromycin) < 1/100 x MIC anti-tumor agent treatment group (Figure 6).

**Discussion**

In previous reports, the effects of *H. pylori* eradication therapy have been deemed to be equal to the evaluation of *H. pylori* itself, which is known to colonize and exert disruptive effects on the gastric mucosa (24-30). However, it has recently been revealed that bacterial eradication therapy is also effective against other gastrointestinal diseases, including *H. pylori*-negative MALTomas (1-3, 31-35). It is, therefore, difficult to attribute all the effects of bacterial eradication therapy to the elimination of *H. pylori* alone. Nakase et al. (3) reported the possibility that *H. pylori* eradication therapy on *H. pylori*-negative MALTomas may also involve the effects on microorganisms other than *H. pylori*. However, they did not comment on the nature of the other microorganisms. We previously demonstrated that antibiotics directly induced apoptosis in inflammatory-related immunocytes in the gastric mucosa, such as mononuclear cells (36). The present study was undertaken assuming that the antibiotics may act directly on the tumor cells. The results indicate that antibiotics can also induce apoptosis of tumor cells, although the magnitude of this activity may be lower than that of standard anti-tumor agents. Adriamycin, which is currently used as an anti-tumor agent, was originally developed as an antibiotic. It has also been indicated that this action of antibiotics may involve TNFR/TNFR, similar to the case with some anti-tumor agents (37,38). It has been reported that bacterial eradication therapy is effective only for low-grade malignancy MALTomas which do not involve translocation of chromosome t(11, 18) or the API-2 MALT gene (21-23). This previous finding may be explained by the results of the present study indicating that the potential for induction in apoptosis of tumor cells differed between anti-tumor agents and antibiotics. We also consider that the recently highlighted effect of *H. pylori* eradication therapy in cases of ITP and atopic dermatitis may be attributable to the direct actions of the antibiotics.

We think that it is necessary to review the pathogenetic mechanisms involved in *H. pylori*-associated diseases. It is essential to clearly identify the abnormalities attributable to *H. pylori* itself and/or to some unknown factors. Such an approach may lead to a better understanding of mucosal pathophysiology.

**Acknowledgements**

This work was supported by a grant from the Otsuka Pharmaceutical Co. Ltd., Japan.

**References**


