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Ethanol Induces Apoptosis in Human Gastric Carcinoma Cells: The Role of Apoptosis-Related Molecules

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Ethanol triggers apoptosis in a variety of mammalian cultured cells. Here, ethanol-induced apoptosis in five human gastric carcinoma cell lines was examined. Trypan blue-positive cells were detected in more than 77% of 10% ethanol treated cells at 6 h in all the cell lines. The apoptosis, which had a typical morphology and terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) signals were induced with 3% and 5% ethanol treatments. The apoptotic cells were detected in 36% of MKN-74, 54% of MKN-45 which have wild-type *p53*, and 37% of TMK-1 cells at 48 h with 5% ethanol, in contrast to less than 18% of MKN-28 and KATO-III cells. The DNA ladder appeared in the MKN-74, MKN-45 and TMK-1, but not in the MKN-28 and KATO-III. The expression of apoptosis-associated molecules p53, Bcl-2, Bax and c-Myc proteins was analyzed in the ethanol-treated cells. The levels of p53, Bcl-2 and Bax proteins were not changed in the ethanol-induced apoptosis. MKN-28 and KATO-III showed higher expressions of Bcl-2 protein compared to the other cell lines, and the down-regulation of c-Myc protein after 1.5 h of ethanol treatment. Calcium antagonists verapamil or nifedipine decreased apoptotic cells in ethanol treated MKN-74, MKN-45 and TMK-1. These results suggest: i) 3% and 5% ethanol induced apoptosis via the influx of extracellular calcium, but were not changed the expression levels of p53 and c-Myc protein, while 10% ethanol provoked necrosis in these cell lines, and ii) the apoptosis resistance by ethanol might be implicated in the higher expression of Bcl-2 protein and down-regulation of c-Myc protein as shown in MKN-28 and KATO-III.

Key words: apoptosis; gastric carcinoma cell line; ethanol; calcium; c-Myc protein

Apoptosis is a form of cell suicide and plays a major role in embryogenesis, the regulation of the immune system, and carcinogenesis (Kerr et al., 1972, 1994; Wyllie, 1980, 1981; Ucker, 1991; Williams, 1991; Anilkumar et al., 1992; Cohen, 1993; Hockenbery, 1995). The morphological features of apoptosis are cell shrinkage, loss of normal contacts and condensation of the nuclear chromatin. The apoptotic cells with nuclear fragmentation subsequently break down into multiple apoptotic bodies, which are phagocytosed rapidly by adjacent cells or macrophages (Kerr et al., 1972; Wyllie et al., 1984). Biochemically, deoxyribonucleic acid (DNA) isolated from apoptotic cells shows a characteristic “ladder” structure on agarose gel electrophoresis (Wyllie, 1980).

Ethanol has been shown to induce the apoptosis of thymocytes, lymphocytes, hepatocytes, and tumor cell lines in vitro (Lennon et al., 1991; Ewald and Shao, 1993; Slukvin and Jerrells, 1995; Kravtsov and Fabian, 1996; Kikuchi et al., 1997; Kurose et al., 1997). Ethanol affects a signal transduction pathway in thymocytes by altering the cellular calcium level and increasing protein kinase C activity (Shao et al., 1995; Slukvin and Jerrells, 1995). However, the signal transduction pathways

Abbreviations: DAB, diaminobenzidine; DMSO, dimethyl sulfoxide; dUTP, deoxyuridine 5'-diphosphate; 5-FU, 5-fluorouracil; PBS, phosphate-buffered saline; TdT, terminal deoxynucleotidyl transferase; TUNEL, TdT-mediated dUTP-biotin nick end-labeling

underlying ethanol-induced apoptosis still remains obscure. It has also been shown that ethanol causes rat or rabbit gastric mucosal cell damage (Oates and Hakkinen, 1988; Tepperman et al., 1991) and apoptosis (Piotrowski et al., 1997). Tarnawski and colleagues (1990) have suggested that both ethanol penetration and influx of extracellular calcium are necessary for isolated human gastric gland cell injury by ethanol. Livraghi and colleagues (1991) have reported that percutaneous ethanol injection was performed for the treatment of metastatic liver cancer, including gastric carcinoma, and showed reduction in tumor size. It has been also reported that apoptosis occurred in human gastric carcinomas in both *p53* gene-dependent and -independent manners (Kasagi et al., 1994; Ishida et al., 1997; Ikeda et al., 1998). However, it has not been known whether ethanol induces apoptotic cell death in human gastric carcinoma cells. In addition, the roles of apoptosis-related genes in the process of ethanol-induced apoptosis are as yet poorly understood.

In the present study, the induction of apoptosis with ethanol in gastric carcinoma cell lines was studied. The apoptosis-related molecules were analyzed to define the mechanism and regulation of the ethanol-induced apoptosis.

Materials and Methods

Cell lines

MKN-74 and MKN-28 (Motoyama et al., 1986) were each derived from a well-differentiated adenocarcinoma, MKN-45 (Motoyama et al., 1986) and TMK-1 (Ochiai et al., 1985) from poorly differentiated adenocarcinomas, and KATO-III (Sekiguchi et al., 1978) from a signet ring cell carcinoma. MKN-74 and MKN-45 have wild-type *p53* genes, while MKN-28 and TMK-1 carry mutated *p53* genes (Matter et al., 1992). The *p53* gene of KATO-III is completely deleted (Matter et al., 1992). All cell lines were cultured in RPMI 1640 medium (Cosmo Bio, Tokyo, Japan), supplemented with 200 mmol/L glutamine, 100 U/mL penicillin and 10% fetal bovine serum (Bio Whittaker, Walkersville, MD) at 37˚C in a humidified 5% CO_2 atmosphere.

Induction of cell death

The cells were plated to each experiment at a density of 2×10^5 cells/mL. After 24 h of culture, the medium was replaced with fresh medium containing various concentrations (1%, 3%, 5% and 10%) of ethanol. The cells were then incubated at 37˚C in a humidified 5% CO_2 atmosphere. The cells without ethanol were served as controls. All experiments were performed on cells in exponential phase of growth.

Trypan blue staining

The cells were plated at 5×10^4 cells/mL. For a count of the number of dead cells, the trypan blue-positive cells were measured in 3 different cultures with the trypan blue exclusion test, and the mean value of percentage of positive cells was calculated.

Papanicolaou stain

For morphological examinations, cells were prepared with Auto Smear CF-120 (Sakura, Tokyo) and evaluated with Papanicolaou-staining. Apoptotic and necrotic cells were identified using the criteria described by Kerr and colleagues (1972), Wyllie and colleagues (1980) and Searle and colleagues (1982).

TdT-mediated dUTP-biotin nick end-labeling (TUNEL) method

DNA breaks in cells were detected with a modified TUNEL method (Kasagi et al., 1996). The cells were scraped using a cell scraper (Falcon, Lincoln Park, NJ) and suspended in 100% methanol at 4˚C for 30 min and then dropped onto silane-coated glass slides (Muto Pure Chemicals, Tokyo). Preparations were treated with 1% H_2O_2 solution for 20 min. The cells on glass slides were covered with TdT buffer solution (100 mmol/L potassium cacodylate, 2 mmol/L cobalt chloride, 0.2 mmol/L dithiothreitol, pH 7.2) containing 0.3 U/μL TdT

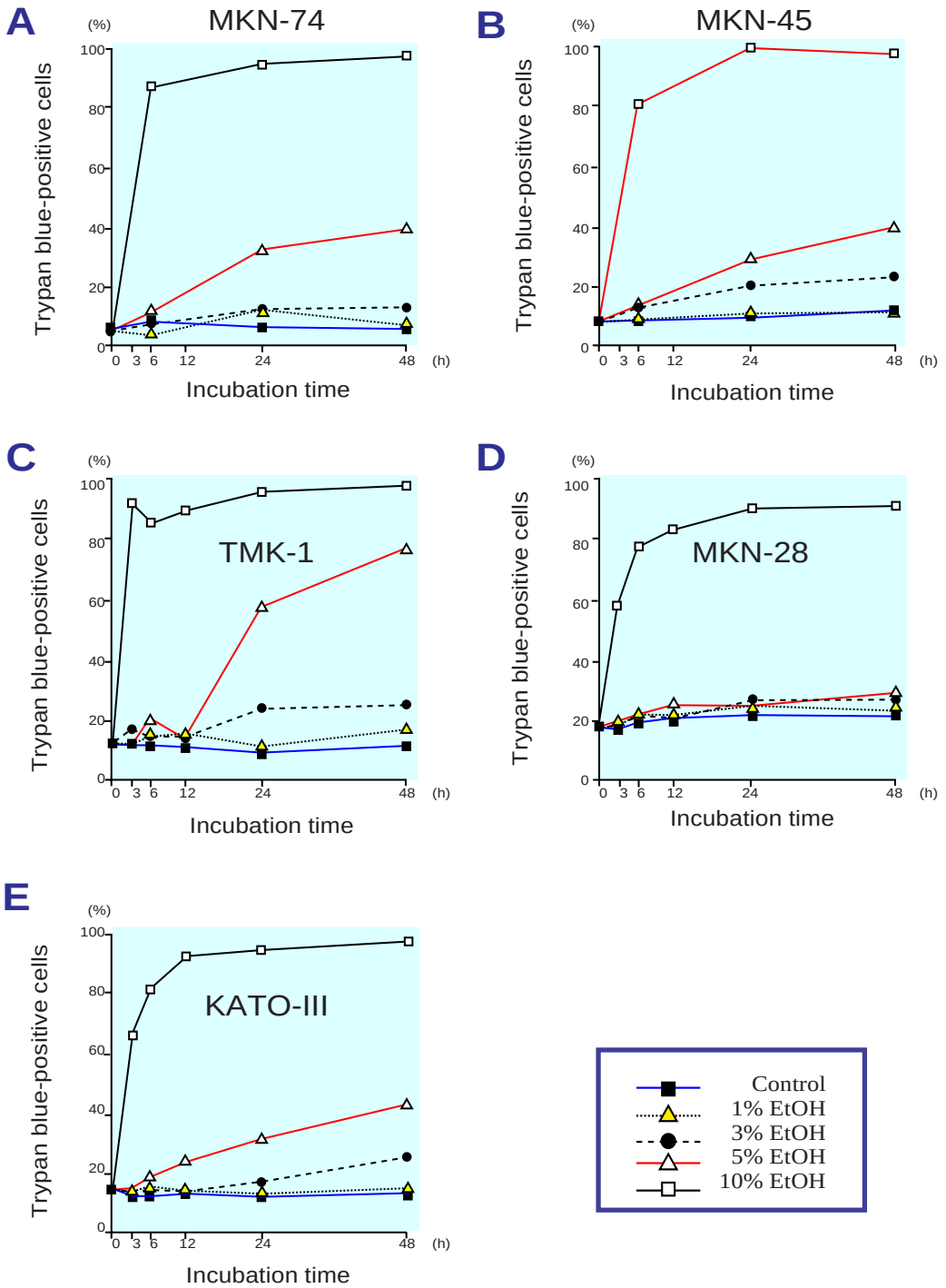

Fig. 1. Induction of cell death in the human gastric carcinoma cell lines MKN-74 (**A**), MKN-45 (**B**), TMK-1 (**C**), MKN-28 (**D**) and KATO-III (**E**) treated with ethanol. Each cell line was exposed to 0% (control), 1%, 3%, 5% and 10% ethanol and cultured for the indicated time. Results are expressed as percentages of trypan blue-positive cells, as determined by the cells' ability to exclude trypan blue dye. Data are the means of three separate experiments.

(Gibco BRL Life Technologies, Gaithersburg, MD) and 0.04 nmol/μL biotinylated dUTP (Boehringer Mannheim, Mannheim, Germany) and incubated in a humidified atmosphere at 37˚C for 90 min. Cells were then washed with TB buffer (300 mmol/L sodium chloride, 30 mmol/L sodium citrate) and then with phosphate-buffered saline (PBS). After incubation with peroxidase-labeled streptavidin for 30 min, the cells were developed in a mixture of 0.02% diaminobenzidine (DAB) and 0.02% H_2O_2 solution, and counterstained with Alcian green. As the negative control, cells on glass slides were treated with only TdT buffer solution.

Cells were considered positive for TUNEL when their nuclei were labeled on high-power fields except for faint signals. The proportion of TUNEL-positive cells was calculated in at least 1,000 cells and expressed as a percentage. The results were expressed as the mean and SD in 3 separate experiments.

DNA isolation and electrophoresis

Cells were collected and suspended at a density of 2×10^6 cells/mL in lysis buffer (10 mmol/L Tris-HCl buffer, pH 8.0, 5 mmol/L EDTA, 100 mmol/L sodium chloride) containing 10 μg/mL proteinase K (Wako Pure Chemicals, Osaka, Japan) and 0.2% sodium dodecyl sulfate and incubated at 37˚C overnight. The crude DNA preparations were extracted with phenol:chloroform:isoamyl alcohol (25:24:1), and then with chloroform:isoamyl alcohol (24:1). RNase A (Sigma, St. Louis, MO) was then added to a concentration of 100 μg/mL followed by incubation at 37˚C for 2 h. The DNA preparations were extracted again and precipitated in ethanol at –80˚C. The DNA precipitates were resuspended in TE buffer (10 mmol/L Tris-HCl buffer, pH 7.4, 1 mmol/L EDTA).

DNA samples of 1 μg were then loaded onto a 2% agarose gel, and electrophoresis was carried out for 1.5 h at 50 V. As the size marker, 123 base pair DNA Ladder (Gibco BRL Life Technologies) was used. DNA was stained with 1 μg/mL ethidium bromide and visualized by fluorescence in UV light.

Western blot analysis

Cells were solubilized in lysis buffer (50 mmol/L Tris-HCl buffer pH 7.4, 125 mmol/L NaCl, 0.1% NP-40, 5 mmol/L NaF, 1 mmol/L PMSF, 1 ng/mL leupeptin, 10 ng/mL soybean trypsin inhibitor, 1 ng/mL aprotinin, 10 ng/mL *N*-tosyl-L-phenylalanyl chloromethyl ketone) for 60 min on ice. The protein concentration was determined by means of the Bradford protein assay (Bio-Rad Laboratories, Richmond, CA) with bovine serum albumin as the standard. Samples (50 μg) were resolved by electrophoresis in a sodium dodecyl sulfate-polyacrylamide gel and electroblotted onto a polyvinylidene difluoride filter (Millipore, Bedford, MA). The filters were incubated with the primary antibodies at 4˚C overnight and then with peroxidase-labeled anti-mouse IgG (diluted 1:1,000; Medical and Biological Laboratories, Nagoya, Japan) or anti-rabbit IgG antibody (diluted 1:2,000; Medical and Biological Laboratories) in the secondary reaction. The primary antibodies used were anti-p53 (diluted 1:100; 1801; MEDAC, Hamburg, Germany), anti-Bcl-2 (diluted 1:100; 124; Dako, Copenhagen, Denmark), anti-c-Myc (diluted 1:50; Ab-1; Oncogene Research Products, Cambridge, MA) monoclonal antibodies and anti-Bax (diluted 1:1,000; P-19; Santa Cruz Biotechnology, Santa Cruz, CA) polyclonal antibody. The immune complex was detected using an enhanced chemiluminescence system (Amersham, Buckinghamshire, United Kingdom).

Effects of calcium antagonists on ethanol-induced apoptosis

After being seeded in 60 mm tissue culture dishes and cultured for 24 h, cells were treated with verapamil (Sigma) or nifedipine (Sigma) dissolved in dimethyl sulfoxide (DMSO) for 30 min, and then ethanol (3% or 5%) was added. After 12 h or 24 h of culture respectively, the percentage of TUNEL-positive cells was calculated as described above.

For the identification of the optimum concentrations of verapamil and nifedipine, pre-

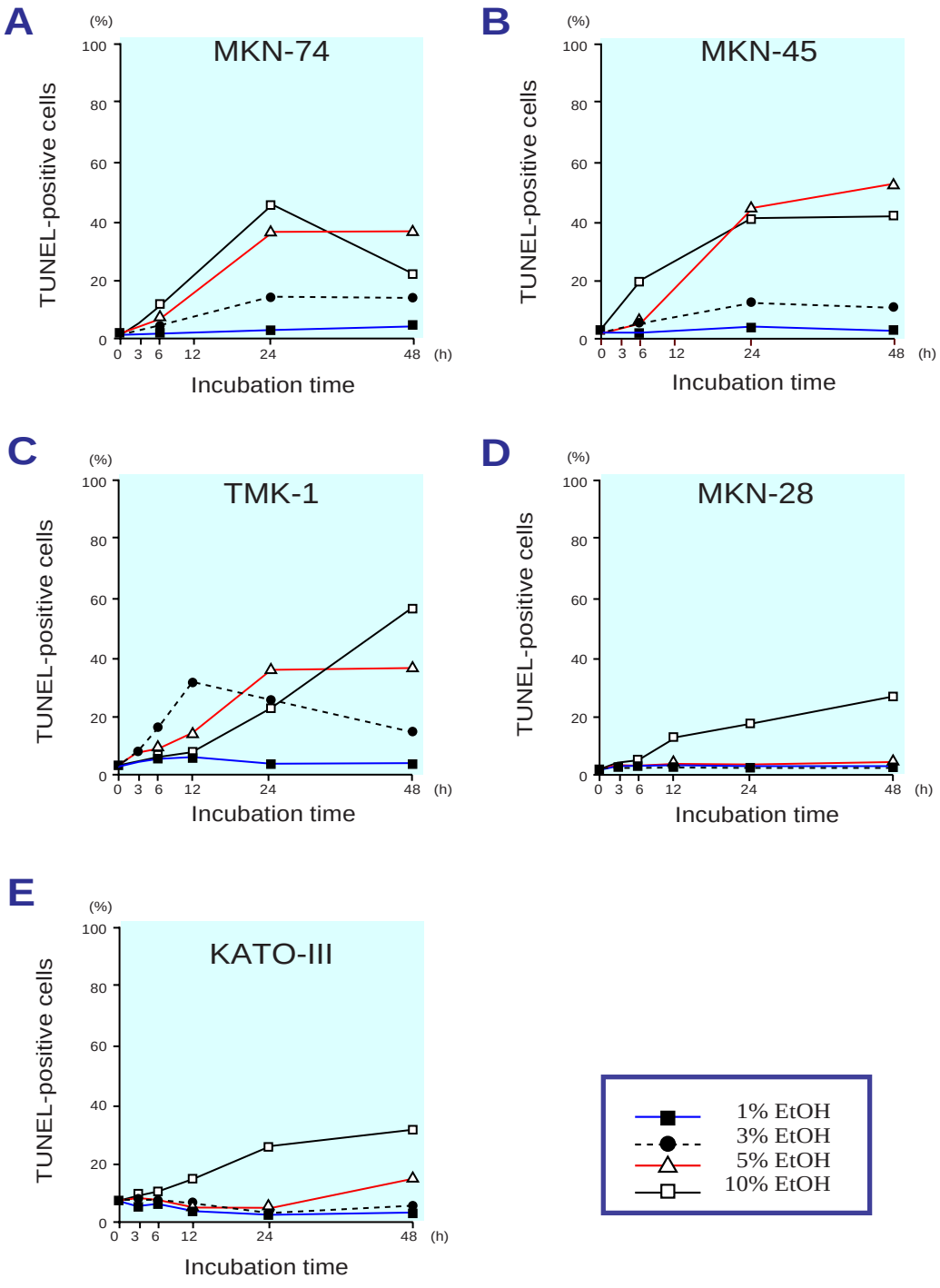

Fig. 2. Time course of TUNEL-positive cells in MKN-74 (**A**), MKN-45 (**B**), TMK-1 (**C**), MKN-28 (**D**) and KATO-III (**E**) treated with ethanol. Each cell line was exposed to 0% (control), 1%, 3%, 5% and 10% ethanol. Results are expressed as percentages of TUNEL-positive cells, as stained by the TUNEL method. Data are the means of three separate experiments.

liminary investigations were carried out which gave minimum trypan blue- and TUNEL-positive cells with or without ethanol. Verapamil or nifedipine was added at the final concentration of 100 μmol/L (TMK-1 and MKN-74) or 200 μmol/L (MKN-45, MKN-28 and KATO-III). Control cultures contained 0.2% DMSO in place of calcium antagonists.

The significance of differences between means was estimated using Student's *t*-test.

Results

Induction of cell death by ethanol treatment

The effects of ethanol on cell death were determined in the gastric carcinoma cell lines at various timepoints of treatment and ethanol concentrations (Fig. 1). The percentages of trypan blue-positive cells increased in a dose-dependent manner in the cell lines. With 10% ethanol treatment, the proportion of trypan blue-positive cells rapidly increased to more than 77% at 6 h of culture in all the cell lines. Treatments with 3% and 5% ethanol induced cell death in a time-dependent manner in all cell lines except MKN-28. No effect was observed with 1% ethanol.

Figure 2 shows the effects of ethanol on the induction of DNA fragmentation, which was estimated by the TUNEL method. In the cell lines MKN-74, MKN-45 and TMK-1, the percentages of TUNEL-positive cells increased in a time-dependent manner when the cells were treated with 5% ethanol. The signals of MKN-74, MKN-45 and TMK-1 were in 35.7 ± 13.8%, 54.2 ± 15.2% and 37.3 ± 13.6% at 48 h of culture, respectively. However, the percentages of TUNEL-positive cells in TMK-1 treated with 3% ethanol were 32.3 ± 10.1% at 12 h and 15.6

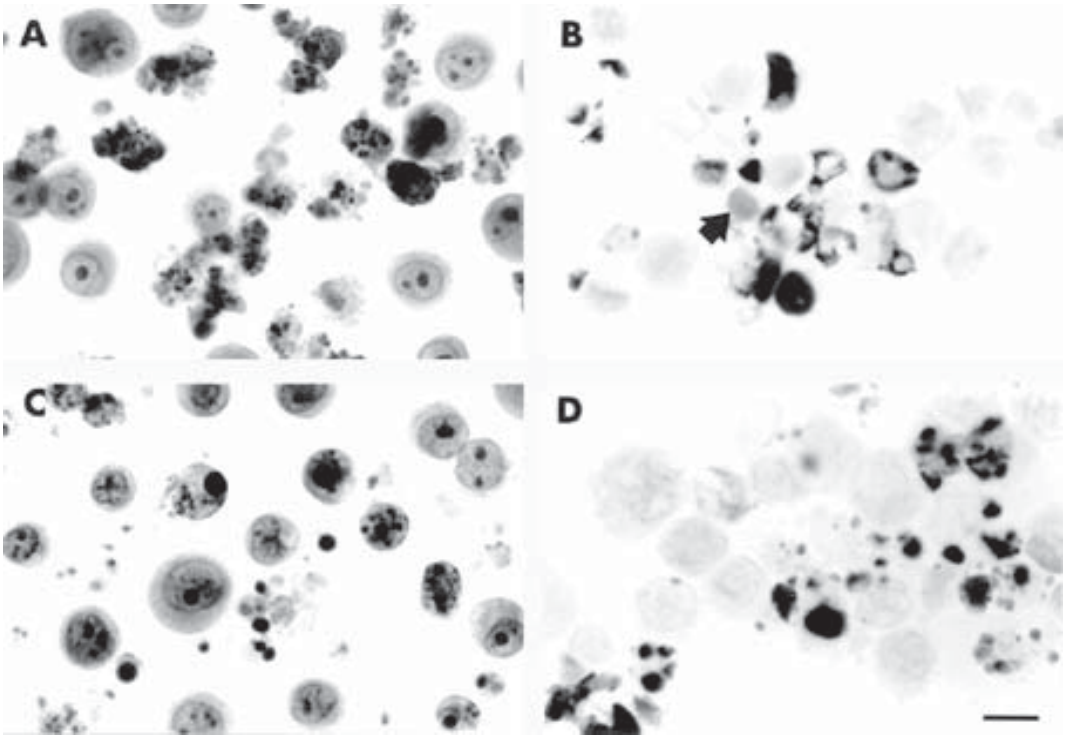

Fig. 3. Morphological features of apoptosis induced by ethanol in MKN-74 (**A, B**) and TMK-1 (**C, D**) cells stained with Papanicolaou stain (**A, C**) and the TUNEL method (**B, D**). MKN-74 cells were exposed to 5% ethanol for 48 h (**A, B**) and TMK-1 cells were exposed to 3% ethanol for 12 h (**C, D**). **A, C**: Cells had nuclear condensation, fragmentation or apoptotic bodies. **B, D**: TUNEL signals were found in the fragmented and condensed nuclei. A cell with a condensed nucleus had no TUNEL signals (arrow). Scale bar = 10 μm.

± 3.7% at 48 h of culture. On the other hand, the TUNEL signals were 4.7 ± 0.8% of MKN-28 and 17.2 ± 7.6% of KATO-III cells at 48 h of culture with 5% ethanol. The percentages of TUNEL-positive cells also increased with 10% ethanol in a time-dependent manner in all cell lines except MKN-74.

Morphologically, the typical features of apoptosis were seen in the cells treated with 3% or 5% ethanol with Papanicolaou stain (Figs. 3A and C). TUNEL signals were found in condensed or fragmented nuclei, and also in some normal-looking nuclei (Figs. 3B and D). A few cells with fragmented or condensed nuclei had no TUNEL signals in the TMK-1 (Fig. 3D) and KATO-III (data not shown) cultures. Cells cultured with 10% ethanol exhibited a necrotic appearance in all of the cell lines. These cells had nuclei that were reduced in size and indistinct vacuolated cytoplasm on Papanicolaou-stained materials. TUNEL signals with various intensity were found in the nuclei (Fig. 4).

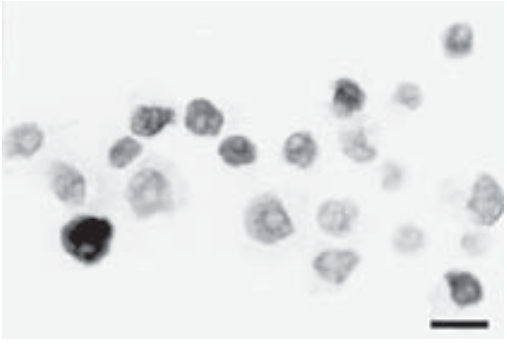

Fig. 4. Necrotic cells stained by the TUNEL method. TMK-1 cells were exposed to 10% ethanol for 48 h and stained by the TUNEL method. They had various intensities of TUNEL signals. Scale bar =10 μm.

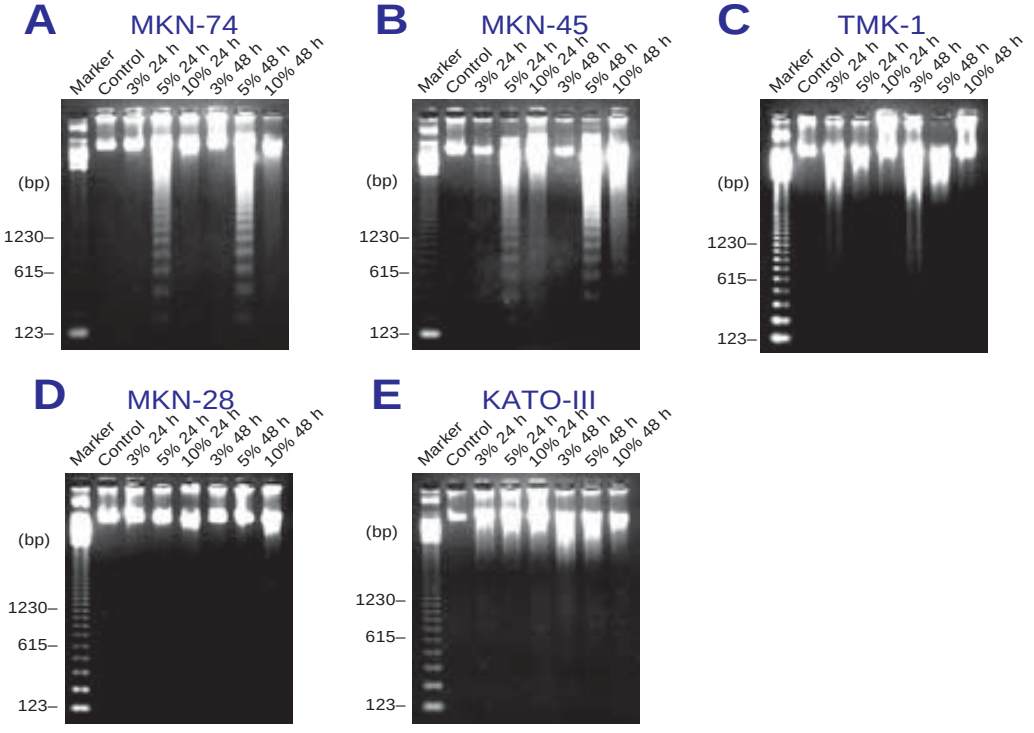

Fig. 5. DNA fragmentation analysis by agarose-gel electrophoresis. DNA fragmentation in MKN-74 (**A**), MKN-45 (**B**), TMK-1 (**C**), MKN-28 (**D**) and KATO-III (**E**) cells treated with ethanol. Each cell line was exposed to 0% (control), 3%, 5%, and 10% ethanol for indicated time. The DNA ladder of apoptosis was seen in MKN-74 (**A:** 5% ethanol 24 h and 48 h), MKN-45 (**B:** 5% ethanol 24 h and 48 h) and TMK-1 cells (**C:** 3% ethanol 12 h and 24 h, 5% ethanol 24 h). The DNA smear pattern of necrosis was seen in cell lines exposed to 10% ethanol. Marker, 123 base pair DNA ladder molecular size marker.

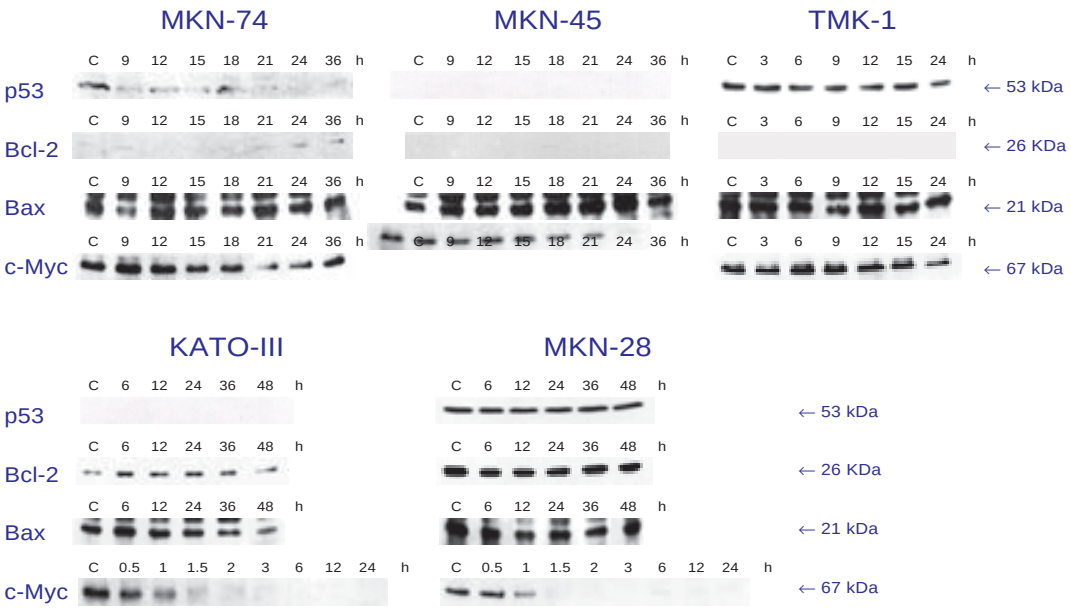

Fig. 6. Western blot analysis of p53, Bcl-2, Bax and c-Myc proteins in MKN-74, MKN-45, TMK-1, KATO-III and MKN-28 cells. MKN-74, MKN-45, KATO-III and MKN-28 cells were incubated with 5% ethanol and TMK-1 cells were incubated with 3% ethanol for indicated times. **C**, without ethanol (control).

Analysis of DNA fragmentation by agarose gel electorophoresis

To determine whether the DNA degradation was apoptosis or necrosis, the DNAs obtained from each cell line were analyzed by agarose gel electrophoresis. The "ladder" pattern, a hallmark of apoptosis, clearly appeared on the electrophoresis of MKN-74 and MKN-45 cells treated with 5% ethanol and TMK-1 cells treated with 3% ethanol (Figs. 5A, B and C). No ladder pattern was observed in all DNAs from MKN-28 and KATO-III cells (Figs. 5D and E). DNA from all five of the cell lines treated with 10% ethanol showed a smear-like, non-specific degradation, regarded as characteristic of necrosis (Fig. 5).

Expression of p53, Bcl-2, Bax and c-Myc protein (Fig. 6)

For the determination of the expression of the apoptosis-related genes, cells were cultured with 3% or 5% ethanol, and then cellular lysates were analyzed by Western blot. The expression levels of p53, Bcl-2 and Bax proteins did not change during the ethanol exposure in any of the cell lines. The expression of p53 protein was higher in the TMK-1 and MKN-28 cells, which carry a mutated *p53* gene, compared to the MKN-45 and MKN-74 cells, which carry the wild-type *p53* gene. The KATO-III cells (with the *p53* gene deleted) did not express p53 protein. The expression level of Bcl-2 protein was higher in the MKN-28 and KATO-III cells than in the other three lines, in the presence or absence of ethanol. MKN-74, MKN-45 and TMK-1 expressed c-Myc protein in the presence or absence of ethanol. The c-Myc protein expressions in MKN-28 and KATO-III were apparently down-regulated after 1.5 h with 5% ethanol treatment and only slightly detected at 24 h.

Effect of calcium antagonists on ethanol-induced apoptosis (Fig. 7)

The role of calcium on the induction of apoptosis was examined using the calcium ion channel

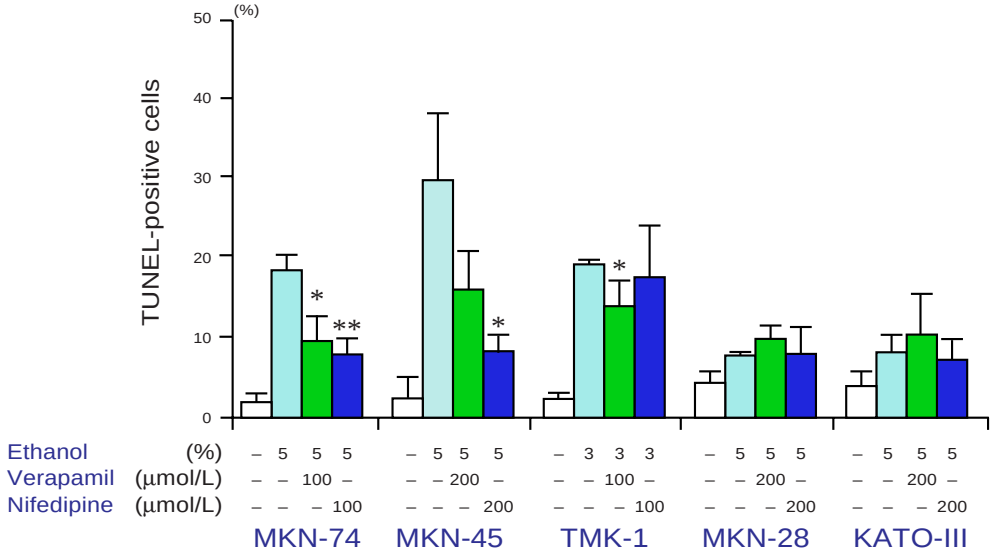

Fig. 7. Effect of calcium ion channel antagonists, verapamil and nifedipine, on apoptosis induced by ethanol. TMK-1 cells were treated with ethanol with/without a calcium ion channel antagonist for 12 h, and the other cell lines were treated for 24 h. Results were shown as the percentage of TUNEL-positive cells. The data are mean ± SD of three separate experiments. **P* < 0.05, ***P* < 0.01 compared with ethanol only-treated cells.

antagonists verapamil and nifedipine (Tepperman and Whittle, 1991). In the MKN-74 cells, incubation with verapamil or nifedipine significantly reduced the percentages of ethanol-induced TUNEL-positive cells. MKN-45 and TMK-1 cells pretreated with verapamil or nifedipine showed decreased TUNEL-positive cells in the ethanol treated cells. The antagonists did not reduce the percentages of TUNEL-positive cells in the MKN-28 and KATO-III cell lines.

Discussion

In this study, it was demonstrated that ethanol induced cell death via apoptosis or necrosis in human gastric carcinoma cell lines in a concentration-dependent manner. The characteristic morphology of apoptosis and TUNEL signals were found in all five cell lines treated with 3% or 5% ethanol, while DNA ladder formation, one of the features of apoptosis (Wyllie, 1980), was confirmed in MKN-74, MKN-45 and TMK-1, but not in MKN-28 and KATO-III cells treated with 5% or 3% ethanol. More than 77% of the cells showed necrotic features with DNA smear patterns in all of the cell lines treated with 10% ethanol at 6 h after the treatment. Trypan blue-positive cells were found in more than 91%, and TUNEL-positive cells were detected in more than 21% of all cell lines treated with 10% ethanol, at 48 h of culture. These results indicated that TUNEL signals are also observed in necrotic cells, as reported by Kressel and Groscurth (1994). The present results could be roughly summarized that 3% or 5% ethanol induces apoptosis, whereas 10% ethanol causes necrotic cell death in gastric carcinoma cells. The results are consistent with the previous reports which described apoptosis induced by ethanol in HL-60 cells, thymocytes and lymphocytes (Lennon et al., 1991; Slukvin and Jerrells, 1995; Kravtsov and Fabian, 1996).

A variety of protein molecules or oncogenes and suppressor genes are involved in the process of apoptosis. p53 gene product has been shown to be required for the induction of the apoptotic pathway triggered by oncogeneous activation and cytotoxic genes (Lowe et al., 1993a, 1993b). The product may sensitize damaged cells to apoptosis, acting to prevent

the propagation of transforming mutations. Fritsche and colleagues (1993) demonstrated that anticancer agents such as cisplatine, mitomycin C, etoposide and 5-fluorouracil (5-FU), as well as energy-rich radiation act on cellular DNA. These agents induced a nuclear accumulation of p53 protein followed by apoptosis. Osaki and colleagues (1997) have reported that 5-FU induced apoptosis of the human gastric carcinoma cell lines MKN-45 and MKN-74 (the wild-type *p53* gene), but not in MKN-28 (the mutated p53 gene) and KATO-III (lacking the *p53* gene). The induction of p53 protein expression was noted in the former two, but not in the latter two cell lines. In the present study, p53 protein expression levels in all five of the cell lines were unchanged in the process of ethanol-induced apoptosis. Therefore, the results have indicated that p53 protein does not play the role in ethanol-induced apoptosis.

Ethanol also did not alter the expression levels of Bcl-2 and Bax proteins in the process of apoptosis induction in any of the cell lines. What's interesting is that 3% or 5% ethanol hardly induced apoptosis in MKN-28 and KATO-III cells, which showed higher expression levels of Bcl-2 protein compared to the other three cell lines. Our results indicate that higher expression of Bcl-2 protein in these gastric carcinoma cells may be associated with the inhibition of ethanol-induced apoptosis. A higher expression of Bcl-2 protein inhibited the induction of apoptosis with various agents in a variety of myeloid leukemia cells (Campos et al., 1993; Lotem and Sachs, 1993).

Overexpression of the *c-myc* gene has been shown to be strongly associated with various human cancers. The constitutive activation of c-Myc protein of cultured cells leads to proliferation and cellular transformation. Moreover, overexpression of c-Myc protein might accelerate cell death via apoptosis (Askew et al., 1991; Evan et al., 1992) in a p53-dependent or -independent mechanism in epithelial cells (Sakamuro et al., 1995). In the present study, the expression level of c-Myc protein did not change throughout the course of apoptosis in MKN-74, MKN-45 and TMK-1 cells treated with 5% ethanol. Thus, the results do not indicate that c-Myc protein plays an important role in the apoptosis signal pathway in these cell lines. On the other hand, the c-Myc protein expression was reduced at 1.5 h and the reduction was sustained up to 24 h in the two apoptosis-resistant cell lines MKN-28 and KATO-III. Dong and colleagues (1997) reported that the lower expression of c-Myc protein is strongly associated with the resistance to etoposide-induced apoptosis in human monocytic leukemia cells, and that c-Myc protein plays a role in cellular susceptibility to death receptor-mediated and chemotherapy-induced apoptosis. It may be that the reducing c-Myc protein expression is also related to the resistance to ethanol-induced apoptosis.

Tarnawski and colleagues (1990) have reported that the influx of extracellular calcium is necessary for human gastric gland cell injury. Alterations in intracellular calcium ion homeostasis are commonly observed during apoptosis, and insights into how calcium ions might regulate the caspases and other components of the pathway are now emerging (McConkey and Orrenius, 1997). The presence of extracellular calcium was found to be necessary for the induction of apoptosis with ethanol (Lennon et al., 1991; Slukvin and Jerrells, 1995), and the cytosolic free calcium increase triggered by ethanol may be a part of the signal pathways leading to apoptosis (Shao et al., 1995). In the present study, verapamil and nifedipine, which inhibit calcium entry into the cell, partially inhibited the induction of apoptosis by ethanol in MKN-74, MKN-45 and TMK-1 cells, which have a lower expression of Bcl-2 protein. It is reasonable to assume that a calcium influx through calcium ion channels by ethanol triggers apoptosis, and that cytosolic free calcium in apoptotic cells increases and regulates several steps in the apoptotic pathway. In addition, the calcium antagonists did not inhibit the induction of apoptosis by ethanol in MKN-28 and KATO-III cells, which have a higher Bcl-2 protein expression. Lam and colleagues (1994) have shown that an overexpression of Bcl-2 protein interferes with thapsigargin-induced calcium ion mobilization from the endoplasmic reticulum in the mouse lymphoma W.Hb12

cells. Additionally, Marin and colleagues (1996) reported that Bcl-2 protein blocks the accumulation of calcium ion in the nucleus in cells exposed to tumor necrosis factor or thapsigargin. Therefore, the overexpression of Bcl-2 protein in MKN-28 and KATO-III cells might interfere with an ethanol-induced calcium ion mobilization or the accumulation of calcium ion in the nucleus.

In summary, this initial study indicated that less than 5% ethanol induced apoptosis by the influx of calcium ions across the plasma membrane and that p53 and c-Myc protein were not associated with the process of apoptosis, while 10% ethanol provoked necrosis in five human gastric carcinoma cell lines. The two ethanol-induced apoptosis-resistant cell lines showed a higher expression of Bcl-2 protein, the down-regulation of c-Myc protein, and no effects of calcium ion channel antagonists. The precise role of these molecules and intracellular signal transduction in the process of ethanol-induced apoptosis await further investigation, for the clarification of the mechanism and regulation of apoptosis in human gastric carcinomas.

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