Summary. We developed a chronic drinking rat model to investigate the long-term effects of ethanol feeding on cell proliferation and apoptosis in rat stomach. Adult male Sprague-Dawley (SD) rats received either an isocaloric control or drinking water containing 6% (v/v) ethanol as their only water intake for 1, 3, 7, 14 and 28 days. At the end of each feeding period, animals were sacrificed and the stomach was dissected for the sample preparation. The cell proliferation and apoptosis in gastric mucosa of rats in different groups were analyzed by flow cytometry, immunohistochemistry and computer image analysis. In the flow cytometric study, compared with the control, the cell apoptosis in gastric mucosa of the rats was enhanced during the exposure to the ethanol in 3rd to 28th day. Otherwise the cell proliferation was increased in 3rd to 14th days, and decreased in 28th days, respectively. The results were confirmed by immunohistochemistry and computer image analysis studied. This finding suggested that short-term chronic adequate alcohol intake may enhance the cell turnover of gastric mucosa and may be one of the mechanisms underlying the gastric pathology associated with alcohol abuse.

Key words: Apoptosis, Proliferation, Ethanol, Stomach, Rat

Introduction

Ethanol is one of the most widely used and abused drugs. Excess alcohol consumption may induce or aggravate various types of disease. In particular, it is regarded as an important factor for ulcerative and inflammatory lesion gastric mucosa (Luis, 2000). However, the pathogenesis of this lesion remains poorly understood. The gastric epithelial cells are continuously renewed in the whole extension of the gland (Alvares, 1992; Alvares and Gama, 1993). The homeostatic regulation of cell numbers in normal tissues reflects a highly regulated balance between cell proliferation and cell death (McDonnell, 1993). The imbalance of cell proliferation and apoptosis has been implicated in the pathogenesis of gastric mucosal lesions (Thompson, 1995; Potten and Booth, 1997). Chronic ethanol consumption resulting in gastric mucosal lesions might thus be expected to influence the kinetic balance between cell proliferation and cell death. Some reports have indicated that ethanol-associated gastric cell proliferation may involve changes in epidermal growth factor receptors (Wang et al., 1996, 1997) and oxidative stress and lipid peroxidation (Hernandez-Munoz et al., 2000).

Animal investigations with various kinds of ethanol feeding have been performed. For example, Lieber and DeCarli (1994) reported that ethanol was fed in the diet with 5% concentration (w/v). Turchan et al. (1999) added the ethanol in the drinking water with the concentration of 6% (v/v). In this study, we have developed a chronic drinking rat model with continuous ethanol ingestion for 28 days using a modified method of Turchan’s (Turchan et al., 1999). Using this model, we investigated the relationship between chronic ethanol intake and the cell turnover related to cell proliferation and apoptosis. The cell proliferation and apoptosis were analyzed by flow cytometer. To confirm the cytometric results, we also investigated the expression of cell proliferation and the apoptosis marker in the rat gastric mucosa. For this purpose, double-staining using the antibody to proliferating cell nuclear antigen (PCNA) and cytokeration 18 were performed in the same slide and computer image analysis was used to quantify the PCNA and cytokeration 18 labeling.

Materials and methods

Animals and treatments

Male Sprague-Dawley rats (200-250g) were used in this study. Sixty rats were housed in plastic cages in an
air-conditioned and light controlled room at 24±2°C and 60±5% humidity. After a 3-day adaptation period, animals were divided into six groups with different feeding periods of 1, 3, 7, 14, 28 days. Rats received drinking water containing 6% (v/v) ethanol as their only water intake by the method of Turchan et al. (1999), control drinking water contained an equal caloric amount of dextrose. Water was offered ad libitum to another group of animals for 28 days as the control group. Each group contained 10 rats. At the end of each feeding period, the rats were anesthetized by urethane and sacrificed. The stomachs were dissected and used for this study. Ethanol consumption averages 6.52 g/kg body weight per day and the plasma ethanol concentrations at time of stomach excision averaged 18.47 mmol/L in this model.

Dissociation of cells from stomach

The stomach cells dissociation was performed as described by Zavros et al. (2000) with slight modification. The stomachs were quickly excised from seven rats in each group, washed in PBS (4°C), and transferred to cold wash buffer. The stomach was opened along the great curvature and rinsed with Hanks’ balanced salt solution (Gibco BRL) with 0.1% bovine serum albumin (BSA). Dissociation of gastric cells was performed by incubating the whole stomach in 1% pronase (Merck USA) dissolved in 0.9% saline for 1.5 hr at 37°C. The solution was removed and replaced with fresh solution every 30 min. The cells were then treated with DNase I at 37°C for 30 min, washed with cold Hanks’ balanced salt solution with 0.1% BSA, and centrifuged at 3000 rpm for 5 min at 4°C. The cells were filtered through a 40-µm nylon cell mesh. Viability and yield were assessed by trypan blue exclusion. Approximately 5x10^6 cells in each rat were analyzed by flow cytometry.

Flow cytometry

Aliquot cells (5x10^6 cells/tube) were washed in ice-cold PBS, fixed in ice-cold 70% ethanol and stored at 4°C. They were then washed with PBS and treated with RNase (500/ml: Sigma) for 15 min at 37°C. Propidium iodide (PI) (Sigma Chemical Co., Missouri) at 50µg/ml in PBS was used to stain DNA. Cell cycle analysis was performed using a Becton Dickinson fluorescence-activated cell analyzer (Becton Dickinson Co, CA) and the Cellfit cell-cycle analysis software program provided by the manufacturer. Approximately 15000 cells were counted for each determination.

Histological and immunohistochemical analyses

The stomachs were excised from three rats in each group and fixed in 10% neutral buffered formalin, embedded in paraffin and sectioned at 5µm for H&E and PCNA-cytokeratin 18 double staining. H&E staining was performed according to the routine standard procedures. The total cell number of each gastric gland was counted from the uppermost parietal cell to the fundus of each gland of every 10th gland of the first 100 glands viewed per slide for a total of 10 glands. Results are expressed as means of numbers±S.D. Slides were examined in a blinded fashion by coding them in a way so the examiner was unfamiliar with the experimental protocol (Schmidt et al., 1985). PCNA-cytokeratin 18 double staining was performed according to the modified technique of Greenwell et al. (1991). Briefly, the sections deparaffinized in xylene, cleared in graded ethanol to PBS. Sections were then quenched in 3% hydrogen peroxide in 0.1% sodium azide to suppress the endogenous peroxidase activity and placed in an antigen retrieval solution consisting of 1% zinc sulfate in deionized water and irradiated for 7.5 min in a 700 W microwave oven on full power. A routine streptavidin–biotin protocol using the DAKO LSAB + Kits (HRP and AP) (Dako Japan, Kyoto, Japan) was applied. The tissue sections mounted on glass slides were first incubated in 0.5% BSA in PBS to reduce nonspecific protein binding, and then sequentially incubated to react with polyclonal anti-PCNA primary antibody (NeoMarkers Lab Vision Corporation, CA) overnight at 4°C. The antibody was then linked with streptavidin conjugated to horseradish peroxidase (HRP). HRP sites were visualized with 3,3’-diaminobenzidine (DAB) and H2O2. The slides were immersed three times with 0.1 M glycine-HCl buffer (pH 2.2) for 30 min. After washing with Milli-Q water once and with PBS three times, the sections were reacted with monoclonal M30 CytoDEATH antibody (clone M30; Roche, Mannheim, Germany) and AP-goat antimouse IgG successively, similar to that described above. The sections were visualized with nitroblue tetrazolium chloride (NBT)/5-bromo-4-chlor-3-indolyl-phosphate (BCIP) in the dark. As a negative control, some sections were reacted with normal mouse IgG or normal rabbit IgG instead of the specific antibody.

Quantitation of PCNA and M30 CytoDEATH labeling

An image analysis system (NYD100) was used for quantitative analysis of cell density (cell number/view field) of the PCNA-positive and M30 CytoDEATH-positive cells in the rat stomach. Three sections from three rats were used. PCNA-positive and M30 CytoDEATH-positive cells were counted in five randomly selected view fields per section at a magnification of x400. At least 15 view fields in each group were analyzed. Results are expressed as means of numbers±S.D.

Statistical analysis

One-way analysis of variance (ANOVA) was used to estimate the significance of data obtained. P value less than 0.05 was considered to be significantly different.
Student's t-test was used for the comparison of data between groups.

Results

Flow cytometric analyses

Compared to the control groups, the percentage of cells in S-phase was significantly increased in the rats treated with 6% ethanol for 3, 7 and 14 days, and decreased in the animals treated with ethanol for 28 days. The percentage of apoptotic cells in gastric mucosa of rats was significantly enhanced during exposure to the ethanol (data described in Table 1, Fig 1).

Histological analyses

There was no inflammatory proliferative change in the gastric mucosa in each group of rats treated with ethanol, even those exposed to the ethanol for 28 days (Fig. 2A). The mean total cell number was described in Table 2.

Immunohistochemical analyses

The positive PCNA-stained cells with uniformly

<table>
<thead>
<tr>
<th>Groups</th>
<th>S phase cells</th>
<th>Apoptosis cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>3.31±0.05 a</td>
<td>0.03±0.00 a</td>
</tr>
<tr>
<td>1st</td>
<td>3.44±0.07 a</td>
<td>0.09±0.01 a</td>
</tr>
<tr>
<td>3rd</td>
<td>5.16±0.10* a</td>
<td>0.40±0.05* a</td>
</tr>
<tr>
<td>7th</td>
<td>6.53±0.04* a</td>
<td>0.43±0.07** a</td>
</tr>
<tr>
<td>14th</td>
<td>7.84±0.09** a</td>
<td>0.60±0.05** a</td>
</tr>
<tr>
<td>28th</td>
<td>2.40±0.01* a</td>
<td>0.33±0.04*a</td>
</tr>
</tbody>
</table>

*: P<0.05; **: P<0.01 VS Controls, a: P<0.05 seven samples were taken from each group and the number of cells in each group were at least more than 10000; (%; mean±SD, n=7).

<table>
<thead>
<tr>
<th>Groups</th>
<th>1st</th>
<th>3rd</th>
<th>7th</th>
<th>14th</th>
<th>28th</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>54.8±0.5</td>
<td>56.1±0.5</td>
<td>55.9±0.1</td>
<td>56.2±0.4</td>
<td>55.7±0.5</td>
</tr>
<tr>
<td>1st</td>
<td>54.8±0.5</td>
<td>56.1±0.5</td>
<td>55.9±0.1</td>
<td>56.2±0.4</td>
<td>55.7±0.5</td>
</tr>
<tr>
<td>7th</td>
<td>54.8±0.5</td>
<td>56.1±0.5</td>
<td>55.9±0.1</td>
<td>56.2±0.4</td>
<td>55.7±0.5</td>
</tr>
<tr>
<td>14th</td>
<td>54.8±0.5</td>
<td>56.1±0.5</td>
<td>55.9±0.1</td>
<td>56.2±0.4</td>
<td>55.7±0.5</td>
</tr>
<tr>
<td>28th</td>
<td>54.8±0.5</td>
<td>56.1±0.5</td>
<td>55.9±0.1</td>
<td>56.2±0.4</td>
<td>55.7±0.5</td>
</tr>
</tbody>
</table>

*: P<0.05, VS controls; (number/gland, mean±SD, n=5).
intense brown nuclei were located in the neck portion of the rat fundic gland in control rats, the position is the same as reported by Yang et al. (1997) labeled gastric mucosa stem cells by Brdu. M30 CytoDEATH positive cells with intense blue cytoplasm were scattered in the surface of the rat fundic gland (Figure 2-B). Significantly increased numbers of PCNA and M30 CytoDEATH positive cells were observed in the fundic gland of the rats treated with the ethanol for 3 to 14 days (Fig. 2C and Table 2). The decreased PCNA positive cells and increased M30 CytoDEATH positive cells were detected in the gland of the rats treated with the ethanol for 28 days (Fig. 2D and Table 3).

Discussion

Cell proliferation can be measured by tritiated thymidine incorporation followed by autoradiography (Langer et al., 1985; Eldridge et al., 1990; Lok et al., 1990), bromodeoxyuridine (Brdu) incorporation followed by anti-Brdu immunohistochemistry (Langer et al., 1985; Kamata et al., 1989; Eldridge et al., 1990; Arbern et al., 1994), flow cytometric cell cycle analysis (Langer et al., 1985; Lu et al., 1991; Beppu et al., 1994), and immunohistochemical labeling of proliferating cell nuclear antigen (PCNA) (Sarrat et al., 1991; Dietrich, 1993; Beppu et al., 1994). All these methods can identify the DNA synthesizing S-phase cells (Langer et al., 1985) in the cell cycle. The data obtained from the labeling of S-phase cell in the cell cycle provide information about activity of cell proliferation in the tissues. The level of apoptosis was often assessed using propidium iodide (PI) staining (Koopman et al., 1994; Kieslinger et al., 2000), and the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay, which identifies DNA strand breaks by labeling free 3’OH termini with modified nucleotides (Nagarkatti, 2000; Moulian et al., 2001). Recently, a monoclonal antibody by M30 CytoDEATH antibody directed against a neo-epitope of cytokeratin 18, which is formed after cleavage of this cytoskeletal protein by caspases, was shown to be of advantage over other tests for the detection of cell apoptosis (Leers et al., 1999; Austgulen et al., 2002; McPartland et al., 2005). The immunoreactivity of the M30 antibody is confined to the cytoplasm of apoptotic cells. The present study is designed to investigate, by immunohistochemical labeling of PCNA and M30 CytoDEATH combined flow cytometric cell cycle analysis, the effect of ethanol on cell proliferation and apoptosis in the rats that received 6% ethanol for 28 days, using an alcohol administration animal model, allowed to consume alcohol voluntarily. This method is commonly used in the study of long-term alcohol intake (Turchan et al., 1999).

To our knowledge, the present study is the first report on the effect of long-term treatment with ethanol on cell proliferation and apoptosis in rat gastric mucosa in vivo. The present study demonstrated a clear increase in the apoptosis cells in the stomach of animals subjected to ethanol treatment for 3 to 28 days. The proliferative cell numbers were increased in the ethanol

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**Table 3. Numbers of the PCNA and M30 CytoDEATH positive cells in the gastric Mucosa of the chronic drinking rats.**

<table>
<thead>
<tr>
<th>Groups</th>
<th>PCNA cells</th>
<th>M30 CytoDEATH cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>21.6±0.8</td>
<td>3.5±0.8</td>
</tr>
<tr>
<td>1st</td>
<td>19.8±0.5</td>
<td>4.3±0.6</td>
</tr>
<tr>
<td>3rd</td>
<td>34.9±1.0*</td>
<td>12.7±0.9*</td>
</tr>
<tr>
<td>7th</td>
<td>42.3±0.7**</td>
<td>16.8±0.5**</td>
</tr>
<tr>
<td>14th</td>
<td>39.7±0.8**</td>
<td>18.1±0.1**</td>
</tr>
<tr>
<td>28th</td>
<td>4.0±0.5**</td>
<td>24.1±1.1**</td>
</tr>
</tbody>
</table>

*: P<0.05; **: P<0.01 VS Controls; (numbers/view field, mean±SD, n=15).
treated rats for 3 to 14 days, and decreased at 28 days. No inflammatory proliferative change was found in the gastric mucosa in each group of rats treated with ethanol, even those exposed to the ethanol for 28 days (Fig. 2A). These results suggested the enhancement of the cell turnover of gastric mucosa in the period of ethanol treatment for 3 to 14 days, in which both cell proliferation and cell apoptosis is increased and the total number of cells have no change in the ethanol treated rats in this duration. These results also showed a decrease of cell turnover in the gastric mucosa of the rats treated with the ethanol for 28 days by a reduction in cell proliferation and enhanced cell death by apoptosis and a decrease in the total number of cells was found in the gastric mucosa of these rats. The imbalance of cell proliferation and apoptosis has been implicated in long-term ethanol treatment.

The ability of the gastric mucosa to resist injury by endogenous secretions (acid, pepsin, and bile) and by ingested irritants (e.g., alcohol, NSAIDs) can be attributed to a number of factors that have been collectively referred to as “mucosal defense” (Wallace and Granger, 1996). The gastric mucosal barrier is a complex system made up of submucosal epithelial and mucus elements (Allen et al., 1993). Epithelial cell proliferation and migration leads to re-epithelialization of the ulcer crater, and the reconstruction and differentiation of glands. The functional integrity of the gastric mucosa and its secretory pit-gland units is maintained through the constant renewal of the epithelium. The ongoing generation of new epithelial cells is a fundamental mechanism in mucosal protection of the stomach, suggesting that undamaged epithelial renewal function is required and coordination activities of cell proliferation and apoptosis would be necessary for normal progression of post-wounding epithelial repair and successful mucosal recovery (Silen and Ito, 1985). The current study clearly demonstrates that ethanol was involved in the regulation of in vivo regeneration of rat gastric epithelial cells. Increased cell proliferation of the stomach in short-term ethanol feeding (3-14 days) is a regenerative change subsequent to ethanol-induced chronic mucosal injury (Hernandez-Munoz et al., 2000), and an adaptive cytoprotection in gastric mucosa was correlated with the enhanced cell turnover rate (data not shown). Long-term (28 days) stimulus with the low concentration (6%) ethanol may reduce the cell turnover of the gastric mucosa and decrease the capacity of regeneration, which leads to the pathogenesis of mucosal injuries such as ulcer and gastritis. This finding is in agreement with those of Clayman (Clayman and Moncada, 1968), who reported that atropic gastric mucosa has an increased susceptibility towards damaging effects.

Growth factors promote the proliferation of epithelial cells and their migration over the ulcer crater (Tarnawski et al., 1992; Szabo et al., 1994; Li et al., 1999). Chronic ethanol treatment could cause an alteration of epidermal growth factor receptor in the stomach (Wang et al., 1996, 1997). Without further examination we can not rule out the possibility that the increased cell proliferation of the gastric mucosa is a result of the changes of growth factors and their receptors. The ethanol apparently can directly stimulate cell apoptosis in the stomach of the rats because the M30 CytoDEATH labeled cells were observed sporadically in the control animals and diffusely in the rats treated with ethanol for 3 to 28 days (Fig. 2B, C, D). More investigations are needed to delineate the specific intracellular signaling pathways involved in the ethanol modulation of gastric epithelial proliferation and apoptosis. Moreover, further evaluation will be required to draw real clinical implication of long-term ethanol drinking.

In conclusion, long-term chronic ethanol feeding (28 days) resulted in decreases of cell proliferation and increases of cell apoptosis. This impairment of the cell turnover function of gastric mucosa may be associated with a weakening of the cytoprotective function of the stomach and be related to ethanol-induced gastric lesions, which may be one of the mechanisms underlying the gastric pathology associated with alcohol abuse.

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