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Chapter 10

Studies on the Stable Cell Lines (Gastric Cancer, Hepatoma, Colorectal Cancer, Mouse Myeloma)

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10.1. Introduction

The original term “gastric cytoprotection” described by Robert in 1979 (Robert, 1979; Robert et al., 1979) has new meaning nowadays. The “cytoprotection” expression has been limited to the level of cells, and new concepts have been defined, such as histoprotection and organoprotection (gastroprotection), which are widely accepted and recently studied in experiments searching for other fields of gastroenterology (e.g., hepatology). In these in vivo investigations, many environmental factors, such as central nervous system (Grijalva and Novin, 1990), vagal nerve (Mózsik et al., 1993a, b, 1992a, b, c, d), blood flow (Szabó et al., 1985; Guth et al., 1984), vascular permeability (Szabo et al., 1985) and rapid epithelial restitution of neck cell (Lacy et al., 1984), were studied as a part of defense mechanisms of the gastric mucosa.

The in vivo and in vitro experiments essentially differ from each other. In vitro investigations on isolated cells have many advantages. All of the environmental effects originated from other organs, tissues can be eliminated, so the behavior of a single cell can purely be examined. Unfortunately, these kinds of studies might have harmful consequences. The acutely isolated cells are less suitable for pharmacological studies than the gastric mucosa in vivo experiments because of their isolation procedure (Nagy et al., 1994).

The stress may reduce the cell responsiveness; surface receptors are damaged during the digestive stages of the isolation procedure. These effects are eliminated by using stable cultured cells in the experiment, though they are few in number and some properties of the tumor origin can be seen in their behavior.

Ethanol (EtOH) is widely used as a toxic agent in the gastric cytoprotection investigations in vivo (Nagy et al., 1994; Djahanguirí, 1969; Brodie et al., 1970; Karádi et al., 1994; Lacy et al., 1982). Indomethacin (IND) is also generally used for experiments (Djahanguirí, 1969; Brodie et al., 1970; Nagy et al., 1994). It inhibits prostaglandin synthesis, which is responsible for the maintenance of gastric mucosal integrity due to the stimulation of mucosal blood flow,
preservation of cellular-ion transport and protection of the mucosal proliferative zone (Lacy and Ito, 1982). These chemical agents have been widely studied in vivo experiments, but only a few experiments have been carried out in vitro on isolated cells or cell lines with these toxic agents.

Various chemicals have been known and used as cytoprotective agents in the investigation of gastric, liver, pancreatic and large bowel protection; quite a few of them are scavengers (β-carotene, DMSO, DMPO, GSH and mannitol).

10.2. The aims of this present study were the followings

• To analyze the toxic effect of 5-minute EtOH treatment in vitro on acutely isolated mixed gastric mucosal cells (GMC) and on stable cultured cell lines;

• To compare the differences between the GMC and stable cultured cells – myeloma (Sp2/0-Ag14) and hepatoma (Hep G2) cell lines;

• To evaluate the differences between the myeloma (Sp2/0-Ag14) and hepatoma (Hep G2) cell lines;

• To study the effect of IND on GMC and Sp2/0-Ag14 cells;

• To examine the combined effect of EtOH and IND on these two types of cells;

• To analyze the toxic effect of EtOH after an alcohol-dehydrogenase enzyme inhibitor treatment (we used pyrazole for inhibition);

• To examine the scavenger effect of dimethyl sulfoxide (DMSO), glutathione (GSH) and β-carotene on these two types of stable cultured cells.

10.3. Materials and methods

10.3.1. Preparation of mixed gastric mucosal cells

Gastric mucosal cells from Sprague-Dawley rats were isolated by the method of Nagy et al. (1994). The segments of the glandular stomach were separated from the blood vessels and the surrounding connective tissue and were incubated in a physiological solution containing 0.5 mg/mL pronase E (type XXV, Sigma Chemical Co.) and 10^{-3} mol/L EGTA. After several washings, the cells were resuspended in a solution (0.157 mol/L, pH 7.4) produced freshly with the following ingredients: 98.0 mmol/L NaCl, 5.8 mmol/L KCl, 2.5 mmol/L Na_{2}PO_{4}, 5.1 mmol/L sodium pyruvate, 6.9 mmol/L sodium fumarate, 2.0 mmol/L glutamine, 24.5 mmol/L HEPES-Na, 1.0 mmol/L Trizma base, 11.1 mmol/L D-glucose, 1.0 mmol/L CaCl_{2}, 1.0 mmol/L MgCl_{2} and 2.0 mg/mL (w/v) bovine serum albumin. AH examinations were carried out in this solution.

10.3.2. Stable cultured cells

Sp2/0-Ag14 (CRL 1581) is a non-secreting mouse myeloma; Hep G2 is a human hepatocellular carcinoma cell line obtained from the American type culture collection (ATCC). Cells were
cultured and the examinations were carried out in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum in a humidified incubator that held 95% air and 5% CO₂ at 37°C.

10.3.3. Toxicological studies

The cells were incubated with different concentrations of EtOH (1, 5, 10, 15, 20 and 50%) (v/v), IND (10⁻⁸–10⁻³ mol/L dissolved in 5% NaHCO₃, pH 7.4 with 5 N HCl) and their combination (15% EtOH and 10⁻³ mol/L IND) for 5 minutes in a shaking water bath at 37°C. Each study used 10⁵ cells. Alcohol-dehydrogenase inhibitor pyrazol was used during and after the EtOH treatment in the concentration of 10⁻⁵ mol/L. After 5 minutes of incubation, the cells were separated from the supernatant by centrifugation (500 g, 10 minutes), washed out (10 minutes water bath and centrifugation again) and resuspended in a toxic-free medium.

10.3.4. Cytoprotective studies

In the cytoprotective studies, the DMSO was used in the concentration of 10⁻³ mol/L. The GSH was applied to our medium in two concentrations: 10⁻⁴ mol/L and 10⁻³ mol/L. The 10% water-soluble β-carotene from Sigma was used in three concentrations: 10⁻⁸, 10⁻⁷ and 10⁻⁶ mol/L.

1.3.5. Trypan blue exclusion test

Trypan blue is taken up by damaged cells, staining the cytoplasm blue; viable cells can resist this staining. Trypan blue (0.2%) was mixed with the same volume of cell suspension and, after 5-minute latency, the numbers of stained (dead) and unstained (viable) cells were calculated as percentages in a hemocytometer. In comparison with stable cultured cells, we examined the viability for longer periods (5 minutes, 60 minutes, 4 hours and 24 hours) after the 5-minute EtOH incubation.

Statistics

Values in figures and text are expressed as means ± SEM. Comparisons were performed using the unpaired Student’s t-test, and P-values were considered significant at P < 0.05.

10.4. Results

10.4.1. Result of the toxicological studies

10.4.1.1. Effect of EtOH on GMC

EtOH (1, 5, 10, 15, 20 and 50%) concentration-dependently decreased the viability of GMC. The EC₅₀ was 13.5% (Figure 286).

10.4.1.2. Effect of EtOH on the Sp2/0-Ag14 cell line

The EtoH decreased the viability of the stable cultured cells in concentration-dependently. In the case of Sp2/0-Ag14 cells, there is no significant difference between the viability values
obtained at 5 and 60 minutes, but 4 hours after the incubation with 10 and 15% of EtOH, a significant cell loss (secondary cell destruction) could be detected (Figure 287).

Figure 286. Changes in the viability of acutely isolated rat gastric mucosal cells after 5 minutes of incubation with 1–50% ethanol, detected by Trypan blue exclusion test (percentage of control). The results are expressed as means ± SEM ($n=5$).

Figure 287. Changes in the viability of Sp2/0-Ag14 cells after 5 minutes of incubation with 1–20% ethanol (EtOH) counted at 5 minutes, 60 minutes, 4 hours and 24 hours by Trypan blue exclusion test. The results are expressed as means ± SEM ($n=5$). *: $P<0.01$ compared to 4-hour value. [Szabo et al. (1997a) Inflammopharmacology 5:20–28 (with kind permission).]
10.4.1.3. Effect of EtOH on the Hep G2 cell line

In the case of Hep G2 cell line, a higher resistance was found up to 15% of EtOH concentration than in the case of Sp2/0-Ag14 cells. Above that concentration, a similar level of cell destruction occurred. During the 5-minute incubation of Hep G2 cells with various concentration of EtOH (1–20%), secondary cell destruction could not be detected (Figure 288).

Figure 288. Changes in the viability of Sp2/0-Ag14 cells after 5 minutes of incubation with 1–20% ethanol (EtOH) counted at 5 minutes, 60 minutes, 4 hours and 24 hours by Trypan blue exclusion test. The results are expressed as means ± SEM (n=5). *: P < 0.01 compared to 4-hour value. [Szabo et al. (1997a) Inflammopharmacology 5:20–28 (with kind permission).]

10.4.1.4. Comparing gastric mucosal cells(GMCs) and stable cultured cells

At all concentrations, the EtOH decreased the viability of GMC much more potently than the viability of the stable cultured cells. The EC\textsubscript{50} for GMC was 13.5%; the EC\textsubscript{50} for Sp2/0-Ag14 was 16% (Figures 287 and 288).

10.4.1.5. Effect of IND

Five minutes of incubation with 10^{-8}–10^{-3} mol/L IND had no effect on the viability of Sp2/0-Ag14 cells. In the case of GMC, only the highest dose (10^{-3} mol/L) of IND decreased the number of viable cells significantly (P < 0.02) (Figure 289).

10.4.1.6. Combined effect of EtOH and IND

After the combined treatment, greater cell destruction could be detected. While using different concentrations, the 10^{-3} mol/L dose was the most aggressive; the amount of necrotic cell loss
was concentration-dependent (Figure 290). Comparing the response of the GMC with the myeloma cells, the GMC are much more vulnerable than Sp2/0-Ag14 cells after the EtOH treatment and after the combined treatment too (Figure 291).

**Figure 289.** Changes in the viability of GMC and Sp2/0-Ag14 cells after 5 minutes of incubation with $10^{-8}$–$10^{-3}$ mol/L indomethacin, detected by Trypan blue exclusion test (percentage of control). The results are expressed as means ± SEM ($n = 6$–8). *: $P < 0.02$ compared with GMC. [Szabo et al. (1997a) Inflammopharmacology 5:20–28 (with kind permission).]

**Figure 290.** Changes in the viability of gastric mucosal cells (GMCs) after combined incubation with 15% ethanol and $10^{-8}$–$10^{-3}$ mol/L indomethacin, detected by Trypan blue exclusion test (percentage of control). The results are expressed as means ± SEM ($n = 5$). *: $P < 0.02$, **: $P < 0.01$, ***: $P < 0.001$ compared with 15% EtOH alone. [Szabo et al. (1997a) Inflammopharmacology 5:20–28 (with kind permission).]
10.4.1.7. Comparison of myeloma (Sp2/0-Ag14) cells and hepatoma (Hep G2) cells after pyrazole treatment

The viability values of the Sp2/0-Ag14 cells, compared to the values of EtOH treatment, did not change during the 5-minute co-incubation of the alcohol-dehydrogenase inhibitor pyrazole and EtOH (Figure 292).

In the case of Hep G2 cells, adding pyrazole to the incubation medium decreased the viability values, subduing the high resistance observed during the EtOH incubation (Figure 293).

Figure 291. Changes in the viability of GMC and Sp2/0-Ag14 cells after 5 minutes of incubation with 15% ethanol (EtOH), $10^{-8}$–$10^{-5}$ mol/L indomethacin (IND) and 15% EtOH and $10^{-5}$ mol/L IND combined, detected by Trypan blue exclusion test (percentage of control). The results are expressed as means ± SEM ($n = 6$–8). + $P < 0.01$ compared with GMC. [Szabo et al. (1997a) Inflammopharmacology 5:20-28] with kind permission.

10.4.1.7. Comparison of myeloma (Sp2/0-Ag14) cells and hepatoma (Hep G2) cells after pyrazole treatment

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In the case of Hep G2 cells, adding pyrazole to the incubation medium decreased the viability values, subduing the high resistance observed during the EtOH incubation (Figure 293).

Figure 292. Changes in the viability of Sp2/0-Ag14 cells after 5 minutes of incubation with 1–20% ethanol (EtOH) and continuous $10^{-5}$ mol/L pyrazole treatment counted at 5 minutes, 60 minutes, 4 hours and 24 hours by Trypan blue exclusion test. The results are expressed as means ± SEM ($n = 5$).
Figure 293. Changes in the viability of Hep G2 cells after 5 minutes of incubation with 1–20% ethanol (EtOH) and continuous $10^{-4}$ mol/L pyrazole treatment counted at 5 minutes, 60 minutes, 4 hours and 24 hours by Trypan blue exclusion test. The results are expressed as means ± SEM ($n = 5$). [Szabo et al. (1997a) Inflammopharmacology 5:20-28] with kind permission.

Figure 294. Changes in the viability of Sp2/0-Ag14 cells after 5 minutes of incubation with 15% ethanol (EtOH) and continuous $10^{-3}$ mol/L dimethyl sulfoxide (DMSO) counted at 5 minutes, 60 minutes, 4 hours and 24 hours by Trypan blue exclusion test. The results are expressed as means ± SEM ($n = 5$). *$P < 0.05$ compared to 24-hour value of 15% EtOH incubation. [Szabo et al. (1997b) Usage of scavengers on stable cultured (Sp2/0-Ag14 and Hep G2) cell lines as an approach to reveal the mechanisms of cytoprotection. In: Mózsik Gy., Nagy L., Király Á. (Eds). Twenty Five Years of Peptic Ulcer Research in Hungary (1971–1995). Akadémiai Kiadó, Budapest. pp. 275–283 (with kind permission).]
10.4.2. Results of the cytoprotective studies

10.4.2.1. Effect of DMSO

A $10^{-3}$ mol/L DMSO was added to the incubation medium containing 15% of EtOH (for 5 minutes); the viability values of the Sp2/0-Ag14 cells were tested at 4 and 24 hours of incubation. The $10^{-3}$ mol/L DMSO significantly decreased the secondary cell destruction of the Sp2/0-Ag14 cell line (Figure 294).

10.4.2.2. Effect of GSH

Both concentrations ($10^{-4}$ and $10^{-3}$ mol) of GSH used during and after the EtOH incubation significantly increased the viability of the Sp2/0-Ag14 cells at the time period of 4-hour toxic-free incubation (Figure 295). Four hours after the incubation of EtOH, the GSH effect to protect was lost, probably due to its short-life span.

10.4.2.3. Effect of β-carotene

During the co-incubation with 15% of EtOH and $10^{-8}$–$10^{-6}$ mol/L – β-carotene, we could not find any protective effect. The viability values of the Sp2/0-Ag14 cells were much lower than they were obtained in the 15% EtOH incubation. The $10^{-7}$ mol/L dose of β-carotene significantly aggravated the 15% EtOH-induced cell destruction of the myeloma cells ($P < 0.01$) (Figure 296).

Figure 295. Changes in the viability of Sp2/0-Ag14 cells after 5 minutes of incubation with 15% ethanol (EtOH) and continuous $10^{-4}$–$10^{-3}$ mol/L glutathione counted at 5 minutes, 60 minutes, 4 hours and 24 hours by Trypan blue exclusion test. The results are expressed as means ± SEM $(n = 4–6)$. *$P < 0.05$ compared to 4-hour value of 15% EtOH incubation. [Szabo et al. (1997b) Usage of scavengers on stable cultured (SP2/0-Ag14 and Hep G2) cell lines as an approach to reveal the mechanisms of cytoprotection. In: Mózsik Gy., Nagy L., Király Á. (Eds). Twenty Five Years of Peptic Ulcer Research in Hungary (1971–1995). Akadémiai Kiadó, Budapest. pp. 275–283 (with kind permission).]
10.5. Discussion

In these studies freshly isolated rat gastric mucosal cells and two types of stable cultured cells were used to evaluate the effects of EtOH, IND and their combination in toxicological studies. The mixed population of isolated rat GMC contained at least three types of cells: parietal (20–25%), chief (40%) and epithelial (45%). A viability of 80–95% could be maintained for 6–7 hours. These cells do not have the potential for proliferation. Cultured cells were always kept in the same conditions, tests are reproducible and cells can survive for a longer time. During the calculation of viability, it must be remembered that the two cell lines have different proliferation rates. In particular, this should be borne in mind during the interpretation of the different viability values of longer incubation times. Our results indicate that the acutely isolated cells are more vulnerable than cultured cells. After EtOH treatment, in the case of Sp2/0-Ag14 cells, the EC$_{50}$ (16%) was higher than that of GMC (13.5%). IND had no damaging effect on stable cultured cells. The combined treatment reduced the viability in both types of cells, but this effect was much smaller in Sp2/0-Ag14; almost all the GMC were destroyed. These results show that cultured cells are more resistant to toxic agents than acutely isolated cells. Though different types of cells were used in this study, the differences in behavior may derive from the differences in isolation procedure rather than their differences in type. In vivo experiments have shown that gastrointestinal ulceration can be produced by IND administration (Nagy et al., 1994; Djahanguire et al., 1969). It is known that IND inhibits the activity of cyclooxygenase, producing less prostaglandins and excessive vasoconstrictor leukotrienes (Rainsford, 1992).

Figure 296. Changes in the viability of Sp2/0-Ag14 cells after 5 minutes of incubation with 15% ethanol (EtOH) and continuous 10^{-8}–10^{-6} mol/L β-carotene counted at 5 minutes, 60 minutes, 4 hours and 24 hours by Trypan blue exclusion test. The results are expressed as means ± SEM (n = 6). *P < 0.05 compared to 24-hour value of 15% EtOH incubation. [Szabo et al. (1997b) Usage of scavengers on stable cultured (SP2/0-Ag14 and Hep G2) cell lines as an approach to reveal the mechanisms of cytoprotection. In: Mózsik Gy., Nagy L., Király Á. (Eds). Twenty Five Years of Peptic Ulcer Research in Hungary (1971–1995). Akadémiai Kiadó, Budapest. pp. 275–283 (with kind permission).]
and decreases the mucosal level of adenosine triphosphate (Rainsford, 1987). These factors reduce the gastric mucosal resistance to acid. In the in vitro study described here, IND was applied without any other aggressive factor, such as EtOH, and was not toxic for these cells. However, after the combination treatment, cell viability was considerably decreased compared with the effect of EtOH alone. It is likely that the decreased levels of endogenous prostaglandins might play a role in the enhanced toxic effect of EtOH. These results are in good agreement with those of Tarnawski et al. (1988) who observed a protective effect of exogenous prostaglandins on human-isolated gastric glands against IND and EtOH injury.

In our experiment, two types of stable cultured cells were used for cytoprotective studies with DMSO, GSH and β-carotene. In our experiment, when the Hep G2 cells were incubated with an alcohol-dehydrogenase inhibitor, their higher resistance against ethanol disappeared. This observation indicates that the alcohol-dehydrogenase enzyme might have an important role in the high resistance of the Hep G2 cells.

In the case of the Sp2/0-Ag14 cells, a great cell loss (secondary cell destruction) was derived after a long period (4 and 24 hours of incubation time). In the background of this phenomenon, the role of oxygen-free radicals had been suspected and proved (Bódis et al., 2000). Therefore, we tested the effect of the applications of some commonly known scavengers. Dimethyl sulfoxide and glutathione were effective, but the 10% water-soluble β-carotene had no cytoprotective effect. It was possible that the β-carotene with high molecular weight did not have enough time to be taken up by the cells in our experiments. In order to support this assumption, we have studied the effect of a 24-hour preincubation of $10^{-7}$ mol/L β-carotene, but in this case, we also found similar toxic behavior.

The tissue level (organoprotection) and cellular level (cytoprotection) mechanisms of the stomach “cytoprotection” have many similarities, but they also have many differences (e.g., dosages). The details of these differences are not clearly known yet. If we accomplish the tissue and cellular level examinations by choosing different toxic and protective agents, then we will easily be able to understand the effects of toxic agents and the cellular and tissue level protecting reactions generated by them.

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