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APOPTOSIS IN GASTRIC MUCOSA WITH STRESS-INDUCED GASTRIC ULCERS

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The maintenance of gastric mucosal integrity depends upon the interplay between epithelial cell proliferation and apoptosis (programmed cell death). The Bcl-2 family of proteins plays a central role in the regulation of apoptotic cell death by suppressing the apoptosis while some others such as Bax proteins promote this process. Stress-induced gastric ulcerations are accompanied by the fall in gastric mucosal cell proliferation but little is known about the influence of the stress on the apoptosis in gastric mucosa. In the present study, the gastric epithelial apoptosis was determined by means of expression of Bax and Bcl-2 mRNA in the gastric mucosa following acute stress. Wistar rats were exposed to mild water immersion and restraint stress (WRS) for 3.5 h and then sacrificed at 0, 2, 4, 6, 12 and 24 h after the termination of WRS. At each time interval after WRS, the gastric blood flow (GBF) and the proliferating cell nuclear antigen (PCNA) labeling were determined. The apoptosis rate in the gastric mucosa was determined by the terminal deoxynucleotidyl transferase (TDT) mediated 2-deoxyuridine 5-triphosphate (dUTP)-biotin nick end-labeling (TUNEL) staining method and the expression of Bax and Bcl-2 mRNA was analyzed by RT-PCR and southern blot hybridization. WRS produced multiple erosions accompanied by the fall in GBF and PCNA index and by a dramatic enhancement in gastric epithelial apoptosis rate reaching maximum at 4 h after exposure to WRS. Following 6 and 12 h after the end of WRS the apoptosis declined but even 24 h after WRS it failed to reach the value recorded in intact gastric mucosa. The PCNA index was still significantly inhibited at 2 h after WRS but then showed significant rise at 6 and 12 h to reach at 24 h after WRS, the level similar to that measured in intact gastric mucosa. The expression of Bax mRNA was detected in intact gastric mucosa and gradually increased in first 4 h after WRS to decline at 24 h to the level not significantly different from that observed in the intact mucosa. In contrast, the expression of Bcl-2 mRNA was almost undetectable during first 4 h but showed strong signal at 6 and 12 h to decline to the control level 24 h after WRS. We conclude that: 1. Healing of WRS lesions involves an increase in GBF and mucosal cell proliferation and 2. the enhancement in gastric epithelial apoptosis accompanies the mucosal damage induced by stress and this appears to be triggered by the shift from the cell death effector Bax to the cell death repressor Bcl-2 protein.

Key words: *stress, apoptosis, cell proliferation, mucosal repair, gastric blood flow, reverse-transcription polymerase chain reaction, Bax, Bcl-2.*

INTRODUCTION

The preservation of gastric mucosal integrity is a complex biological process involving the balance between epithelial cell proliferation and programmed cell death termed apoptosis (1—3). This apoptosis is considered to be a physiological process leading to cell death. In this process the cell becomes more compact, blebbing occurs at the membranes, chromatin becomes condensed and DNA is fragmented. The pathway is active and depends on RNA and protein synthesis by the dying cell. In contrast to necrosis-induced cell death, the naturally occurring apoptosis results in the elimination of the dying cells without involvement of an inflammatory response (3).

The precise mechanism and the cause of apoptotic death are still unknown. But it is accepted, that various signals originating from either within or outside the cells can influence the decision between the life or death of these cells (4). For example, *Helicobacter pylori* (Hp) infection leads to an increased apoptosis of gastric epithelial cells, while the eradication of Hp or dietary supplementation (vitamin C and beta-caroten) tends to reduce apoptosis (5). This increased Hp-induced apoptosis was proposed to activate a complementary hyperproliferative mucosal response (6).

Recently, several pro- and anti-apoptotic factors involved in the regulation of apoptosis have been identified including the proteins promoting and suppressing this apoptosis. The key role in the regulation of programmed cell death is played by Bcl-2 family of peptides that inhibit programmed cell death, whereas others such as Bax protein promote this death (7, 8). The Bcl-2 protein suppressing the apoptosis and the gene encoding this protein were first discovered because of its involvement in the chromosomal translocation commonly found in B-cell lymphomas, where it contributes to neoplastic cell expansion by preventing cell turnover due to programmed cell death (9). The actions of the Bcl-2 protein can be opposed by other proteins sharing with it significant sequence homology. The Bax protein promoting apoptosis, has been found to share 21% amino acid sequence homology with Bcl-2. The balance between pro-apoptotic Bax proteins and anti-apoptotic Bcl-2 proteins was proposed to determine the susceptibility of the cell to apoptosis (10).

Both Bax and Bcl-2 proteins are expressed normally in gastrointestinal tract, but their patterns of expression are different. The expression of Bcl-2 protein is restricted to the stem cell and the proliferation zone of gastric glands. In contrast, Bax is detected mainly in the epithelial cells lining the gastric pits of the stomach (11). It is known that exposure of animal to a stress of cold and immobilization usually results in appearance of acute gastric lesions (12). The mechanism responsible for the induction of stress lesions has not been fully clarified but it appears to include the increase in gastric acid secretion, the fall in DNA synthesis and mucosal cell proliferation as well as the disorders in

mucosal protective mechanisms such as mucus alkaline secretion, gastric blood flow (GBF) and prostaglandin (PG) biosynthesis (12—19).

The purpose of this study was to determine the gastric epithelial apoptosis in the rat gastric mucosa exposed to stress and to study the changes in the GBF, cell proliferation and in the expression of Bax and Bcl-2 proteins during the mucosal recovery from stress lesions.

MATERIAL AND METHODS

Induction of stress lesions and determination of GBF

All experiments were carried out on male Wistar rats weighing 200—250 g. The rats were maintained under a 10:14 hour light:dark. The rats were fasted for 24 h before the experiment and allowed free access only to water. This study was approved and supervised periodically by the Institutional Animal Care and Use Committee of Cracow University School of Medicine. The animals were placed in restraint cages using modified technique described originally by Takagi *et al* [20] and immersed vertically to the level of the xiphoid process in a water bath of 23°C for 3.5 h. After this water immersion and restraint stress (WRS), the animals were sacrificed either immediately (0 h) or at 2, 4, 6, 12 or 24 h upon the withdrawal from this 3.5 h WRS. Each experimental group consisted of 8—10 rats. At each time point of the study, the rats were lightly anesthetized with ether, the abdomen was opened and the stomach was exposed. The GBF was measured in the oxyntic gland area of the stomach by means of local H₂-gas clearance method using an electrolytic regional blood flow meter (Biomedical Science, Model RBF—2, Osaka, Japan) as described previously (21). Briefly, the double needle electrodes were inserted through the serosa into the mucosa; one electrode being used for local generation of gaseous H₂ and another for the measurement of tissue content of H₂. With this method, the H₂ generated locally is carried out by flow of blood, while the polarographic current detector reads out decreasing tissue H₂. The clearance curve of tissue H₂ was used to calculate an absolute flow rate (ml/100g-min) in the oxyntic gland area. The GBF was measured in three areas of macroscopically intact oxyntic mucosa and the mean values of these measurements were calculated and expressed as percent changes from those recorded in intact animals. The total length of GBF measured including the opening of abdominal cavity usually did not exceed 5 min.

The stomach was then removed, opened along the greater curvature and placed flat to count the number of gastric lesions by two investigators unaware of the treatment given as described in our previous studies (19, 21). The stress lesions were defined as round or linear mucosal defects of at least 0.1 mm in diameter.

RNA extraction and reverse-transcription polymerase chain reaction (RT-PCR) for detection of messenger RNA (mRNA) for Bax and Bcl-2

After removal of the stomachs and counting the number of mucosal erosions, the mucosa was scraped of using a slide glass and immediately snap frozen in liquid nitrogen and stored at -80°C until analysis. Total RNA was extracted from the mucosal specimens using a guanidium isothiocyanate/phenol chloroform single step extraction kit from Stratagene (Heidelberg, Germany). Following precipitation, RNA was resuspended in RNase-free water and its concentration was estimated by absorbency at 260 nm wavelength. RNA samples were stored at -80°C until analysis.

Single stranded cDNA was generated from 5 µg of total cellular RNA using StrataScript reverse transcriptase (Stratagene, Heidelberg, Germany) and oligo-(dT)-primers (Stratagene,

Heidelberg, Germany). Briefly, 5 µg of total RNA was uncoiled by heating (65°C for 5 min) and then reverse transcribed into complementary DNA (cDNA) in a 50 µl reaction mixture that contained 50 U Moloney murine leukemia virus reverse transcriptase (MMLV-RT), 0.3 mg oligo-(dT)-primer, 1 ml RNase Block Ribonuclease Inhibitor (40 U/µl), 2 µl of a 100 mmol/l mixture of deoxyadenosine triphosphate (dATP), deoxyribothymidine triphosphate (dTTP), deoxyguanosine triphosphate (dGTP) and deoxycytidine triphosphate (dCTP), 5 µl 10 × RT buffer (10 mmol/l Tris-HCl, pH = 8.3, 50 mM KCl, 5 mM MgCl₂). The resultant cDNA (2 µl) was amplified in a 50 µl reaction volume containing 0.3 µl (2.5 U) Taq polymerase, 200 mM (each) dNTP (Pharmacia, Germany), 1.5 mM/l MgCl₂, 5 µl 10 × polymerase chain reaction buffer (50 mM KCl, 10 mM Tris-HCl, pH = 8.3) and primers used at final concentration of 0.5 µM. The mixture was overlaid with 25 µl of mineral oil to prevent evaporation. The polymerase chain reaction mixture was amplified in a DNA thermal cycler (Perkin-Elmer-Cetus, Norwalk, CT) for 30 cycles, each of which consisted of 45 s at 95°C for denaturation, 45 s at 60°C for annealing and 2 min at 72°C to ensure full extension of the product. The nucleotide sequence of the primers for actin, Bax and Bcl-2 were accepted on the basis of the published cDNA encoding actin, Bax and Bcl-2, respectively (22–24) and were purchased from Maxim Biotechnology Inc. (San Francisco, USA). The primers sequences were as follows: β-actin: 5' TT GTA ACC AAC TGG GAC GAT ATG G 3' (sense); 5' GAT CTT GAT CTT CAT GGT GCT AGG 3' (antisense), Bax: 5' TGG CAG CTG ACA TGT TTT CTG AC 3' (sense), 5' CGT CCC AAC CAC CCT GGT CT 3' (antisense); Bcl-2: 5' CAG ATG CAC CTG ACG CCC TT 3' (sense); 5' CCC AGC CTC CGT TAT CCT GGA 3' (antisense). Polymerase chain reaction products were detected by electrophoresis on a 1.5% agarose gel containing ethidium bromide. Location of a predicted products was confirmed by using GIBCO 100-bp ladder (Gibco BRL/Life Technologies, Eggenstein, Germany) as a standard size marker. For quantitation of amplified DNA, southern blot analysis was conducted with β-actin, Bax and Bcl-2 probes purchased from Maxim Biotechnology Inc. (San Francisco, CA, USA).

Southern hybridization

PCR products were run on 1.5% agarose gel with a molecular weight marker in the presence of ethidium bromide to detect amplified products. Following the electrophoresis, the gel was denaturated by soaking for 45 min in the denaturing buffer (1.5 M NaCl and 0.5 M NaOH) and neutralized for 45 min in neutralizing buffer (1 M Tris, 1.5 M NaCl, pH = 7.4) at room temperature. The PCR products were blotted by capillary transfer onto positively charged nylon membrane (Boehringer Mannheim, Germany). Hybridization proceeded at 50°C for 6 h with 10 pM/ml digoxigenin-11-ddUTP (DIG-ddUTP)-labeled internal oligonucleotide probe in DIG Easy Hyb hybridization buffer (Boehringer Mannheim GmbH, Germany). For labeling of the internal probe DIG Oligonucleotide 3'-End Labeling Kit from Boehringer Mannheim GmbH (Germany) was used. According to the kit protocol, reagents of the 3'-End-Labeling Kit (5 × reaction buffer, CoCl₂ solution, terminal transferase and DIG-ddUTP) were incubated at 37°C for 15 min with the oligonucleotide probe. The internal probes for actin Bax and Bcl-2 were purchased by Maxim Biotechnology Inc. (San Francisco, CA, USA). The reaction mixture was placed then on ice and the labeling reaction was terminated by adding EDTA. After hybridization and stringency washes the hybridized probe was detected using DIG Luminescent Detection Kit (Boehringer Mannheim GmbH, Germany). Briefly, membrane was rinsed for 3 min in washing buffer (0.1 M maleic acid, 0.15 M NaCl, 0.3% Tween 20, pH 7.4), and then incubated for 30 min. in 1 × blocking reagent (1% blocking reagent in 0.1 M maleic acid, 0.15 M NaCl, pH 7.4) according to manufacturer recommendation. In the next step, the membrane was incubated for 30 min with anti-DIG-AP-conjugated antibodies diluted 1:10000 in 1 × blocking solution. Then, the membrane was washed twice for 15 min in washing buffer and equilibrated for 5 min in detection buffer (0.1 M Tris-HCl, 1 M NaCl, pH 9.5). Following washing, the membrane was placed in disodium

3-(4-methoxyspiro {1,2-dioxetane-3,2'(5'-tricyclo decan)-4-yl}phenyl phosphate (CSPD solution, Boehringer Mannheim GmbH, Germany). After 5 min incubation in this solution, the excess of CSPD was soaked up and the membrane was sealed in fresh hybridization bag to avoid the complete dry-out of the membrane. Finally, the hybridized membrane was exposed for 15–20 min at room temperature to X-ray film (Kodak X-OMAT, Sigma, Deisenhofen, Germany). The intensity of bands was quantified using densitometry (Kodak Science, US). Quantities of each product were normalized according to control levels of β -actin and expressed as a percentage of the expression observed in control rats not exposed to water immersion and restraint stress.

Assessment of apoptosis

The transferase-mediated dUTP-biotin nick-end-labeling (TUNEL) staining method was performed using modified procedure originally described by Gavrieli *et al* [25]. About 5 μ m sections were cut from formalin fixed, paraffin wax embedded tissue blocks and mounted on microscope slides. The sections were deparaffinized through xylene and alcohol, washed and then digested with proteinase K (20 μ g/ml) (Sigma-Aldrich, Deisenhoffen, Germany) for 15 min at room temperature, followed by further washing and blocking of endogenous peroxidase with 2% hydrogen peroxide (Sigma) for 5 min. After preincubation with terminal transferase buffer containing 200 mM potassium cacodylate, 0.2 mM EDTA, 25 mM TRIS-HCl, bovine serum albumine (0.25 mg/ml) pH 6.6 for 10 min, the sections were incubated at 37°C for 90 min with the same buffer containing, in addition, 1 mM cobalt chloride, terminal transferase (0.5 U/ μ l), and 0.4 μ M digoxigenin-11-deoxyuridine triphosphate (dUTP), all purchased from Boehringer, Mannheim, Germany. Detection of incorporated digoxigenin-11-dUTP was carried out with anti-digoxigenin-peroxidase-conjugated antibody at a concentration of 1:300 in 100 mM TRIS-HCl, 150 mM sodium chloride, pH 7.5 at room temperature for 30 min. The sections were then washed with buffered saline, stained with 0.05% diaminobenzidine hydrochloride (DAB) solution with nickel enhancement for 5 min, followed by counterstaining with methyl green, dehydration and mounting. In addition, negative controls were prepared in accordance with the manufacturer instructions. For negative controls either terminal deoxynucleotidyl transferase or digoxigenin-dUTP were omitted, resulting in uniformly negative staining. At least 300 mucosal cells (100 in surface epithelium, 100 in neck region and 100 in basal portion of gastric glands) were counted in each section and mean number of positive cells per 100 cells was expressed as apoptotic index (in percentage). The number of cells showing positivity for apoptosis was counted per total of 100 cells was expressed as a percentage of apoptotic cells. Counting was performed by a two independent observers unaware of slide coding and the mean from two measurements in the same microscopical field was calculated and considered as an apoptic index (%).

Assessment of cell proliferation by PCNA

For determination of cell proliferation the mucosal biopsy was taken about 5 mm from the area of gastric lesions and the immunostaining for PCNA was performed as described in detail in our previous report (26). Briefly, 5 μ m thick sections were cut, placed on coated slides and deparaffinized. They were then exposed to microwave pretreatment (in 10 mM citrate buffer, pH 6 at 850 W for two periods of five min) to improve antigenicity. Non-specific binding was blocked with 1:10 normal horse serum (ABC Kit, Dianova) and sections were then incubated with the specific monoclonal primary antibody against PCNA (1:40, Oncogene Science) for 1 h in the room temperature. Detection of binding of primary antibody was achieved using a biotinylated antimouse IgG (Oncogene Science) and the streptavidin-peroxidase complex (ABC kit, Oncogene Science) with diaminobenzene as a chromogen (Sigma). The number of cells with positive PCNA staining was counted in 300 consecutive cells in the proliferative zone of the gastric mucosa and

expressed as PCNA labeling index (%). Negative sections were processed immunohistochemically after replacing the primary antibody with irrelevant monoclonal antibody or with PBS. As a positive control, the sections from gastric lymphoma were used. Counting was conducted by two observers unaware of slide coding.

Statistical analysis

For statistical analysis the Mann-Whitney U test was used to compare the scores between the groups. All data are expressed as means \pm SEM. With all analyses, an associated probability (p-value) of less than 5% was considered significant.

RESULTS

WRS damage, the GBF and recovery from WRS lesions

Exposure to WRS for 3.5 h resulted in an immediate (time 0 h) appearance of multiple gastric lesions in the oxyntic mucosa averaging 21 ± 2 per rat (*Fig. 1*) with no macroscopic damage found in the forestomach. The time course of the recovery of gastric mucosa from the stress injury and the disappearance of gastric lesions is shown in *Fig. 1*. Microscopical

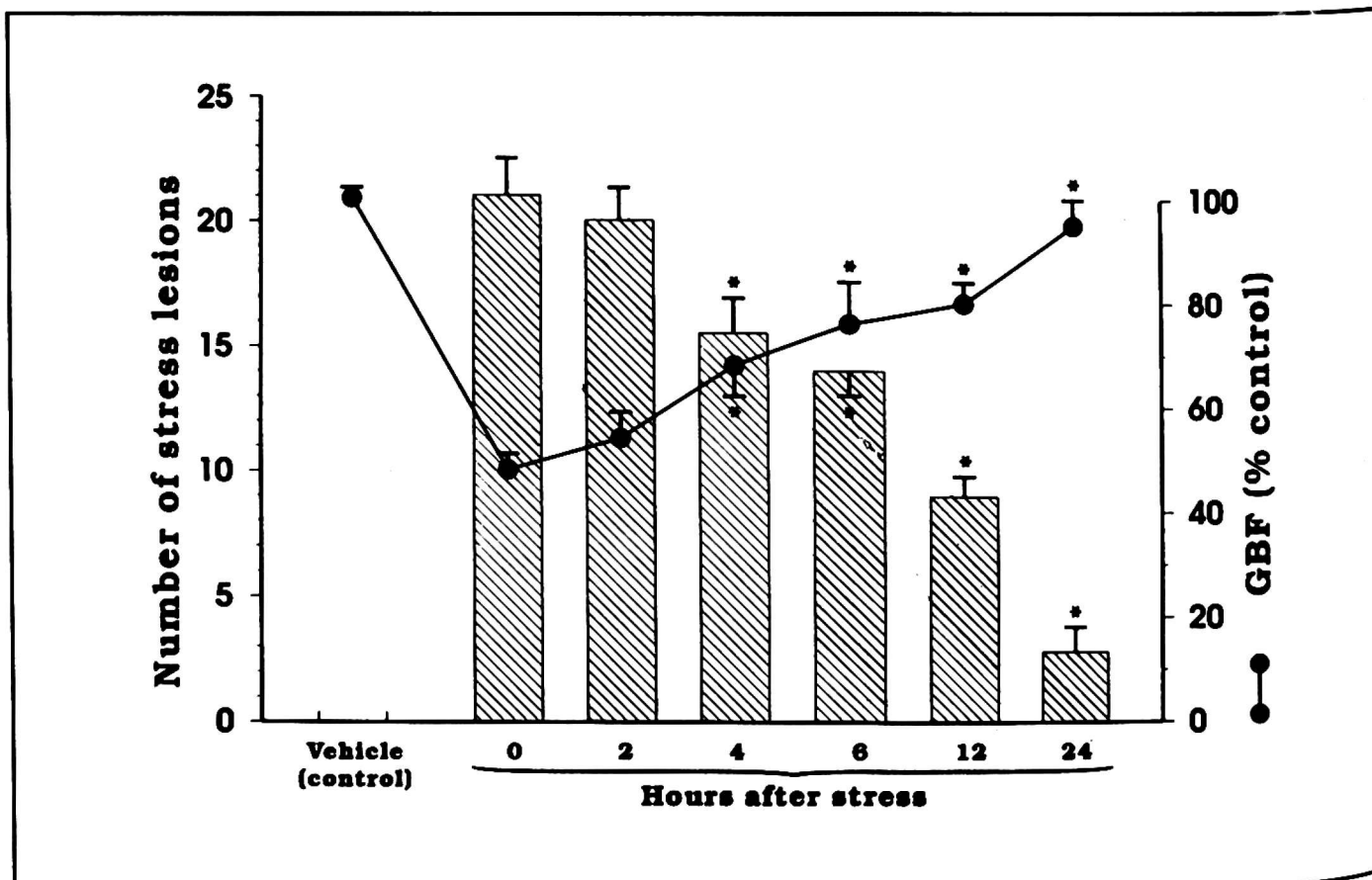


Fig. 1. The number of gastric lesions and gastric blood flow (GBF) in intact rats and in those exposed to 3.5 h of WRS and observed immediately (time 0) and 2, 4, 6, 12 and 24 after stress. Mean \pm SEM of 8–10 rats. Asterisk indicates significant change as compared to that obtained in vehicle-treated control and in stressed rats immediately (time 0) after WRS.

examination of the mucosa after 3.5 h of WRS revealed widespread damage of the surface epithelium with numerous cells sloughed off into the gastric lumen that was significantly reduced already at 4 h after WRS and then further significantly declined at 12 h by about 50% to disappear by about 87% at 24 h after the withdrawal from the WRS. Immediately after the WRS (time 0), the GBF fell to about 50 % of the value recorded in the intact stomach and then gradually increased to reach first significant increment at 4 h and to return to the control value 24 h after the WRS (*Fig. 1*).

Determination of apoptosis and cell proliferation

In the control rats not exposed to WRS, the apoptotic cells were seen only occasionally at the surface epithelium (*Fig. 2A*). In contrast, gastric mucosa exposed to WRS showed many apoptotic cells at the surface epithelium as detected by TUNEL technique (*Fig. 2B*). The apoptotic index rose significantly at 2 and 4 h to reach a peak at 4 h after the termination of WRS (10.1 ± 2.0 % vs. 0.4 ± 0.1 % in the intact rats)

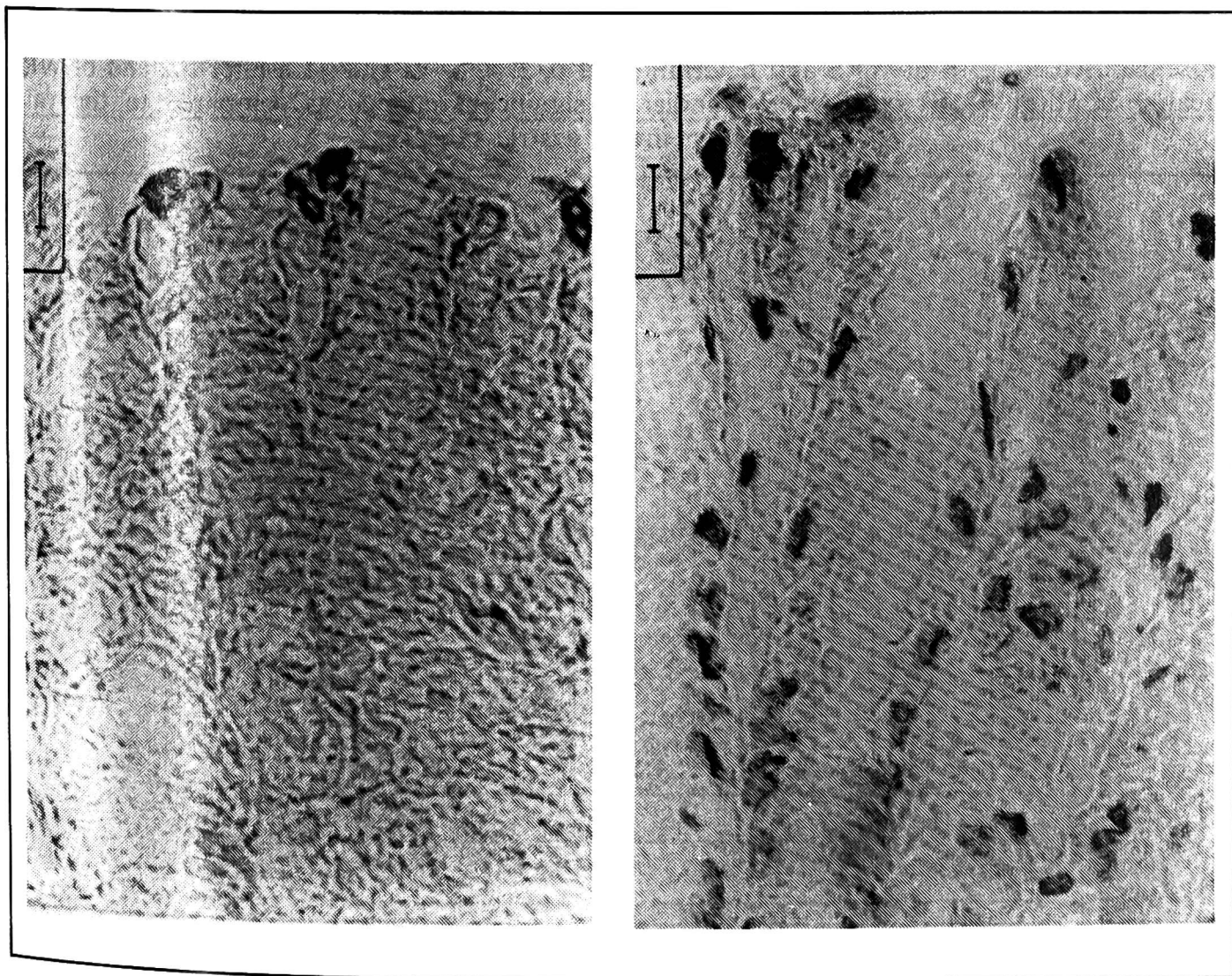


Fig. 2 A. Normal (control) gastric mucosa with epithelial apoptotic cells (arrows) detected by TUNEL histochemical method. *2.B.* The gastric mucosa at 4 h after exposure to 3.5 h of WRS with numerous apoptotic epithelial cells (arrows).

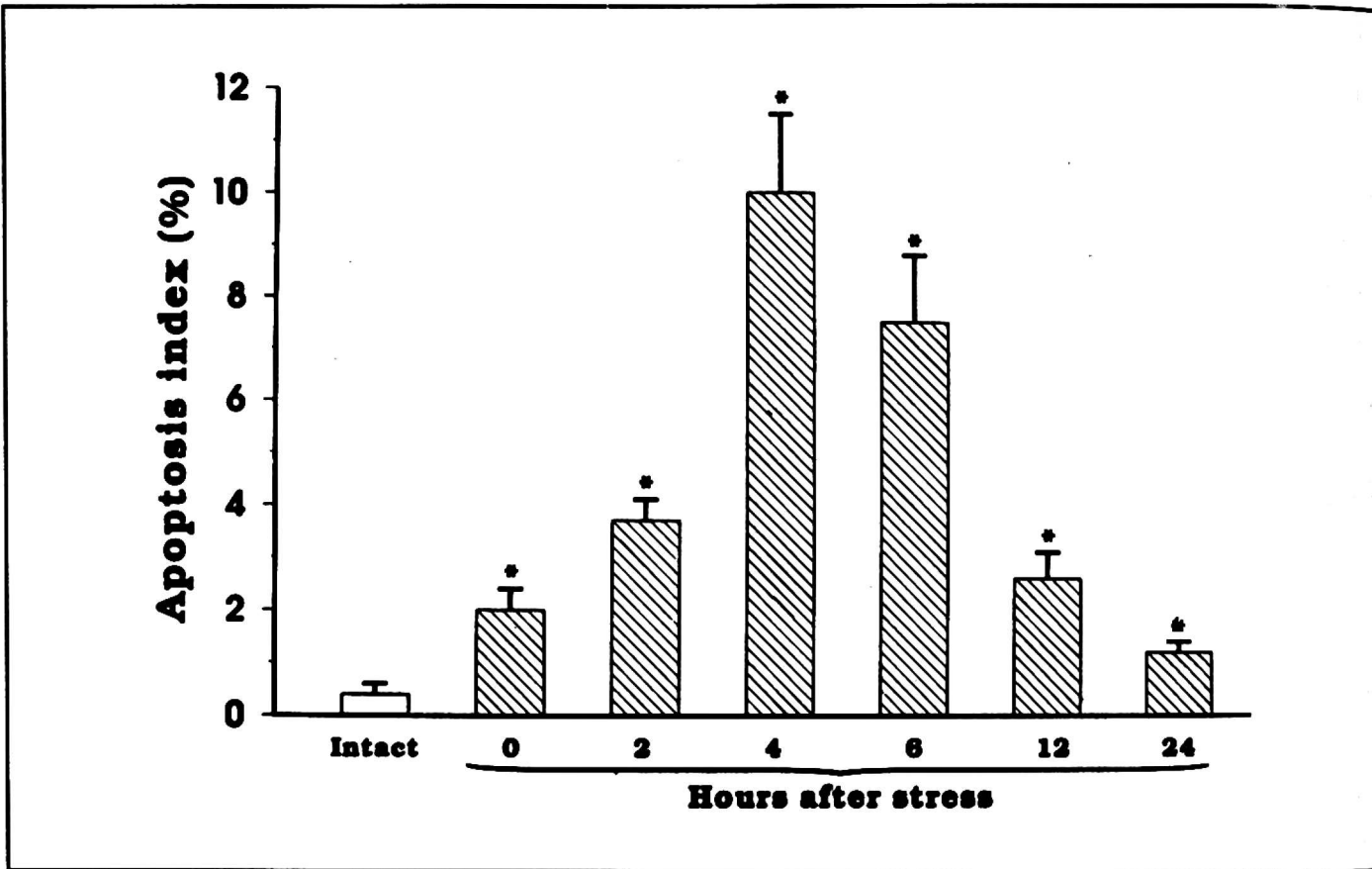


Fig. 3. Apoptosis index (%) of TUNEL stained sections of gastric mucosa from intact rats and those exposed to 3.5 h of WRS and determined at 0, 2, 4, 6, 12 and 24 h after the end of WRS. Mean \pm SEM of 8–10 rats. Asterisk indicates significant change as compared to the value obtained in intact gastric mucosa.

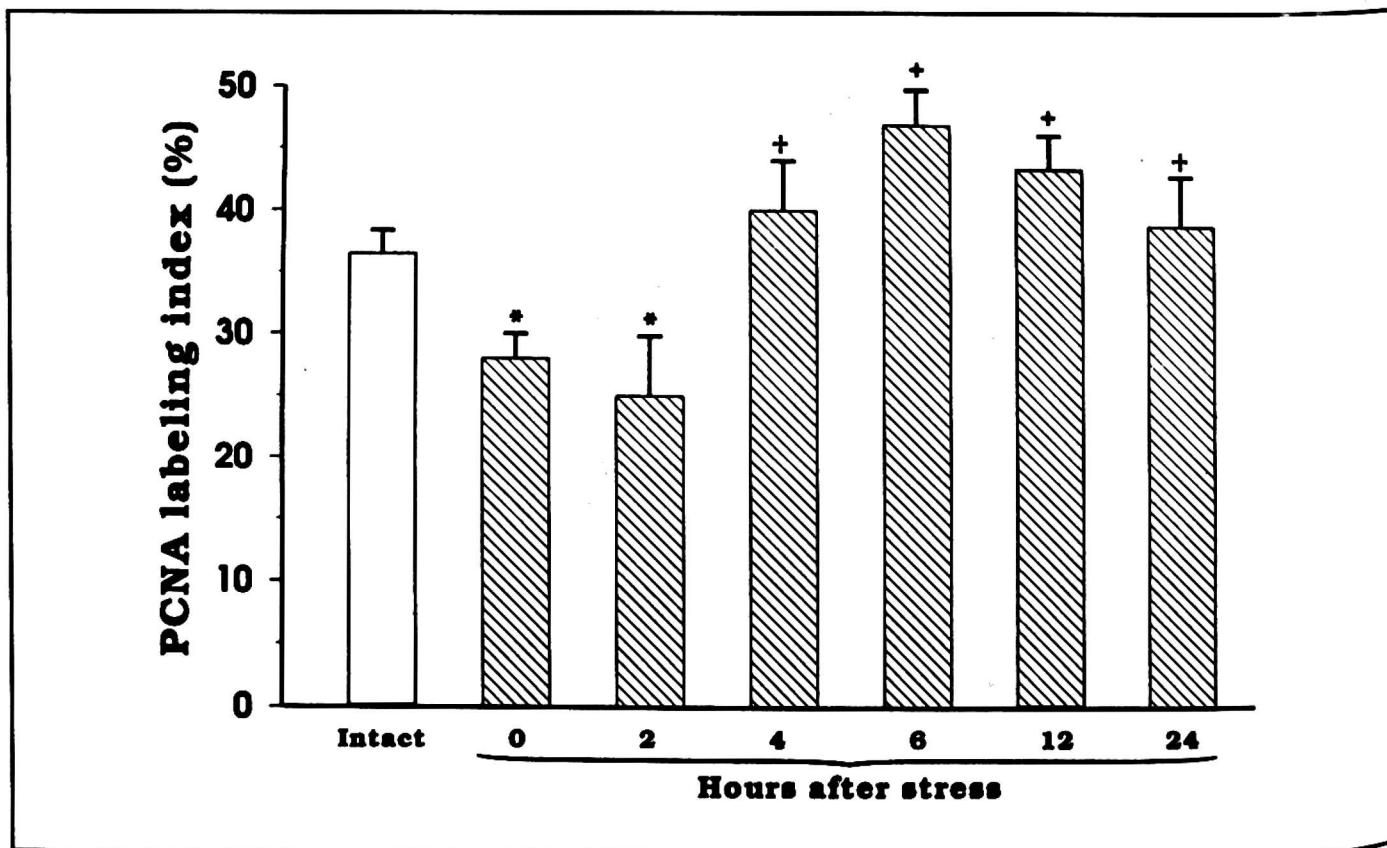


Fig. 4. Proliferating cell nuclear antigen (PCNA) expression in gastric mucosa in intact rats and those sacrificed at 0, 2, 4, 6, 12 and 24 h after single exposure to WRS (3.5 h). Mean \pm SEM of 8–10 rats. Asterisk indicates significant change as compared to the value obtained in the intact gastric mucosa. Cross indicates significant change as compared to the value obtained in gastric mucosa at 0 h after the WRS.

(Fig. 3). With the duration of the interval after the termination of WRS, the apoptotic index gradually declined but failed to return even at 24 h to the level recorded in the intact mucosa. In separate group of rats (8 animals) with WRS but without GBF measurement, the apoptotic index was not significantly different from that observed in rats (8 animals) with GBF measured and these results are not included for the sake of clarity.

The PCNA cells, which in intact mucosa predominantly occurred in the proliferate zone of the gastric glands, decreased significantly immediately after the end of WRS (time 0) and in the first 2 h after exposure to WRS. Later, the PCNA labeling index returned to normal level (at 4 h) and then increased to reach a peak at 6–12 h followed by the decline at 24 h towards the value observed in the intact rats (Fig. 4).

Determination of Bcl-2 and Bax mRNA expression by RT-PCR

The expression of both Bax and Bcl-2 mRNA using RT-PCR and Southern hybridization was detected only as a relative weak signals in the intact gastric mucosa. The intense signals for Bax mRNA were detected in the gastric mucosa at 0 to 4 h, whereas the Bcl-2 mRNA was almost absent during the first 4 h of recovery of the mucosa from WRS (Fig. 5). Quantitative analysis by

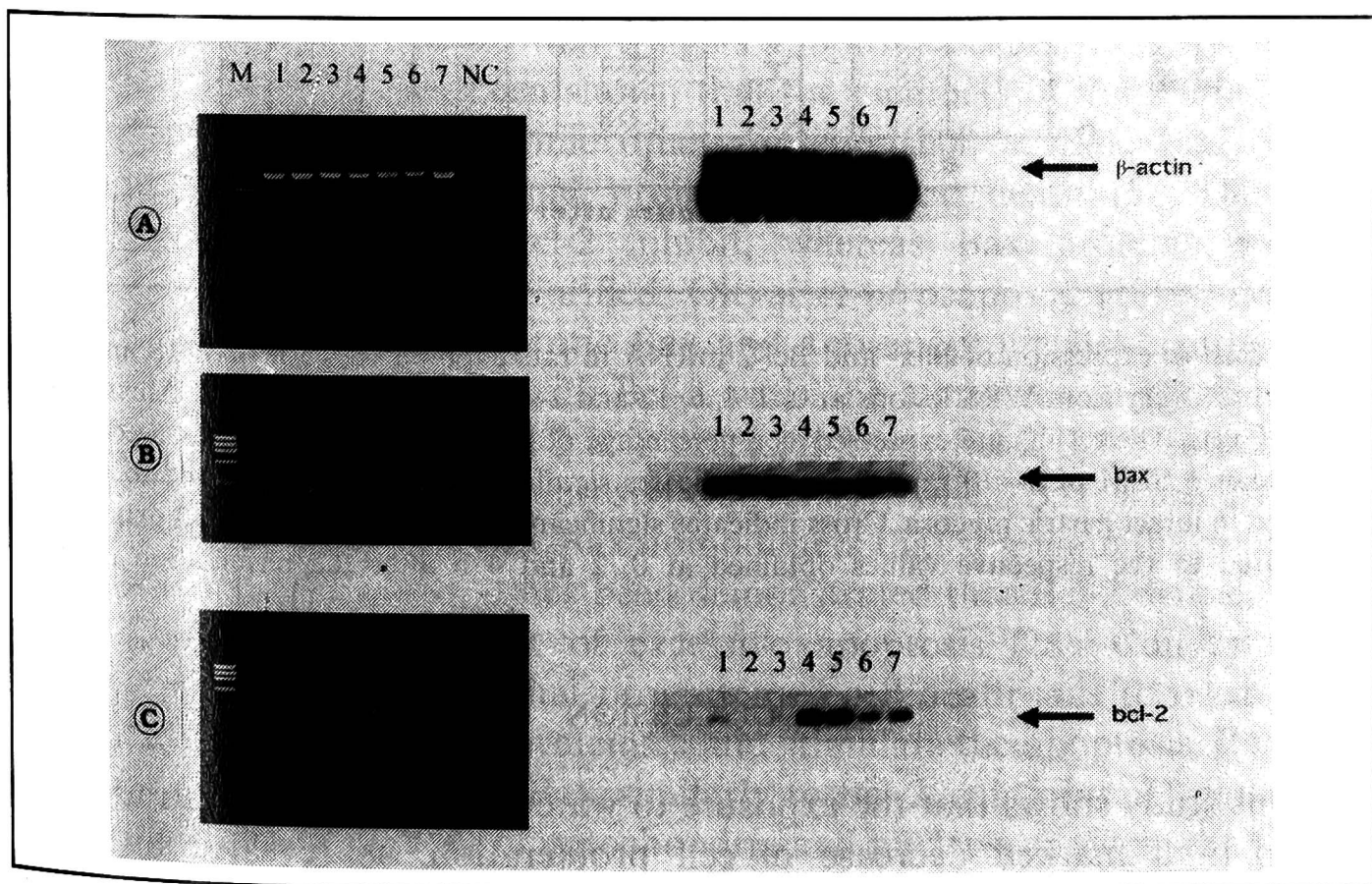


Fig. 5 A, B and C. Analysis of polymerase chain reaction (PCR) — amplified β -actin (A), Bax (B) and Bcl-2 (C) products in ethidium bromide stained agarose gel and with southern blot method, respectively. M = DNA size marker, lane 1 = 0 h, lane 2 = 2 h, lane 3 = 4 h, lane 4 = 6 h, lane 5 = 12 h, lane 6 = 24 h after exposure to 3.5 h WRS, lane 7 = intact (control group), NC = negative control.

Southern hybridization confirmed that the expression of Bax was increased from 0 to 4 h after stress and at the same time Bcl-2 expression was significantly decreased (Fig. 6). Thereafter, that is at 6 and 12 h after WRS, the expression of Bcl-2 was again significantly increased and this was accompanied by a progressive decrease in the expression of Bax mRNA (Figs 5 and 6). After 24 h, the expression of Bax mRNA remained significantly lower but Bcl-2 remained still significantly elevated over that observed immediately (time 0) after the termination of WRS (Figs. 5 and 6).

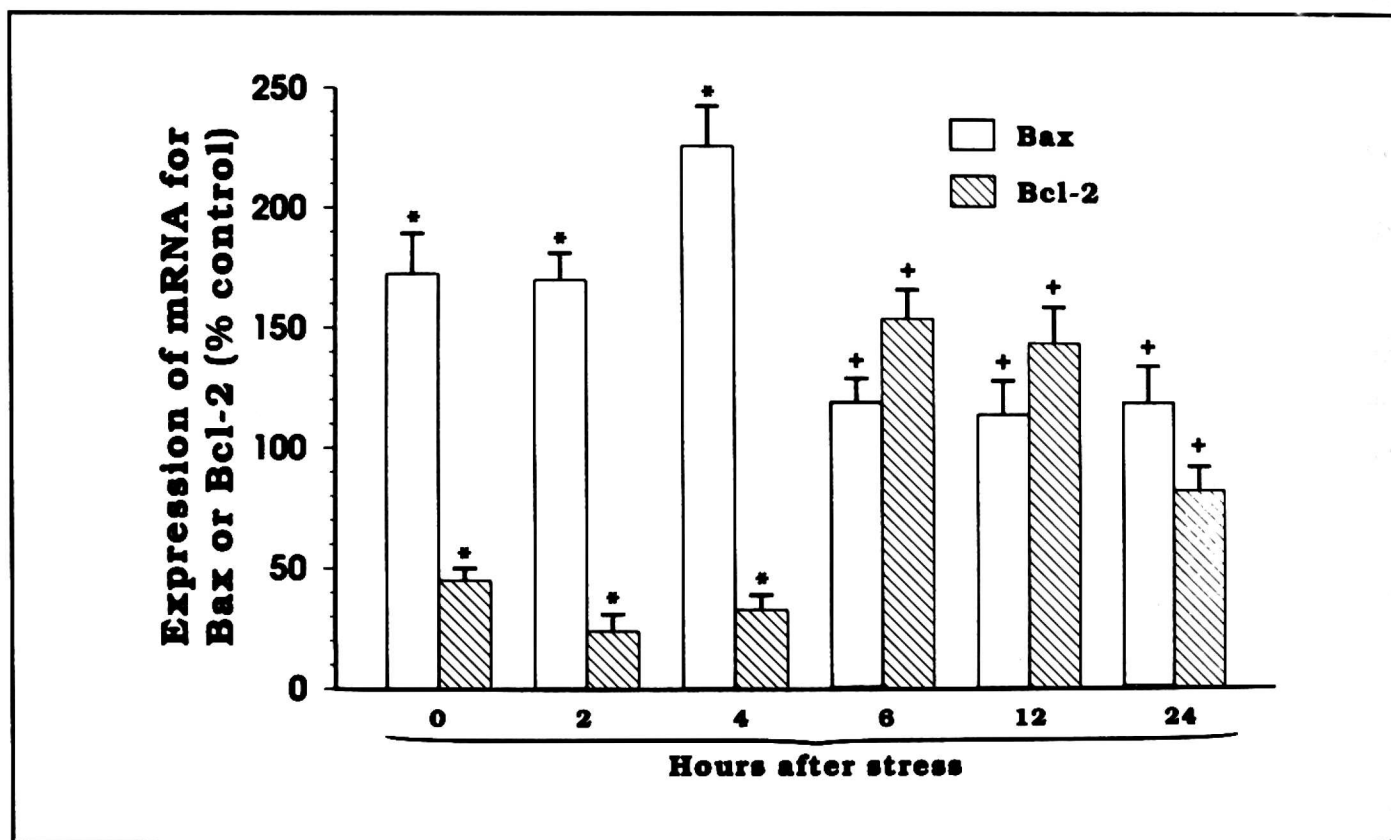


Fig. 6. Gastric expression of Bax- and Bcl-2 mRNA in rats exposed to 3.5 h of WRS and during mucosal recovery from WRS lesions at 0, 2, 4, 6, 12 and 24 h after the end of WRS. Mean \pm SEM of 8–10 rats. The results are expressed as a percentage of the apoptotic change observed in intact rats. Mean \pm SEM of 8–10 rats. Asterisk indicates significant change as compared to the value obtained in intact gastric mucosa. Cross indicates significant change in Bax and Bcl-2 expression as compared to the respective values obtained at 0, 2 and 4 h after the termination of WRS.

DISCUSSION

This study shows that the exposure to water immersion and restraint stress results in a marked decrease of cell proliferation, as revealed by PCNA labeling, the impairment of gastric microcirculation (GBF) measured by H_2 -gas clearance as well as a significant increase in the rate of apoptosis in the gastric mucosa. The apoptotic index increased time-dependently in the first hours after withdrawal from the stress reaching the peak at 4–6 h but then progressively declining while the cellular proliferation exhibited a marked rise

reaching peak at 6—12 h after termination of this stress. After 24 h, when the acute stress lesions almost completely disappeared, both the rate of apoptosis and mucosal proliferation returned to the values similar to those observed in the non-stressed control rats. Our detailed time sequence analysis suggests an involvement of the apoptosis only in the early phase of recovery from stress lesions that was followed by augmented mucosal cell proliferation. Therefore, our study provides new insight into the pathomechanism of acute stress lesions by showing that the recovery of gastric mucosa from these lesions depends predominantly upon the mucosal cell proliferation probably triggered by a marked initial cell apoptosis similarly as that observed after Hp infection [6].

Apoptosis represents a physiological cell death, which involves the activation of pathway leading to suicide of the cell by characteristic process in which the cell becomes more compact, blebbing occurs of the membranes, chromatin becomes condensed and DNA is fragmented [3]. The method employed in this study to detect DNA fragmentation, using the terminal dUTP nick end labeling technique (TUNEL), provides a sensitive method of visualization of apoptotic cells before the morphologic changes can be detected [23].

Although the mechanisms of molecular regulation of apoptotic process has not been clarified, several apoptosis-related proteins have been recently identified. The major pathway that triggers apoptosis occurs *via* the properties of the Fas receptor. It has been shown that the members of Bcl-2 family may inhibit the apoptotic death while other proteins such as Bax, Bad or Bak oppose the effect of Bcl-2 and promote apoptotic death (5). The exact mechanism by which the Bcl-2 inhibit, whereas Bax proteins promote apoptosis has not been fully clarified. Oltvai *et al* proposed (10) a model of relationship between pro-apoptotic Bax and anti-apoptotic Bcl-2 proteins, in which Bax form heterodimers with Bcl-2, with one monomer antagonizing the function of the other. When Bcl-2 is in excess, it forms Bcl-2/Bcl-2 homodimers that protect the cells from apoptosis. Conversely, when Bax is produced in excessive amounts, it forms Bax/Bax homodimers that stimulate the apoptosis. To support this hypothesis, it has been demonstrated that Bcl-2 deficient mice die as embryos as the result of excessive apoptosis (28). Conversely, in Bax-knockout mice some normal programmed cell death fails to occur (27).

It is still unclear how Bax proteins could stimulate the apoptosis. Recently, it has been demonstrated that Bax, similarly to Bcl-2, can form pH-dependent channels in lipid membranes of mitochondria and nucleus, allowing the passive fluxes of ions and small molecules across the intracellular membranes of organelles in which the proteins are localized (7, 8). The presence of Bax in mitochondrial membranes may trigger a membrane permeability followed by the disruption of the transmembrane potential, an important event occurring during early stages of apoptosis (27, 28). The activity of Bax is antagonized by

the anti-apoptotic Bcl-2 protein, which inhibits mitochondrial permeability transition (29).

The present study suggests, that the activation of apoptosis, as assessed by the *in situ* TUNEL-method, was triggered, at least in part, by upregulation of apoptosis-promoting Bax mRNA and by the downregulation of Bcl-2 mRNA as demonstrated in the first 4 hours after exposure to stress. The significant increase of Bcl-2 mRNA expression observed at 6 and 12 h after stress, suggests that some compensatory mechanisms are activated in gastric mucosa cells in attempt to maintain cell survival. Parallel to the increase of Bcl-2 expression, a progressive reduction in apoptosis in the gastrointestinal epithelium was observed.

The alterations in the expression of Bax and Bcl-2 do not seem to be the only factors responsible for the development of apoptosis. We hypothesize, that the sudden reduction in gastric blood flow and local mucosal ischemia, which have been demonstrated in the gastric mucosa in this and previous studies (32) almost immediately after exposure to WRS, might play some role in the induction of apoptosis. Ischemia is known to be a cause of apoptosis in different organs (33—35), although the exact mechanism how stress-induced ischemia could contribute to the apoptosis in the gastric mucosa is still unknown. Recent studies demonstrated, that ischemia may contribute to the changes in the expression of Bax and Bcl-2 proteins in the liver and to lead to the shift from the cell death repressor Bcl-2 to cell death effector Bax (33, 34).

Another important candidate contributing to the induction of apoptosis after exposure to stress may be an excessive release of nitric oxide (NO) in gastric mucosa due to overexpression of inducible NO synthase (iNOS) after exposure to stress (36). Excessive amounts of NO produced, for instance, due to upregulation of iNOS, have been demonstrated to induce apoptosis *via* disruption of the mitochondrial transmembrane potential, followed by increased generation of reactive oxygen species (37). These free radicals, which are known to be generated in the gastric mucosa after exposure to stress have also been proposed to contribute to the programmed cell death (38). One of the possible ways by which stress could induce apoptosis is the action of highly cytotoxic and apoptotic agent such as peroxynitrate formed due to interaction between NO and superoxide anion (39—41). It is likely that NO and peroxynitrate acting as reactive nitrogen species such as occurring in the Hp infected mucosa (5, 6) or following exposure to stress accompanied by high expression and activity of iNOS, might contribute to the DNA damage and apoptosis.

Taken together, our findings indicate that the peak in apoptosis occurs during stress-induced ulcerogenesis suggesting that these two processes are interrelated. The increased apoptosis in the gastric mucosa after stress may be

triggered by the shift in the ratio of cell death effector Bax proteins to cell death repressor Bcl-2 proteins. On the other hand, the initial apoptosis appears to activate the mucosal cell proliferative response leading to the recovery of the mucosa from the stress damage.

Acknowledgment: This study was supported in part by a grant from Interdisciplinary Center for Clinical Sciences at the University Erlangen-Nuremberg, Erlangen, Germany.

REFERENCES

1. Benedetti A, Mancini R, Marucci L, Paolucci F, Jezequel AM, Orlandi F. Quantitative study of apoptosis in normal rat gastroduodenal mucosa. *J Gastroenterol Hepatol* 1990; 5 (4): 369—374.
2. Hall PA, Coates PJ, Ansari B, Hopwood D. Regulation of cell number in the mammalian gastrointestinal tract: the importance of apoptosis. *J Cell Sci* 1994; 107: 3569—3577.
3. Que FG, Gores GJ. Cell death by apoptosis: basic concepts and disease relevance for the gastroenterologist. *Gastroenterology* 1996; 110: 1238—1243.
4. Savill J. Apoptosis in disease. *Eur J Clin Invest* 1994; 24: 715—723.
5. Mannick EE, Bravo LE, Zarma G, Reaple JL, Ruiz XJ, Fonthan ET *et al*. Inducible nitric oxide synthase, nitrotyrosines and apoptosis in *Helicobacter pylori* gastritis: effect of antibiotics and antioxidants. *Cancer Res* 1996; 56: 3238—3243.
6. Moss SF, Calam J, Agrawal B, Wang S, Holt PR. Induction of gastric epithelial apoptosis by *Helicobacter pylori*. *Gut* 1996; 38: 498—501.
7. Reed JC. Bcl-2 and the regulation of programmed cell death. *J Cell Biol* 1994; 124: 1—6.
8. Antonsson B, Conti F, Ciavatta A, Montesuit S, Lewis S, Martinou I *et al*. Inhibition of Bax-forming activity by Bcl-2. *Science* 1997; 277: 370—372.
9. Tsujimoto Y, Cossman J, Jaffe E, Croce CM. Involvement of the Bcl-2 gene in human follicular lymphoma. *Science* 1985; 228: 1440—1443.
10. Oltvai Z, Milliman C, Korsmeyer SJ. Bcl-2 heterodimerizes in vivo with a conserved homolog Bax that accelerates programmed cell death. *Cell* 1993; 74: 609—619.
11. Krajewski S, Krajewska M, Shabaik A, Miyashita T, Wang HG, Reed JC. Immunohistochemical determination of in vivo distribution of Bax, a dominant inhibitor of Bcl-2. *Am J Pathol* 1994; 145: 1323—1336.
12. Moody FG, Cheung LY, Simons MA, Zalewsky C. Stress and the acute mucosal lesions. *Dig Dis* 1976; 21: 148—154.
13. Kim Y, Kerr RJ, Lipkin M. Cell proliferation during the development of stress erosions in the mouse stomach. *Nature* 1967; 215: 1180—1181.
14. Lahtiharju A, Rytomaa T. DNA synthesis in fore and glandular stomach and in skin after nonspecific stress in mice. *Exp Cell Res* 1967; 46: 593—596.
15. Imondi AR, Balis ME, Lipkin M. Nucleic acid metabolism in the gastrointestinal tract of the mouse during fasting and restraint stress. *Exp Mol Path* 1968; 9: 339—348.
16. Ludwig WM, Lipkin M. Biochemical and cytological alterations in gastric mucosa of guinea pigs under restraint stress. *Gastroenterology* 1969; 56: 895—902.
17. Takeuchi K, Johnson LR. Pentagastrin protects against stress ulcerations. *Gastroenterology* 1979; 76: 327—334.
18. Kuwayama H, Eastwood GL. Effects of water immersion restraint stress and chronic indomethacin ingestion on gastric antral and fundic epithelial proliferation. *Gastroenterology* 1985; 88: 362—365.

19. Konturek PC, Brzozowski T, Konturek SJ, Dembinski A. Role of epidermal growth factor, prostaglandins and sulfhydryls in stress-induced gastric lesions. *Gastroenterology* 1990; 99: 602—615.
20. Takagi KY, Kayuya Y, Watanabe K. Studies on the drugs for peptic ulcer. A reliable method for producing stress ulcers in rats. *Chem Pharm Bull* 1964; 12: 465—72.
21. Brzozowski T, Konturek PC, Sliwowski Z, Drozdowicz D, Hahn EG, Konturek SJ. Importance of nitric oxide and capsaicin-sensitive afferent nerves in healing of stress lesions induced by epidermal growth factor. *J Clin Gastroenterol* 1997; 25 (Suppl 1): S28—38.
22. Cleary ML, Smith SD, Sklar J. Cloning and structural analysis of cDNAs for Bcl-2 and hybrid Bcl-2/immunoglobulin transcript resulting from the t(14:18) translocation. *Cell* 1986; 47: 18—19.
23. Steller H. Mechanisms and genes of cellular suicide. *Science* 1995; 267: 1445—1449.
24. Nudel U, Zakut R, Shani M, Neuman S, Levy Z, Vaffe D. The nucleotide sequence of the rat cytoplasmic β -actin gene. *Nucleic Acids Res* 1983; 11: 1759—1771.
25. Gavrieli Y, Sherman Y, Ben-Sasson SA. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J Cell Biol* 1992; 119: 493—501.
26. Konturek PC, Brzozowski T, Konturek SJ, Ernst H, Drozdowicz D, Pajdo R. Expression of epidermal growth factor and transforming growth factor alpha during ulcer healing. *Scand J Gastroenterol* 1997; 32: 6—15.
27. Veis DJ, Sorenson CM, Shutter JR, Korsmeyer SJ. Bcl-2 deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys, and hyperpigmented hair. *Cell* 1993; 75: 229—240.
28. Knudson CM, Tung KS, Tourtellotte WG, Brown GA, Korsmeyer SJ. Bax-deficient mice with lymphoid hyperplasia and male germ cell death. *Science* 1995; 270: 96—99.
29. Marchetti P, Hirsch T, Zamzami N, Castedo M, Decaudin D, Susin SA *et al.* Mitochondrial permeability transition triggers lymphocyte apoptosis. *J Immunol* 1996; 157(11): 4830—4836.
30. Hennet T, Bertoni G, Richter C, Peterhans E. Expression of Bcl-2 protein enhances the survival of mouse fibrosarcoma cells in tumor necrosis factor-mediated cytotoxicity. *Cancer Res* 1993; 53: 1456.
31. Susin SA, Zamzami N, Castedo M, Hirsch T, Marchetti P, Macho A *et al.* Bcl-2 inhibits the mitochondrial release of anapoptogenic protease. *J Exp Med* 1996; 184: 1331—1342.
32. Konturek SJ, Brzozowski T, Majka J, Dembinski A, Slomiany A, Slomiany BL. Transforming growth factor alpha and epidermal growth factor in protection and healing of gastric mucosal injury. *Scand J Gastroenterol* 1992; 27: 649—655.
33. Shimatsu K, Wanless IR. Role of ischemia in causing apoptosis, atrophy, and nodular hyperplasia in human liver. *Hepatology* 1997; 145(6): 1323—1336.
34. Gillardon F, Lenz C, Waschke KF, Krajewski S, Reed JC, Zimmermann M *et al.* Altered expression of Bcl-2, Bcl-X, Bax, and c-fos colocalizes with DNA fragmentation and ischemic cell damage following middle cerebral artery occlusion in rats. *Brain Res Mol Brain Res* 1996; 40(2): 254—260.
35. Krajewski S, Mai JK, Krajewska M, Sikorska M, Mossakowski MJ, Reed JC. Upregulation of bax protein levels in neurons following cerebral ischemia. *J Neurosci* 1995; 15(10): 6364—6376.
36. Qiu BS, Pfeiffer CJ, Cho CH. Effects of chronic nitric oxide synthase inhibition in cold-restraint and ethanol-induced gastric mucosal damage in rats. *Digestion* 1996; 57: 60—66.
37. Hortelano S, Dallaporta B, Zamzami N, Hirsch T, Susin SA, Marzo I *et al.* Nitric oxide induces apoptosis via triggering mitochondrial permeability transition. *FEBS-Lett* 1997; 410(2—3): 373—377.
38. Salim AS. Protection against stress-induced acute gastric mucosal injury by free radical scavengers. *Intensive Care Med* 1991; 17: 455—460.
39. Konturek PC, Konturek SJ, Brzozowski T, *et al.* Gastroprotective activity of melatonin and its precursor, L-tryptophan, against stress-induced and ischemia-induced lesions is mediated by scavenge of oxygen radicals. *Scand J Gastroenterol* 1997; 32(5): 433—438.

40. Wood K, Youle RJ. Apoptosis and free radicals. *Ann NY Acad Sci* 1994; 738: 400—407.
41. Sandoval M, Zhang XJ, Liu X, Mannick EE, Clarck D. A84 and RAW 264.7 cells attenuation by L-ascorbic acid. *Free Radical Biol Med* 1997; 22(3): 489—495.

Received: January 25, 1999

Accepted: April 13, 1999

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