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Review article

I. SZABÓ, A.S. TARNAWSKI

APOPTOSIS IN THE GASTRIC MUCOSA: MOLECULAR MECHANISMS, BASIC AND CLINICAL IMPLICATIONS

Medical Service, Department of Veterans Affairs Medical Center, Long Beach, Department of Medicine, University of California, Irvine, California, USA,

Apoptosis a programmed cell death, is an essential mechanism of eliminating damaged or aged cells and thus to maintain tissue integrity. There are two central pathways that lead to apoptosis: a) the positive induction by ligands (death factors) binding to plasma membrane receptors (death factor receptors) and b) negative induction by the loss of suppressor activity. The common execution mechanisms of apoptosis consist of the activation of cytosolic aspartate-specific proteases (ICE-proteases) termed caspases, which can be activated via various intracellular pathways. In the stomach, mucosal surface epithelial cells are constantly exfoliating to the gastric lumen and completely replaced within 3—5 days under physiological conditions. Apoptosis has been reported to take place in all regions of the stomach with apoptotic cells occurring predominantly in the superficial parts of the gastric glands, at a rate of 2—3% for all cells. Following mucosal injury (e.g. ulcer development), apoptosis rapidly increases and remains elevated for 2—3 months. In a 3-month old ulcer scar, the apoptosis rate of mucous, parietal, chief and endocrine cells was found to be similar to that of normal gastric mucosa. *Helicobacter pylori* (*H. pylori*) infection induces apoptosis in the gastric mucosa and this action appears to be independent of VacA cytotoxin of *H. pylori* strains. Nonsteroidal anti-inflammatory drugs (NSAIDs), especially cyclooxygenase-2 (COX-2) inhibitors are potent inductors of gastric epithelial cell apoptosis. However, they can abrogate apoptosis or proliferation effects induced by *H. pylori*. Many details of the exact intracellular and molecular mechanisms regulating apoptosis in gastric mucosa remain to be elucidated.

Key words: *apoptosis, gastric mucosa, Helicobacter pylori, NSAIDs, caspases.*

INTRODUCTION

Tissue integrity depends on a balance between the cell renewal and the death of damaged or aged cells. Gastric mucosa is a tissue with a high renewal

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rate. For example, it takes approximately 3—5 days to replace all surface epithelial cells (1). Apoptosis — a programmed cell death — is the last stage of a cell destiny (2—4). It is a controlled disassembly of a cell, which does not affect neighbouring cells and does not induce an inflammatory responses (1). Apoptosis can be triggered by a variety of factors e.g. UV or γ-radiation, heat shock, DNA injury, protease inhibitors, and other factors listed in *Fig. 1.*

Factors and injurious stimuli inducing apoptosis

Proinflammatory cytokines: TNFα, IL-1, IL-16, Lymphotoxin β
Other death factors: Fas Ligand, TRAIL
Genetic factors: Sek1 null mutation
UV- and γ-radiation
Heat shock, hypoxia
Withdrawal of hormones and growth factors
DNA damaging agents and anticancer drugs: 5 fluorouracil, cisplatin, doxorubicin, daunorubicine, vincristine, nocodazole, colchicine, arabinofuranosylocytosine,
Metabolic changes: intracellular acidification
Reactive oxygen species: H_2O_2, NO donors (S-nitrooglutathione), oxidized dopamin
Other metabolic drugs: polyphenols, 2-methoxyestradiol, monochloramine, lovastatin, amylin, ceramide, arsenite, curcumin, calphostin C

Fig. 1. Factors and injurious stimuli inducing apoptosis.

There are two central pathways that lead to apoptosis: a) the positive induction by ligands (death factors) binding to plasma membrane receptors (death factor receptors) (*Fig. 3*) and b) negative induction by the loss of suppressor activity. The common execution mechanisms of apoptosis consist of the activation of cytosolic aspartate-specific proteases (ICE-proteases) termed caspases (5, 6), which can be activated via various intracellular pathways. The activated caspases cause endonucleolytical cleavage of DNA (7), and subsequent cleavage of several cellular substrates such as poly(ADP-ribose) polymerase, gelsolin, actin, lamin, and fodrin (8—11). The caspase enzymes (caspase-1–12), the members of the interleukin-1β converting enzyme (ICE) family, are divided based on their sequence homology into three groups: ICE-, CPP32-, and Ich-1-like proteases. The activation of caspases is accompanied by characteristic morphologic changes of the cell, including membrane blebbing, cell shrinkage, margination of nuclear matrix, chromatin condensation, DNA cleavage and formation of apoptotic bodies (*Fig. 2*).

Positive induction of apoptosis involves ligands such as tumor necrosis factor (TNF), TRAIL (TNFα-related apoptosis inducing ligand), or CD95 ligand/Fas ligand (CD95L/FasL) (*Fig. 3*), which are able to induce programmed cell death by acting on the surface cell death factor receptors [TNF receptor-1 (TNFR1), death receptor 3—6 (DR-3-6) and CD95/Fas receptor]. The ligands are predominantly trimeric and bind to cell surface causing the

aggregation of cell surface receptors (12). Receptor oligomerization orients their cytosolic 80 amino acid death domains (DD) into a configuration that recruits adapter proteins (13). The adapter complex in turn recruits caspase-8, causing its activation and initiating the cascade of caspase-mediated cell disassembly.

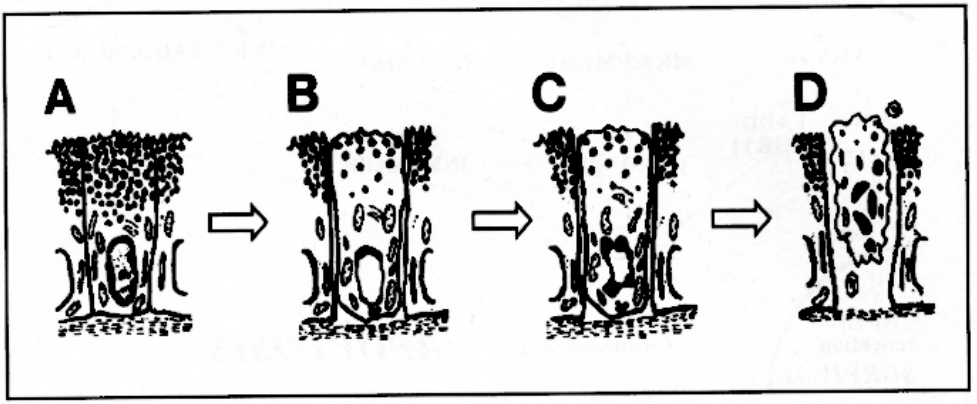

Fig. 2. Diagrammatic presentation of cellular changes during apoptosis: (A) normal cell, (B) translocation of chromatin toward the nuclear envelope, (C) condensation of the cytoplasm, followed by (D) nuclear fragmentation and the formation of membrane-bound apoptotic bodies.

The death ligand, TNFα, can lead to the activation of caspases in many cell types through the activation of the two members of mitogen activated protein kinase (MAPK) superfamily; c-Jun N-terminal kinase/stress-activated protein kinases (JNK1/SAPK) (14—16) or p38 MAPK family (17, 18). The upstream mediator of p38 is MAPK kinase 3/6 (MKK3/MKK6), which can be activated by apoptosis signal regulating kinase (ASK1). ASK1 also activates stress signaling kinase (SEK1/MKK4), the inducer of JNK1/SAPK after genotoxic stress (*Fig. 3*). One apoptotic pathway initiated through TNFR1 uses caspase-8 pathway through the interaction of TNFR-associated death domain protein (TRADD) and Fas-associated death domain protein (FADD/MORT1) (19). TRADD additionally recruits receptor interacting protein (RIP), which may trigger a second pathway (*Fig. 3*).

In CD95/Fas-induced signaling pathway (20), the activation of JNK1/SAPK has been described through MKK7 (21) (*Fig. 3*). Binding of the ligand (CD95L/FasL) is thought to induce oligomerization of the receptor with transduction of the death signal by recruitment of FADD/MORT1, which in turn binds to caspase-8, initiating the protease cascade of apoptosis (22). Fas has been shown to be constitutively expressed at a low level in most tissues, including the gastric mucosa and other parts of the gastrointestinal tract (23, 24).

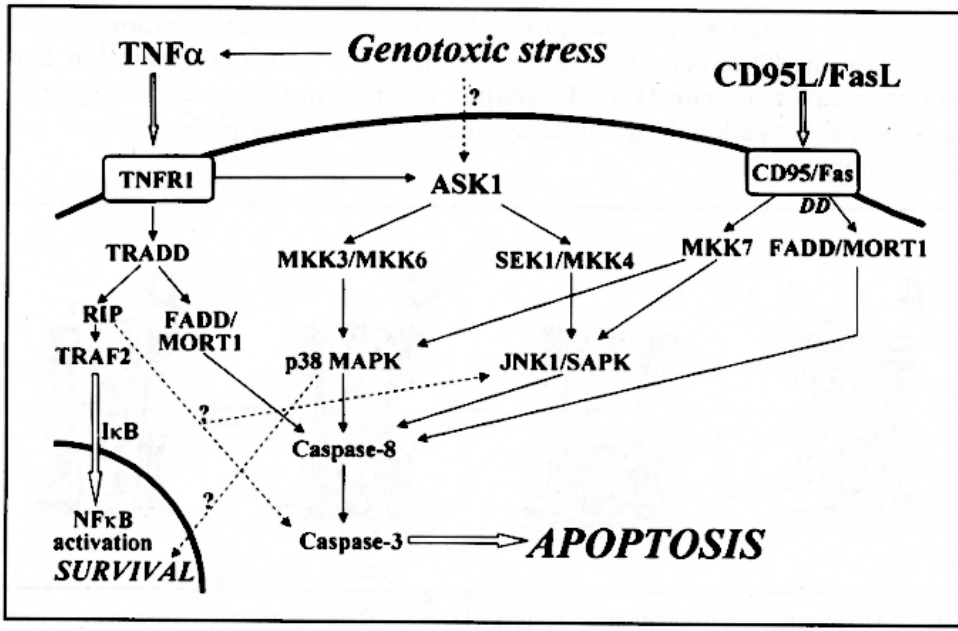

Fig. 3. Diagrammatic presentation of general pathways of positive induction of apoptosis. Abbreviations: ASK1, apoptosis signal regulating kinase-1, DD, death domain; JNK1/SAPK, C-Jun N-terminal kinase or stress-activated protein kinase; CD95L/FasL, CD95 ligand or Fas ligand; FADD/MORT1, Fas-associated death domain protein; MAKP, mitrogen activated protein kinase; MKK, mitogen activated protein kinase kinase; NFκB, nuclear factor-κB; RIP, receptor interacting protein; SEK1, stress signal kinase-1; TNFα, tumor necrosis factor receptor-1; TRADD, TNFR-associated death domain; TFAF2, TNF receptor associated factor-2.

The recently identified death receptors, DR-3, -4, -5 and -6 mediate cell death in a wide variety of malignant cell lines and primary tumor cells (25—26), where they exert action similar to TNFR1 but not to Fas (27). They have not been shown to be present in gastric mucosal cells.

Negative induction of apoptosis is generated by the loss of suppressor activity and involves the mitochondrial mechanisms. Release of cytochrome C from mitochondria into the cytosol serves as a trigger to activate caspases (28) (*Fig. 4*). Proteins of Bcl-2 family regulate the permeability of mitochondrial outer membrane to ions and to cytochrome C. At least 16 genes of Bcl-2 family have been identified (29), which either promote (Bax, Bad, Bak) or antagonise (Bcl-2, Bcl-x) apoptosis. The ratio of apoptosis promoting factors to those inhibiting apoptosis in the cell determines whether the apoptotic cell death will be turned on and the cascade of caspases will be activated. In the cytosol, cytochrome C binds to apoptosis protease activator protein-1 (APAF-1) in the presence of ATP and activates caspase-9, which induces effector caspases such as caspase-3 (30).

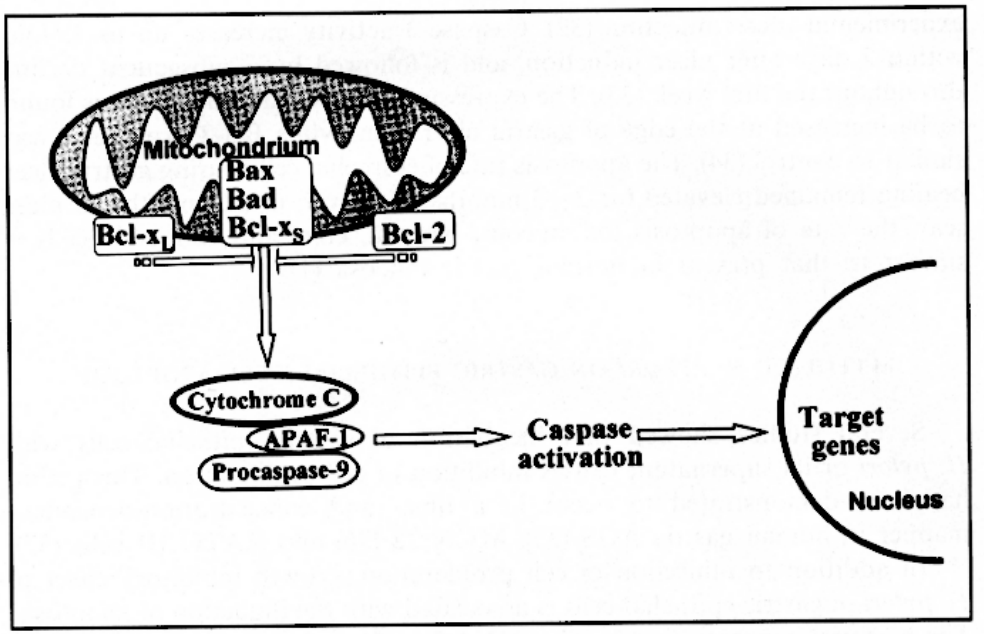

Fig. 4. Diagrammatic presentation of negative induction of apoptosis. Abbreviation: APAF-1, apoptosis protease activator protein-1.

The pathway by which apoptosis is induced clearly vary, depending on stimulus and cell types. The sequence of intracellular signaling and events involved in apoptosis of gastric mucosa has been only partly elucidated.

APOPTOSIS IN THE GASTRIC MUCOSA

The surface mucosal cells are constantly exfoliating into the gastric lumen, with a 3—5 day renewal rate under normal physiological conditions. The fasting of the animal prolongs the cell renewal rate, while feeding and injury causes faster renewal. Apoptosis has been reported to take place in all regions of the stomach, occurring predominantly in the upper part of the gastric glands and involving 2—3% of all epithelial cells (31). Exfoliated surface mucosal cells are replaced by cells migrating from the glandular foveolar and neck area (1). During migration these cells differentiate, mature and eventually degenerate. The loss of parietal, chief, and endocrine cells of oxyntic mucosa is much slower than the loss of surface epithelial cells (1).

Apoptosis has been shown to be induced following acute mucosal injury and during gastric ulcer healing (1, 32). At the onset of gastric ulceration, the rate of apoptosis rapidly increases. The 3.9-fold increase in mucosal expression of caspase-3 (effector caspase) activity can be detected as early as 2 hrs after

experimental ulcer induction (32). Caspase-3 activity increases up to 33-fold within 2 days after ulcer induction, and is followed by a subsequent decline throughout the first week (33). The expression of Bax, Bak and p53 was found to be increased at the edge of gastric ulceration, while Bcl-2 expression was similar to control (34). The apoptosis rate of epithelial cells during gastric ulcer healing remained elevated for 2—3 months. However, in a 3-month old ulcer scar, the rate of apoptosis for mucous, parietal, chief and endocrine cells is similar to that present in normal gastric mucosa (1).

EFFECT OF *H. PYLORI* ON GASTRIC EPITHELIAL CELL APOPTOSIS

Several studies showed that incubation of gastric epithelial cells with *H. pylori* or its supernatant causes inhibition of cell proliferation. This action has been demonstrated to occur in a time- and concentration-dependent manner in human gastric AGS (35), MKN 28 (36) and KATO III cells (37).

In addition to inhibition of cell proliferation, growth inhibitory effect of *H. pylori* in gastric epithelial cells is associated with the induction of apoptosis. The bacterial concentration causing DNA fragmentation and reduction of cell number was found to be 100-fold lower than the concentration that causes inhibition of DNA synthesis (31). Using terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labelling (TUNEL) technique to detect apoptosis in gastric mucosal sections, Moss et al. found that the apoptotic index was increased up to 16% in *H. pylori* infected patients with duodenal ulcers, whereas it is only 2—3% in uninfected healthy mucosa (31). Eradication of *H. pylori* decreased the elevated apoptotic index of the gastric mucosa to the basal level.

Further studies aimed to characterise the anti-proliferative activity of *H. pylori* demonstrated that this effect does not correlate with the presence of vacuolizing cytotoxin (VacA) (32). Knipp et al. (36) found a putative inhibitory protein originated from the cytosol of *H. pylori* (termed proliferation inhibiting protein) responsible for induction of apoptosis in gastric mucosa. Another potential candidate is ammonia, generated by *H. pylori's* urease. Ammonia has been shown to induce apoptosis *in vitro* in rat gastric epithelial cell lines (38). Wagner et al. (39) suggested that the stimulation of apoptosis is a general feature of *H. pylori* that is induced by all strains, and is specific to *H. pylori*, because *C. jejuni* is not able to affect epithelial cell growth and apoptosis. Clarithromycin has the ability to attenuate *H. pylori*-induced apoptosis of gastric mucosa by altering either the metabolism of the bacteria or the mucosal responses induced by bacteria (40).

In vivo studies using proliferating cell nuclear antigen, BrdU labeling and MIB-1 antibody contradicted the results of the above *in vitro* investigations.

H. pylori infection is accompanied by increased epithelial cell proliferation in patients with *H. pylori*-associated gastritis (41—45). Enhanced apoptosis caused by *H. pylori* occurs predominantly in the superficial compartment of the mucosa, where *H. pylori* is localised. The inflammation of the deeper portion of the mucosal glands, such as the neck area, combined with the high rate of apoptosis may cause a compensatory cell hyperproliferation. Piotrowski *et al.* (46) demonstrated that bacterial lipopolysaccharide (LPS) is the virulence factor responsible for the induction of apoptosis by *H. pylori*. Intragastric administration of *H. pylori* LPS induces a concentration dependent apoptosis in rat gastric mucosal cells detected by *in situ* DNA fragmentation assay (46).

In the analysis of other potential mechanisms of *H. pylori*-induced apoptosis, it should be pointed out, that *H. pylori* infection stimulates the release of pro-inflammatory cytokines — interleukin-1, interleukin-2, interleukin-6, interleukin-8, interferon-γ, and TNFα (47—49). These cytokines have been shown to upregulate the expression of CD95/Fas (50). CD95 expression has been shown upregulated and CD95L/FasL mRNA levels increased in gastric surface epithelium of patients infected with *H. pylori*, suggesting that apoptosis involved in *H. pylori*-gastritis includes CD95 and CD95L activation (51, 52). CagA producing *H. pylori* strains are associated with increased cytokine production in the gastric mucosa and in gastric epithelial cells (24, 48, 53—55), which in turn may be responsible for activation of CD95-CD95L system. Incubation of gastric epithelial cells with *H. pylori* supernatant in presence of $F(ab')_2$ anti-Fas fragments is able to reduce (at least in part) apoptosis. In addition, the number of dead cells detected by cytotoxic assay is higher than the fraction of apoptotic cells measured by FACS analysis, indicating that cytosolic cell death might also occur in addition to apoptosis (51). This additional cytolytic cell death is caused by *H. pylori* VacA cytotoxin, which induces cell degeneration by vacuolar-ATPase (56).

Recent experiments have shown changes in the expression levels of mitochondrial factors (negative inductors) during *H. pylori*-induced apoptosis. Upregulation of pro-apoptotic Bax, Bcl-x_S and downregulation of anti-apoptotic Bcl-2 were found *in vivo* in gastric epithelium and *in vitro* in Kato III cells infected with *H. pylori* (57, 58).

NSAIDS AND APOPTOSIS

NSAIDs have been shown to induce apoptosis in isolated rat gastric mucosal cells (59) and in various cell lines derived from gastrointestinal tract (60—63). Indomethacin and sodium diclofenac inhibit cell growth (64) and induce apoptotic DNA fragmentation in gastric cells in a dose- and time-dependent manner (59, 60). Following indomethacin treatment, a 20-fold

increase in cell apoptosis, and 4-fold increase in caspase-3 activity can be detected in rat gastric mucosa (32). The DNA fragmentation induced by COX inhibitors, particularly COX-2 inhibitors, is not affected by the exogenous addition of 16,16-dimethyl prostaglandin E_2, suggesting that the inhibition of prostaglandin production is not responsible for this process. However, NSAIDs-induced apoptosis was significantly reduced by caspase inhibitors (59, 65).

The mechanisms of NSAIDs-inducing apoptosis are not fully elucidated. NSAIDs-induced TNFα release in the gastric mucosa might be responsible for positive induction of apoptosis (33, 66, 67). The expression of apoptosis-related genes, such as Fas, Bcl-2 and Bax, are not affected by indomethacin or selective COX-2 inhibitors (e.g. NS-398). Intra- and extra-cellular calcium chelators, protein tyrosine kinase inhibitor, protein kinase A (PKA) inhibitor and protein kinase C (PKC) inhibitors do not influence indomethacin-induced apoptosis in colon cancer cells. In contrast, NSAIDs induced p21waf-1 transcription rate, suggesting the possible association of NSAIDs-induced apoptosis with cell-cycle control (68). The up-regulation of c-myc proto-oncogene was associated with NSAID-induced apoptosis in gastric epithelial cell lines. In these cell lines indomethacin increased c-myc mRNA and protein expression. Conversely, down-regulation of c-myc with antisense oligonucleotides significantly reduced indomethacin-induced apoptosis (69).

Nitric oxide plays also a role in indomethacin-induced apoptosis in the gastric mucosa. The activity of nitric oxide synthase-2 (NOS-2) positively correlates with the elevation of caspase-3 activity in indomethacin-induced apoptosis in rat gastric mucosa, suggesting the participation of NOS-2 in the amplification of apoptosis signaling (32). Nitric oxide-releasing NSAIDs (NO-NSAIDs), a new class of NSAID derivatives with markedly reduced gastrointestinal toxicity, are non-peptide caspase inhibitors. Inhibition of caspases by these drugs and their ability to increase gastric blood flow likely contribute to their gastroprotective effect (66).

Increased caspase-3 activity can be observed shortly after aspirin treatment in rat gastric mucosa, while chronic administration of aspirin causes only slight elevation of caspase activity, indicating that the resistance to apoptosis might be one of the mechanisms responsible for gastric mucosal adaptation to chronic aspirin administration (70).

In addition to above described mechanisms of NSAID-induced apoptosis, there are certainly many other factors and intracellular pathways involved in this process, which remain to be elucidated.

It remains controversial whether the harmful effects of *H. pylori* and NSAIDs on gastric mucosa are additive. A significantly higher apoptosis rate can be observed in gastric mucosa of human subjects with *H. pylori* infection or treated with NSAIDs, compared to controls. Unlike NSAIDs treated subjects,

patients with *H. pylori* infection show significantly enhanced gastric mucosal proliferation. Interestingly, a study indicates that NSAIDs do not potentiate but rather abrogate the apoptosis induced by *H. pylori* (71).

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ABBREVIATIONS

APAF-1, apoptosis protease activator protein-1; ASK1, apoptosis signal regulating kinase-1; BrdU, bromodeoxyuridine; CagA, cytotoxin associated protein; DD, death domain; JNK1/SAPK, C-Jun N-terminal kinase-1 or stress-activated protein kinase; CD95L/FasL, CD95 ligand or Fas ligand; COX-2, cyclooxygenase-2; FADD/MORT1, Fas-associated death domain protein; FACS, fluorescense-activated cell sorter; ICE, interleukin-1β converting enzyme; IκB, inhibitor of NFκB; LPS, lipopolysaccharide; MAPK, mitogen activated protein kinase; MIB-1, a monoclonal antibody reacting with Ki-67 antigen; MKK, mitogen activated protein kinase kinase; NFκB, nuclear factor-κB; NOS-2, nitric oxide synthase-2; NSAIDs, nonsteroidal anti-inflammatory drugs; NO-NSAIDs, nitric oxide-releasing NSAIDs; RIP, receptor interacting protein; PI-3K, phosphatidylinositol-3 kinase; PKA and PKC, protein kinase A and C, respectively; SEK1, stress signal kinase-1; TNFα, tumor necrosis factor-alpha; TNFR1, tumor necrosis factor receptor-1; TNFR1, tumor necrosis factor receptor-1; TRADD, TNFR-associated death domain; TRAF2, TNF receptor associated factor-2; TRAIL, TNF-related apoptosis inducing ligand; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labelling; VacA, vacuolating cytotoxin.

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Author's address: Andrzej S. Tarnawski, M. D., D. Sc., Gastroenterology Section (111G, DVA Medical Center Long Beach (CA), 5901 East Seventh Street, Long Beach, CA 90822, Tel: (562) 494—5804, Fax: (562) 494—5675.
E-mail: astarnaw@uci.edu