Original Contribution

Protection of Gastric Mucosal Cells from Apoptosis and Necrosis by Induction of HSP and PGE₂

TOHRU MIZUSHIMA*, SHINJI TSUTSUMI,
TATSUYA HOSHINO, WATARU TOMISATO

Faculty of Pharmaceutical Sciences, Okayama University, Okayama, Japan

Abstract: Various gastric irritants induce cell death of gastric mucosal cells, resulting in gastric ulcers *in vivo*. We recently reproduced gastric irritant-induced cell death *in vitro*, using primary cultures of guinea pig gastric mucosal cells. We found that the short-term treatment of cells with relatively high concentrations of irritants or the long-term treatment of cells with relatively low concentrations of irritants induce necrosis or apoptosis, respectively. We also reproduced the spontaneous apoptosis of matured gastric mucosal cells at gastric surface and found that the apoptosis-inducing pathway of this spontaneous apoptosis is different from that of the irritant-induced apoptosis. Heat shock proteins (HSPs) are known to make cells non-specifically resistant to various irritants. We found that pre-induction of HSPs by geranylgeranylacetone or a low concentration of ethanol protects gastric mucosal cells from both apoptosis and necrosis induced by various gastric irritants. Therefore, non-toxic HSP-inducers, such as geranylgeranylacetone, seem to be clinically beneficial for gastric irritant-induced gastric ulcers.

Key Words: heat-shock proteins, gastric mucosal cells, gastric ulcers, apoptosis, necrosis

Introduction

The gastric mucosa can be damaged by various types of gastric irritants (such as alcohols, acids, oxidative stress, and non-steroidal anti-inflammatory drugs (NSAIDs)), resulting in development of gastric ulcers. These gastric irritants were thought to cause gastric mucosal injury by stimulating the onset of necrosis. However, recent studies suggest that apoptosis is also involved in the gastric mucosal injury and development of gastric ulcers *in vivo*¹⁾. The increased rate of apoptosis associated with *Helicobacter pylori* (*H. pylori*) infection was suggested to induce the atrophic gastritis²⁾. In order to understand the molecular mechanism governing gastric irritant-induced necrosis and apoptosis and to establish a clinical protocol for overcoming mucosal damage and gastric ulcers, a system for reproducing such necrosis and apoptosis *in vitro* is necessary. We recently reproduced such necrosis and apoptosis in guinea pig gastric mucosal cells in primary culture and examined the molecular mechanism. In this

Received 24 February 2003, Accepted 13 June 2003. *Corresponding author, Tel & Fax: +81-86-251-7958;
E-mail: mizushima@pharm.okayama-u.ac.jp

paper, we review our recent work on this molecular mechanism.

Heat shock proteins (HSPs) are induced by various irritants and make cells resistant to various irritants. We examined the effect of pre-induction of HSPs on gastric irritant-induced necrosis and apoptosis. In this paper, we also review these our recent work on HSPs and propose that non-toxic HSP-inducers are clinically beneficial for gastric irritant-induced gastric ulcers.

Mechanism of gastric-irritant-induced cell death

In order to understand the molecular mechanism governing gastric irritant-induced cell death, we reproduced such cell death in guinea pig gastric mucosal cells in primary culture and examined its molecular mechanism. As shown in Fig. 1A, treatment cells with various concentrations of indomethacin (one of NSAIDs) for 1 hr or 16 hr decreased cell viability in a dose-dependent manner³⁾. To understand the mechanism of this cell death, we examined the state of chromosomal DNA under these conditions. As shown in Fig. 1B, treatment of cells with indomethacin for 16 hr but not for 1 hr induced apoptotic DNA fragmentation³⁾. Therefore, it seemed that the short-term treatment of cells with relatively high concentrations of indomethacin or the long-term treatment of cells with relatively low concentrations of this drug induce necrosis or apoptosis, respectively. To confirm this idea, we carried out double-staining experiments with propidium iodide (PI) and Hoechst 33342 (Ho 342). Since necrotic cells lose their membrane integrity, PI staining causes pink nuclear staining in necrotic cells, whereas living cells and apoptotic cells are not stained with PI⁴⁾. Control cells and cells treated with indomethacin for 16 hr did not stain with PI (Fig. 2). In contrast, cells treated with 2.5 mM indomethacin for 1 hr showed pink nuclear staining, suggesting that the cell death is mediated by necrosis (Fig. 2). Furthermore, cells treated with indomethacin for 16 hr showed

Fig. 1. Effects of indomethacin on cell viability and chromosomal DNA.

Cultured gastric mucosal cells were incubated with indicated concentrations of indomethacin for 1 hr or 16 hr. Cell viability was determined by the trypan blue exclusion test (A). Chromosomal DNA was extracted and analyzed by 2% agarose gel electrophoresis (B).

chromatin condensation (one of characteristic features of apoptosis) suggesting that the cell death is mediated by apoptosis (Fig. 2). Furthermore, we examined whether or not the pre-treatment of cells with an inhibitor of caspase was able to inhibit the cell death induced by indomethacin. Pre-treatment of gastric mucosal cells with z-VAD-fmk (a broad-spectrum inhibitor of caspases) clearly inhibited the dose-dependent cell death seen following treatment with 1 mM indomethacin for 16 hr but not that with 2.5 mM indomethacin for 1 hr, suggesting that short-term treatment of cells with its relatively high concentrations of NSAIDs or the long-term treatment of cells with its relatively their low concentrations induces necrosis or apoptosis, respectively³⁾. We performed similar experiments for other gastric irritants (ethanol, hydrogen peroxide, and hydrochloric acid) and found that each of all these gastric irritants induces necrosis or apoptosis upon short-term treatment of cells with its relatively high concentrations or the long-term treatment of cells with its relatively low concentrations, respectively⁵⁻⁷⁾. Therefore, we concluded that gastric irritants generally induce both necrosis and apoptosis in gastric mucosal cells.

Next we tried to identify the apoptotic pathway for gastric irritant-induced apoptosis. Apoptotic pathways can be distinguished by their involvement of mitochondria. In a mitochondria-mediated pathway, the apoptotic stimulus causes mitochondrial dysfunction (such as a decrease in mitochondrial membrane potential and release of cytochrome c from mitochondria) and stimulates the release of cytochrome c from mitochondria, which in turn activates the downstream apoptotic pathway⁸⁾. Using an assay system for mitochondrial permeability, we examined whether or not these gastric irritants affect mitochondrial membrane potential. DiPsipher, mitochondrial dye, normally exists in solution as a monomer emitting green fluorescence and forms a dimer emitting red fluorescence in the reaction driven by changes in the mitochondrial membrane potential⁹⁾. Atractyloside, an inhibitor of the ATP-ADP carrier, is known to decrease the mitochondrial membrane potential⁹⁾. As shown in Fig. 3A, red fluorescence was apparent in control cells (in the absence of gastric irritants and atractyloside) especially in peripheral areas of each colony, while the red fluorescence was markedly decreased in atractyloside-treated cells. All of the gastric irritants tested (4% ethanol, 300 μ M hydrogen peroxide, or 20mM hydrochloric acid) decreased the red fluo-

Fig. 2. Induction of apoptosis and necrosis by indomethacin.

Cultured gastric mucosal cells were incubated in the presence or absence of indomethacin for 1 hr or 16 hr. After staining with both Ho 342 and PI, cells were observed under fluorescence microscope.

rescence (Fig. 3A), suggesting that the mitochondrial membrane potential was decreased by each of these gastric irritants⁵⁾. We also monitored the release of cytochrome c from mitochondria. As shown in

Fig. 3. Effects of gastric irritants on mitochondrial functions.

Cultured gastric mucosal cells were incubated with indicated concentrations of gastric irritants for indicated periods (A and B). Mitochondrial membrane potential was analyzed (A). The release of cytochrome c from mitochondria was monitored.

Fig. 3B, all of the gastric irritants tested stimulated the release of cytochrome c from mitochondria⁵⁾. From these observations, we consider that gastric irritant-induced apoptosis is driven by a mitochondria-mediated pathway.

Most apoptotic events are mediated by the sequential activation of caspases¹⁰⁾.

Among them, caspase-3 is located downstream of the pathway. In a mitochondria-mediated apoptosis pathway, mitochondrial dysfunction (such as a decrease in mitochondrial membrane potential) stimulates the release of cytochrome c from mitochondria, which in turn activates caspase-9 in collaboration with Apaf-1 and dATP, resulting in the activation of caspase-3¹⁰⁾. Since results obtained above suggest that gastric irritant-induced apoptosis is caused by a mitochondria-mediated pathway, we predicted that these gastric irritants activate caspase-9 and caspase-3. Since mitochondria-

mediated apoptosis pathways can be further distinguished on the basis of whether or not caspase-8 is activated, we also examined whether these gastric irritants activate caspase-8 in addition to caspase-3 and caspase-9. We found that caspase-3, caspase-8 and caspase-9 were all activated simultaneously by each of all these gastric irritants⁵⁾. Recently, caspase-8 was shown to be able to cause mitochondrial dysfunction and stimulate the release of

cytochrome *c* from mitochondria, which activates caspase-9. For example, in a death-receptor mediated apoptosis pathway, caspase-8 stimulated the release of cytochrome *c* by inducing cleavage of Bid, a bcl-2-related protein ¹¹⁾. These findings suggest that these gastric irritants may at first activate caspase-8 and cause mitochondrial dysfunction to trigger the cytochrome *c*-dependent pathway, in which both caspase-9 and caspase-3 are activated. Furthermore, all of our results supported the notion that these gastric irritants induce apoptosis through a common pathway.

Spontaneous apoptosis associated with a rapid rate of turnover cycle of gastric mucosal cells

Gastric mucosal cells have a rapid rate of turnover, which makes them unique among mammalian cell types. This short turnover cycle is the result of rapid proliferation of progenitor cells at the isthmus, cell maturation that occurs during the upward migration of cells, and rapid apoptotic cell death at the gastric surface. In order to understand the molecular mechanisms governing this short turnover cycle of gastric pit cells, in particular the rapid apoptotic cell death of mature cells at the gastric surface, a suitable *in vitro* model that can adequately reproduce rapid apoptotic cell death is necessary. We found that primary culture of guinea pig gastric mucosal cells spontaneously induce apoptosis in a serum dependent-manner, which is thought to mimic the rapid apoptotic cell death that takes place at the gastric surface *in vivo* ¹²⁾. In order to know whether or not this spontaneous apoptosis share the apoptosis pathway with the irritant-induced apoptosis, we examined the molecular mechanism of this spontaneous apoptosis by examining the activation of caspase. Associating with the spontaneous apoptosis, caspase-3 activity was stimulated strongly (about 10-fold), however, caspase-8 and caspase-9 activities were not significantly activated ¹³⁾. Since caspase-3, caspase-8 and caspase-9 were all activated simultaneously by gastric irritants, it seems that spontaneous apoptosis is mediated by a different pathway from that of gastric irritant-induced apoptosis. Furthermore, these results suggest that some factors in serum are responsible for the spontaneous apoptosis.

We predicted that the factor is TGF- β 1, because TGF- β 1 is known to induce apoptosis in various mammalian cells *in vivo* and *in vitro* ¹⁴⁾¹⁵⁾. We, therefore, examined the effects of recombinant human TGF- β 1 on apoptosis in the absence of serum and found recombinant human TGF- β 1 induced apoptosis (Fig. 4A).

Fig. 4. TGF- β 1 is responsible for the spontaneous apoptosis.

Gastric mucosal cells were cultured with indicated concentrations of recombinant human TGF- β 1 (rhTGF- β 1) under the conditions of 0.5% FCS for 24 hr (A). TGF- β 1 in FCS was immunodepleted using anti-TGF- β 1 antibody and protein A-Sepharose. Gastric mucosal cells were cultured with immunodepleted or control FCS (10%) for indicated periods (B). Chromosomal DNA was extracted and analyzed by 2% agarose gel electrophoresis.

Furthermore, we also showed that the spontaneous apoptosis observed in the presence of serum was diminished by the immunodepletion of TGF- β 1 from serum (Fig. 4B). These data suggest that TGF- β 1 in serum is responsible for the induction of the spontaneous apoptosis.

Effects of HSP-induction on necrosis and apoptosis induced by gastric irritants

As described above, various gastric irritants induce both necrosis and apoptosis, resulting in gastric ulcers. Therefore, in order to prevent gastric ulcers, both apoptosis and necrosis by various gastric irritants should be attenuated. However, since the inducing mechanism of necrosis and apoptosis is different from each other, it is impossible for so-called “specific inhibitor” to attenuate both necrosis and apoptosis induced by various gastric irritants. For example, inhibitors for caspases, which are essential for apoptosis, cannot inhibit necrosis.

When cells are exposed to irritants, they induce so-called “stress proteins” in order to protect themselves from irritants. Ones of representative stress proteins are HSPs.

Cells (from bacteria to human cells) induce HSPs upon exposure to various irritants such as heat, alcohol, and heavy metals and protect themselves from these irritants¹⁶⁾. One of key words for HSP-induction is “non-specific”. HSPs are induced by various (non-specific) irritants and make cells resistant to various (non-specific) irritants¹⁶⁾. This non-specificity made the application of HSP-inducers for development of clinical drugs being difficult, because it is generally thought that clinical drugs must be specific and non-specificity of drug causes its side effects. However, due to the non-specificity of HSPs, we consider that HSP-induction can protect gastric mucosal cells from both necrosis and apoptosis induced by various gastric irritants, in other words, non-toxic HSP-inducers are clinically beneficial as anti-ulcer drugs. One of reasons for this idea is that the most frequently used anti-ulcer drug in Japan, GGA, was reported to be non-toxic HSP-inducer¹⁷⁾. Rokutan and his co-workers showed that GGA induces HSPs by activating HSF1 (a transcription factor for hsp genes) and make gastric mucosal cells resistant to ethanol¹⁷⁾. Therefore, we examined the effect of GGA on necrosis and apoptosis induced by various gastric irritants.

As shown in Fig. 5, pre-treatment of gastric mucosal cells with GGA attenuated both necrosis and apoptosis induced by each of all gastric irritants tested (indomethacin, ethanol, hydrogen peroxide, and hydrochloric acid)^{6)18–20)}. We confirmed that this anti-apoptosis and anti-necrosis effect of GGA is mediated by HSP-induction by following experiments. The time-course and dose-response for the anti-apoptosis and anti-necrosis effect of GGA were well correlated with those for induction of HSPs by GGA.

Pretreatment of cells with cycloheximide (an inhibitor of protein synthesis) completely abolished the anti-apoptosis and anti-necrosis effect of GGA. Furthermore, we showed that induction of HSP by pre-treatment of cells with a low concentration of ethanol also protected gastric mucosal cells from both necrosis and apoptosis induced by various gastric irritants^{6)18–20)}. All these results suggest that pre-induction of HSPs protects cells from irritant-induced both necrosis and apoptosis in general and non-toxic HSP-inducers, such as GGA, are clinically beneficial as anti-ulcer drugs.

We found that GGA also attenuates the spontaneous apoptosis associated with a rapid rate of turnover cycle of gastric mucosal cells²¹⁾. Being different from irritant-induced apoptosis, it is not clear

Fig. 5.

whether or not the spontaneous apoptosis should be attenuated for the protection of gastric mucosa. In conditions of stress, such as exposure to toxic chemicals, the gastric mucosal cells are damaged, and the number of cells may be decreased, which make the balance of aggressive and defensive factors in the worse direction. From this point of view, inhibition of the spontaneous apoptosis would seem to be beneficial for the protection of the gastric mucosa against various gastric irritants, as it would increase the number of gastric mucosal cells. Supporting this notion, growth factors for gastric mucosa were shown to be effective in combating ulcers in rats by increasing the number of gastric mucosal cells present ²²⁾.

As for the mechanism of suppression of apoptosis by HSPs, it seems that there are multiple target molecules depending on species of HSPs. For example, HSP90 inhibits cytochrome *c*-mediated oligomerization of Apaf-1 ²³⁾. HSP70 inhibits apoptosis downstream of cytochrome *c* release and upstream of caspase-3 activation ²⁴⁾. Furthermore, Hsp27 inhibits apoptosis by interacting with cytochrome *c* ²⁵⁾.

B

Fig. 5. Effect of HSP-induction on gastric irritant-induced apoptosis and necrosis.

Cultured gastric mucosal cells were pre-incubated with GGA (1 μ M) or 3% ethanol for 2 hr. Cells were further incubated for 1 hr (A) or 4-16 hr (B) with various concentrations of indomethacin, ethanol, hydrogen peroxide, and hydrochloric acid. Cell viability was determined by MTT method (A). Chromosomal DNA was extracted and analyzed by 2% agarose gel electrophoresis (B).

Effect of Prostaglandin E₂ on gastric irritant-induced cell death

In addition to HSPs, there are a number of factors, which protect the gastric mucosa from various irritants. Prostaglandin E₂ (PGE₂) has a strong protective effect on the gastric mucosa in vivo; however, the molecular mechanism of a direct cytoprotective effect of PGE₂ on gastric mucosal cells is yet to be elucidated. Therefore, we examined the effect of PGE₂ on necrosis and apoptosis induced by various gastric irritants. As shown in Fig. 6, pre-treatment of cells with PGE₂ attenuated apoptosis but not necrosis induced by various gastric irritants²⁶⁾. Therefore, the mechanism of cytoprotection of gastric mucosal cells by PGE₂ seems to be different from that by HSPs.

We further examined the mechanism of anti-apoptotic effect of PGE₂ in gastric mucosal cells. Of the four main subtypes of PGE₂ receptors (EP₁₋₄), we demonstrated, using subtype-specific agonists, that EP₂ and EP₄ receptors are involved in the PGE₂-mediated protection of gastric mucosal cells from ethanol-induced apoptosis²⁷⁾.

Activation of EP₂ and EP₄ receptors is coupled with an increase in cAMP, for which a cAMP analogue was found to inhibit the ethanol-induced apoptosis²⁷⁾. The increase in cAMP is known to

Fig. 6. Effect of PGE₂ on ethanol-induced apoptosis and necrosis.

Cultured gastric mucosal cells were pre-incubated with indicated concentrations of PGE₂ for 2 hr. Cells were further incubated for 4 hr with 3% (A, B) (apoptotic conditions) or 1 hr with 8% (C) (necrotic conditions) ethanol (EtOH). Chromosomal DNA was extracted and analyzed by 2% agarose gel electrophoresis (A). Cell viability was determined by MTT method (B, C).

activate both protein kinase A (PKA) and phosphatidylinositol 3-kinase (PI3K) pathways²⁸). An inhibitor of PKA but not of PI3K blocked the PGE₂-mediated protection of cells from ethanol-induced apoptosis, suggesting that a PKA pathway is mainly responsible for the PGE₂-mediated inhibition of apoptosis. Based on these results, we consider that PGE₂ inhibited gastric irritant-induced apoptosis in gastric mucosal cells via induction of an increase in cAMP and activation of PKA. We also found that PGE₂ inhibited the spontaneous apoptosis, however, its molecular mechanism is unclear at present²⁹).

Conclusions

Based on results described above, we concluded that various gastric irritants damage gastric mucosa by inducing both necrosis and apoptosis and that HSPs can attenuate both necrosis and apoptosis. Therefore, non-toxic HSP-inducers, such as GGA, are useful as anti-ulcer drugs. HSPs can re-fold or degrade mis-folded proteins. It recently became clear that mis-folded proteins cause various diseases (folding diseases), such as neurodegenerative diseases. Therefore, non-toxic HSP-inducers may be useful for various diseases other than gastric ulcers. In order to find more strong non-toxic HSP-inducers than GGA, it is necessary to reveal the mechanism of HSP-induction in mammalian cells, including the mechanism how GGA induces HSPs.

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ネクローシス, 及びアポトーシスに対する, HSP, 及び PGE2 による胃粘膜細胞の保護

水島 徹・堤 信二・星野 竜也・富里 亘

岡山大学薬学部

要 旨: 様々な胃粘膜ストレスは, 胃粘膜細胞死を起こし胃潰瘍の原因となっている. 我々はすべての胃粘膜によって, ネクローシスとアポトーシスの両者が誘導されることを見出した. さらに我々はゲラニルゲラニルアセトン, 及び低濃度エタノール処理による熱ショック蛋白質 (HSP) 誘導が, 様々な胃粘膜ストレスによるアポトーシス, 及びネクローシスから, 胃粘膜細胞を保護することを発見した. 従って, ゲラニルゲラニルアセトンのような毒性のない HSP 誘導薬は, 胃粘膜ストレスによる胃潰瘍に対し有効であると考えられる.