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INVOLVEMENT OF HEAT SHOCK PROTEINS IN THE HEALING OF ACETIC ACID-INDUCED GASTRIC ULCERS IN RATS

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The present study examined the expression of 73-kDa of heat shock cognate protein (HSC70), 72-kDa of heat shock protein (HSP70) and 47-kDa of HSP (HSP47) observed in the ulcer healing process in rats. Gastric ulcers were induced by a luminal application of acetic acid in male Donryu rats. During the ulcer healing process, the expression of HSPs in the ulcerated tissue was determined. A high level of HSC70 expression was observed both in the normal mucosa and ulcerated tissue, but the level did not change upon ulceration and ulcer healing. While HSP70 and HSP47 were markedly expressed in the ulcer base during ulceration, and decreased with ulcer healing. HSP70 expression in the ulcer margin was gradually increased with ulcer healing. Omeprazole accelerated the healing of gastric ulcers with strong inhibition of gastric acid secretion, while indomethactin delayed in ulcer healing despite slight inhibition of gastric acid secretion. Omperazole enhanced the expression of HSP70 both in the ulcer margin and base, but it reduced HSP47 expression in the ulcer base Indomethacin markedly enhanced HSP47 expression only in the ulcer base. In conclusion, the expression of HSP70 and HSP47 is changed during ulcer healing. Furthermore, it was suggested that the enhanced expression of HSP70 is involved in acceleration of ulcer healing, but overexpression of HSP47 is involved in delayed ulcer healing.

Key words: *HSC70, HSP70, HSP47, acetic acid ulcer, ulcer healing, omeprazole, indomethacin.*

INTRODUCTION

Heat shock proteins (HSPs) are necessary for essential cellular events, such as folding, assembly, and transport of proteins, as molecular chaperones, and also serve to protect cells from the cytotoxic effects of aggregated proteins produced by various stresses (1). Expression of HSPs is generally induced following exposure to heat shock, heavy metals, chemical agents, glucose starvation, and pathophysiological stresses in a wide range of living organisms (2). HSC70 (heat shock cognate, 73-kDa) is constitutively expressed in many

cells and has also been shown to have molecular chaperone activity (3). The 72-kDa HSP (HSP70) is the most inducible HSP (4) and the most closely linked to cytoprotection from thermal injury (5). In contrast, HSP47 is known to have a unique collagen binding ability, and is believed to be essential for mature collagen biosynthesis (6—8).

In general, it is considered that healing process of gastric ulcers is resemble for that of wound healing. Laplante *et al.* (9) reported that several HSPs were expressed in mouse skin during wound healing, suggesting that the expression of HSPs in the neoepidermis is related to the proliferation, migration, and differentiation states of keratinocytes within the wound. In the digestive tract, it is known that major HSPs are induced by various treatments. Especially, it was reported that HSP70 is induced by water-immersion stress, hyperthermia and gastric mucosal protecting drugs, suggesting that HSP70 is related to gastric mucosal protection (10—12). Accordingly, it is speculated that HSPs also play important roles in gastric ulcer healing. However, the involvement of HSPs in ulcer healing remains unclear.

Given these backgrounds, the present study investigated HSC70, HSP70 and HSP47 expressions during the gastric ulcer healing process. Furthermore, the expression of these proteins in the normal ulcer healing was compared to those in the healing promoted or delayed by drugs.

MATERIALS AND METHODS

Animals

Male Donryu rats, weighing 250—300 g (Shimizu SLC, Shizuoka, Japan), were used in all experiments. The animals were housed in meshed bottom cages to prevent corprophagy. The animals were fasted for 18 hr before experiments, but water was provided ad libitum. Studies were carried out using 4—5 rats per group.

Ulcer induction

Gastric ulcers were induced by a luminal application of acetic acid solution, according to a previously described method with slight modification (13). Briefly, the animals were fasted for 18 hr, anesthetized with ether, and operated upon to open their abdomens. Each stomach was exposed and the centers of the corpus area were clamped with ring forceps (ID 9 mm). A 60% acetic acid solution was injected into the luminal side at the clamped portion. Sixty seconds later, the acid was removed and the abdomen was closed. The animals were fed normally thereafter. We considered 3 days after the acid application as the day of ulceration (ulcer day 0). To determine the sizes of the ulcers, animals were anesthetized with ether at 0, 1, and 2 weeks after the ulceration, ice-cold saline was systematically perfused. Each stomach was removed and opened along the greater curvature. The stomachs were then rinsed with ice-cold saline and opened with pins on a corkboard.

The area of each ulcer was determined under a dissecting microscope with a square grid (Olympus, Kyoto, Japan). In this study, ulcers were produced both in the anterior and posterior walls. Consequently, the sum of both ulcerated areas was calculated and expressed as ulcer size per animal (13).

In another series of experiments, omeprazole or indomethacin was administered PO or SC at the dose of 60 mg/kg or 2 mg/kg once daily for 2 weeks, starting from the day of ulceration. The dose of drugs is previously reported to accelerate or delay the ulcer healing produced by luminal application of acetic acid solution (14).

Immunoblot analysis for HSPs

After the determination of the ulcer sizes, protein samples from normal mucosa, mucosa from ulcer margin (approximately 1 mm from the ulcer edge) and ulcer base was collected using glass slides. The wet weight of the samples were determined and the samples were then homogenized with 10 volumes of 20 mM Tris-HCl (pH 7.6) containing 150 mM NaCl, 1% triton X-100, 1 mM PMSF, 1 μg/ml leupeptin and 1 μg/ml pepstatin. The samples were then centrifuged at 13,000 rpm for 1 hr at 4°C, and the supernatant was collected as the protein samples. The protein concentration were determined using a BioRad protein assay reagent (BioRad, Osaka, Japan). To analyze HSPs, protein samples (10 μg) were electrophoresed on SDS-10% polyacrylamide slab gels, as described by Laemmli (15), and electrically transferred to a PVDF membrane (Immunobilon, Toyobo, Osaka, Japan). Sequential immunoblotting was performed using a monoclonal anti-HSC70 (Santa Cruz, CA, USA), anti-HSP70 (Stressgen, Victoria, Canada) or anti-HSP47 (Stressgen, Victoria, Canada) antibody, as a primary antibody. The membrane was then reacted with horseradish peroxidase-conjugated goat anti mouse-IgG antibody (Santa Cruz, CA, USA) for 30 min at room temperature. Western blots were visualized by an enhanced chemiluminescence system (Western Blot Chemiluminescence Reagent Plus, NEN, Boston, USA). Protein expression was densitometorically quantified (Quantity One, PDI, NY, USA). Data are expressed as the % changes from normal mucosal levels.

In this study, the protein extract from RGM-1 cells treated with heat shock was used as a positive control. Briefly, RGM-1 cells were treated with heat shock at 42°C for 2 hr. Subsequently, the cells were incubated at 37°C for 20 hr. After washing, total protein was extracted using 0.1% SDS. Using this protein extract, only one band could be detected against each antibody.

Determination of gastric acid secretion

Gastric acid secretion was determined by pylorus ligation method. Briefly, omeprazole or indomethacin was administered for 2 weeks, starting from the day of ulcerations. The animals were fasted for 18 hr after the final administration and the each drug was administered again. Thirty min later, the pylorus was ligated under ether anesthesia through a short midline incision. The animals were killed 3 hr later, and the gastric contents were collected and analyzed for volume and acidity. Acidity was determined by automatic titration of the contents against 0.1 M NaOH to pH 7.0 (Comtite 7; Hiranuma, Tokyo). Total acidoutput (volume × acidity) was expressed as μEq/hr.

Data analysis

All data are presented as means ± SEM for 4—5 animals. Statistical analysis was performed using the student *t-test*, with a P value < 0.05 considered to be significant.

RESULTS

Expression of HSC70, HSP70 and HSP47 during ulcer healing

Upon application of the 60% acetic acid solution onto the gastric mucosa, penetrating ulcers developed in the gastric fundus 3 days after the acid application with 100% incidence. At this time, the ulcerated area was 84.2 ± 3.2 mm^2. These ulcers healed with time, but the healing process was found to be divided into two phases. Rapid healing was observed in the first 7 days, while healing was found to be slow in the last 7 days. The healing rates of early and late phases were 8.9 mm^2/day and 1.9 mm^2/day, respectively.

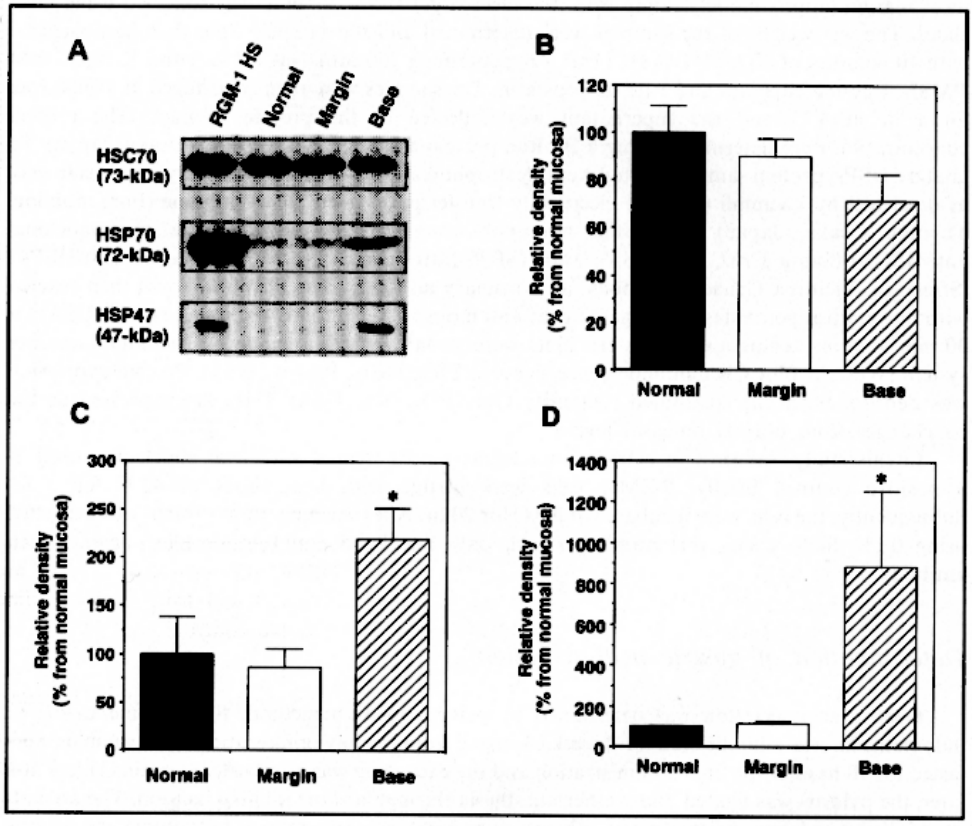

Fig. 1. HSPs expressions at the time of ulcerations. Western blott pattern (A) and densitometory quantification of HSC70 (B), HSP70 (C) and HSP47 (D) were shown. Data are presented as the means ± SEM for 5 animals. * Significantly different from normal mucosa, at $P < 0.05$. RGM-1 HS: positive control.

HSC70 was constitutively expressed in the normal mucosa, and exhibited similar levels in the ulcer margin and base, as compared with normal mucosa (*Fig. 1-A*). In contrast, HSP70 and HSP47 expressions were detectable but

quite weak in the ulcer margin and normal mucosa, it was marked in the ulcer base. Densitometorically quantifying expressions of HSP70 and HSP47 in the ulcer base revealed statistically significant results (*Fig. 1-C, D*). The expression levels of HSP70 and HSP47 were 2.2- and 8.8-fold that of normal mucosa, respectively.

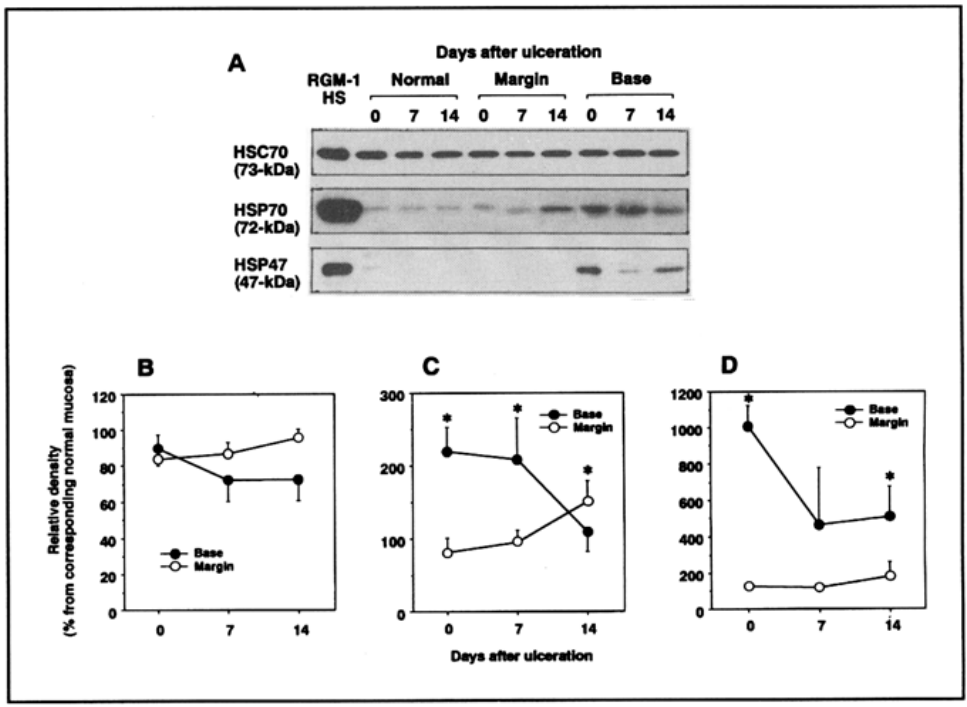

Fig. 2. Course of changes in HSPs expressions during ulcer healing. Western blot pattern (A) and densitometory quantification of HSC70 (B), HSP70 (C) and HSP47 (D) were shown. Data are presented as the means ± SEM for 4—5 animals. * Significantly different from corresponding normal mucosa, at $P < 0.05$. RGM-1 HS: positive control.

HSC70 expression in the ulcer margin and ulcer base was not changed during the ulcer healing process (*Fig. 2-A, B*). HSP70 expression in the ulcer margin gradually increased with ulcer healing (*Fig. 2-A, C*). The 1.5-fold increase in the HSP70 level was observed at day 14, and was found to be statistically significant, as compared with the normal mucosa. In contrast, the HSP70 level in the ulcer base was markedly increased at day 0, but gradually decreased upon healing of the ulcers. HSP47 expression in the ulcer margin was not changed during ulcer healing (*Fig. 2-A, D*). On the other hand, HSP47 expression was dramatically increased upon ulcer development, but decreased upon ulcer healing. Nonetheless, HSP47 expression was maintained at higher levels at 1 and 2 weeks later, as compared with normal mucosa. The increase in

HSP47 at day 0 and 14 were statistically significant in comparison with levels in normal mucosa.

Effects of omeprazole and indomethacin on healing of gastric ulcers

When omeprazole was administered for 1 or 2 weeks starting from the day of ulcerations, the healing of gastric ulcers were significantly promoted (*Fig. 3*). The ulcerated area in the omeprazole treated animals were 20.4 ± 1.1 mm^2 (vs. 26.5 ± 2.6 mm^2 in the control animals) at day 7 and 1.9 ± 0.6 mm^2 (vs. 8.8 ± 2.3 mm^2 in the control animals) at day 14, respectively. On the other hand, indomethacin significantly delayed in ulcer healing. The ulcerated area in the indomethacin treated animals were 44.0 ± 4.2 mm^2 at 1 week and 25.0 ± 6.5 mm^2 at 2 weeks, respectively).

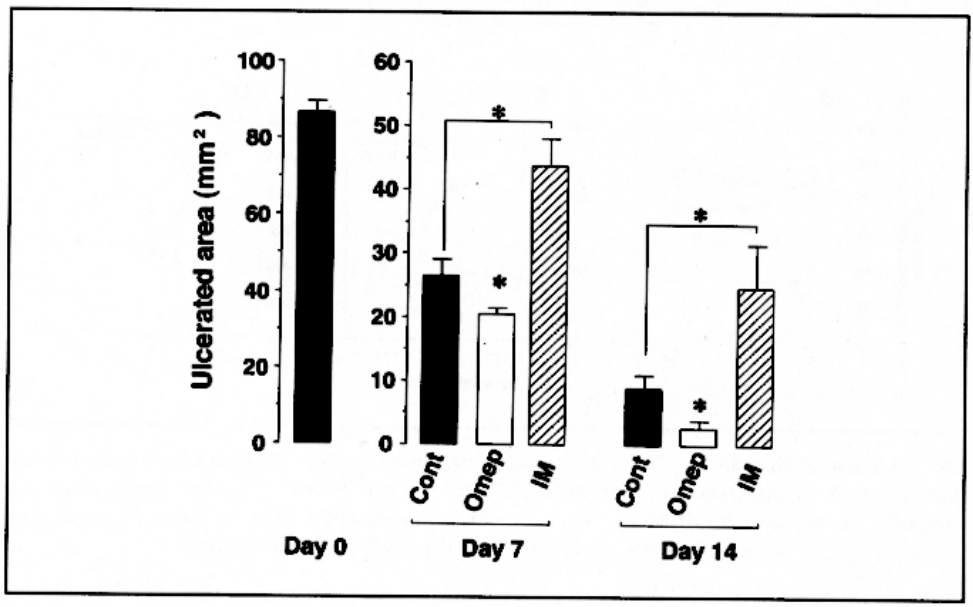

Fig. 3. Effects of omeprazole and indomethacin on healing of gastric ulcers in rats. Omeprazole (Omep, 60 mg/kg, PO) or indomethacin (IM, 2 mg/kg, SC) was administered once daily for 1 or 2 weeks, starting from the time of ulcerations. One and 2 weeks later, the ulcerated area was determined. Data are presented as the mean $\pm$ SEM for 5 animals. * Significantly different from corresponding control group, at $P < 0.05$.

Effects of omeprazole and indomethacin on gastric acid secretion in rats

Gastric acid secretion was determined in the animals with 14 days old ulcers, using pylorus ligated method. Gastric acid output was 232.4 ± 64.9 μEq/hr in the control animals (*Fig. 4*). Omeprazole completely inhibited gastric

acid secretion, the gastric acid output being 0.0 ± 0.0 μEq/hr. While indomethacin inhibited gastric acid secretion at 40% (135.1 ± 21.6 μEq/hr), but this effects was not statistically significant.

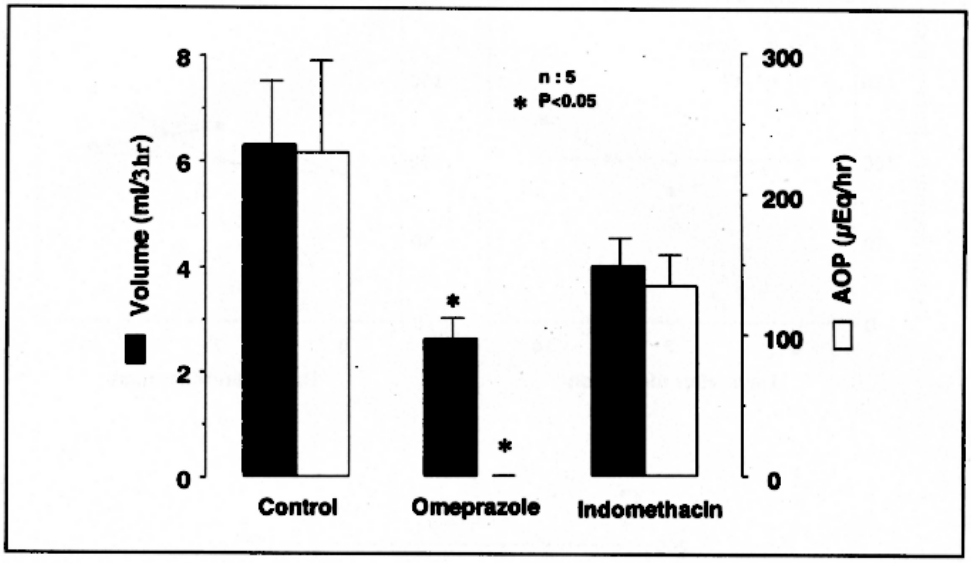

Fig. 4. Effects of omeprazole and indomethacin on gastric acid secretion in pylorus ligated rats. Omeprazole (60 mg/kg, PO) or indomethacin (2 mg/kg, SC) was administered once daily for 2 weeks, starting from the time of ulcerations. After the final administration, the animals were fasted for 18 hr and gastric acid secretion was determined by pylorus ligation. Data are presented as the mean ± SEM for 5 animals. *Significantly different from corresponding control group, at $P < 0.05$.

Effects of omeprazole and indomethacin on the expression of HSC70, HSP70 and HSP47 during ulcer healing

The expression of HSC70 was marked in the normal mucosa, ulcer margin and ulcer base. Omeprazole had little effect on HSC70 expression both in the normal mucosa and ulcerated tissue (*Fig. 5*). Indomethacin significantly increased the HSC70 expression in the normal mucosa and ulcer margin at day 14, but the degrees of increase were only 1.3- and 1.4-fold, respectively.

In regards to HSP70, both omeprazole and indomethacin had no effect on its expression in the normal mucosa (*Fig. 6*). Although the expression of HSP70 in the ulcer margin gradually increased with ulcer healing in the control animals, omeprazole further increased in HSP70 expression at day 7 and 14. In the ulcer base, omeprazole significantly enhanced the expression of HSP70 at day 7. While indomethacin had no effect on the expression of HSP70 in the all portions tested.

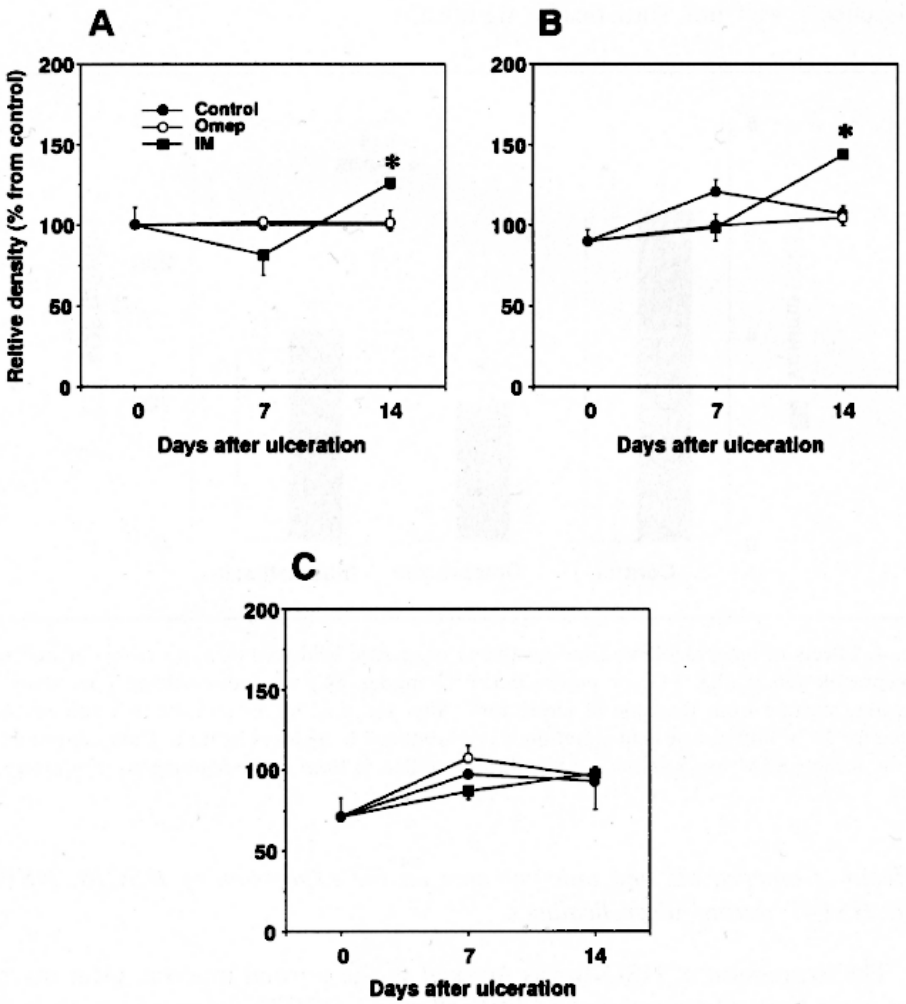

Fig. 5. Effects of omeprazole and indomethacin on HSC70 expression in the rat with ulcers. Omeprazole (Omep, 60 mg/kg, PO) or indomethacin (IM, 2 mg/kg, SC) was administered once daily for 1 or 2 weeks, starting from the time of ulcerations. HSC70 expression in the normal mucosa (A), the mucosa from the ulcer margin (B) and ulcer base (C) was detected by immunoblotting and quantified densitometorically. Data are presented as the means ± SEM for 5 animals, and expressed % changes from the value of corresponding normal mucosa of control animals. * Significantly different from corresponding normal mucosa, at $P < 0.05$.

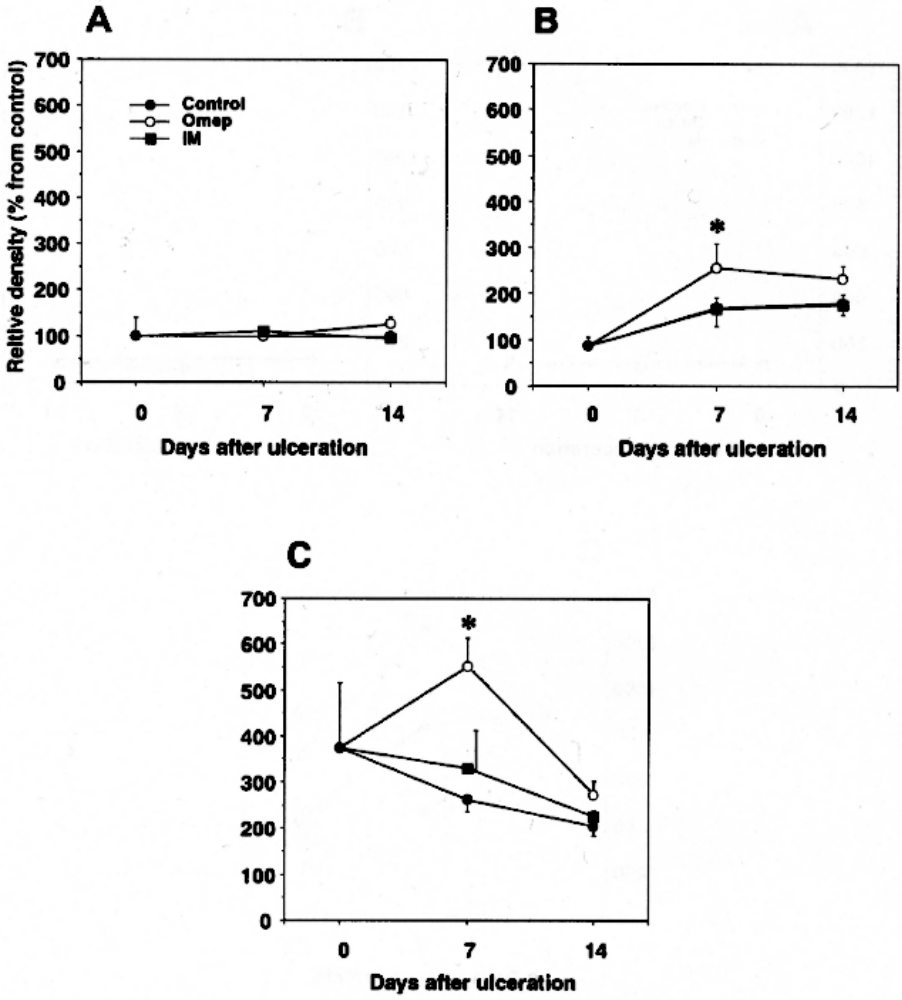

Fig. 6. Effects of omeprazole and indomethacin on HSP70 expression in the rat with ulcers. Omeprazole (Omep, 60 mg/kg, PO) or indomethacin (IM, 2 mg/kg, SC) was administered once daily for 1 or 2 weeks, starting from the time of ulcerations. HSP70 expression in the normal mucosa (A), the mucosa from the ulcer margin (B) and ulcer base (C) was detected by immunoblotting and quantified densitometorically. Data are presented as the means $\pm$ SEM for 5 animals, and expressed % changes from the value of corresponding normal mucosa of control animals. *Significantly different from corresponding normal mucosa, at $P < 0.05$.

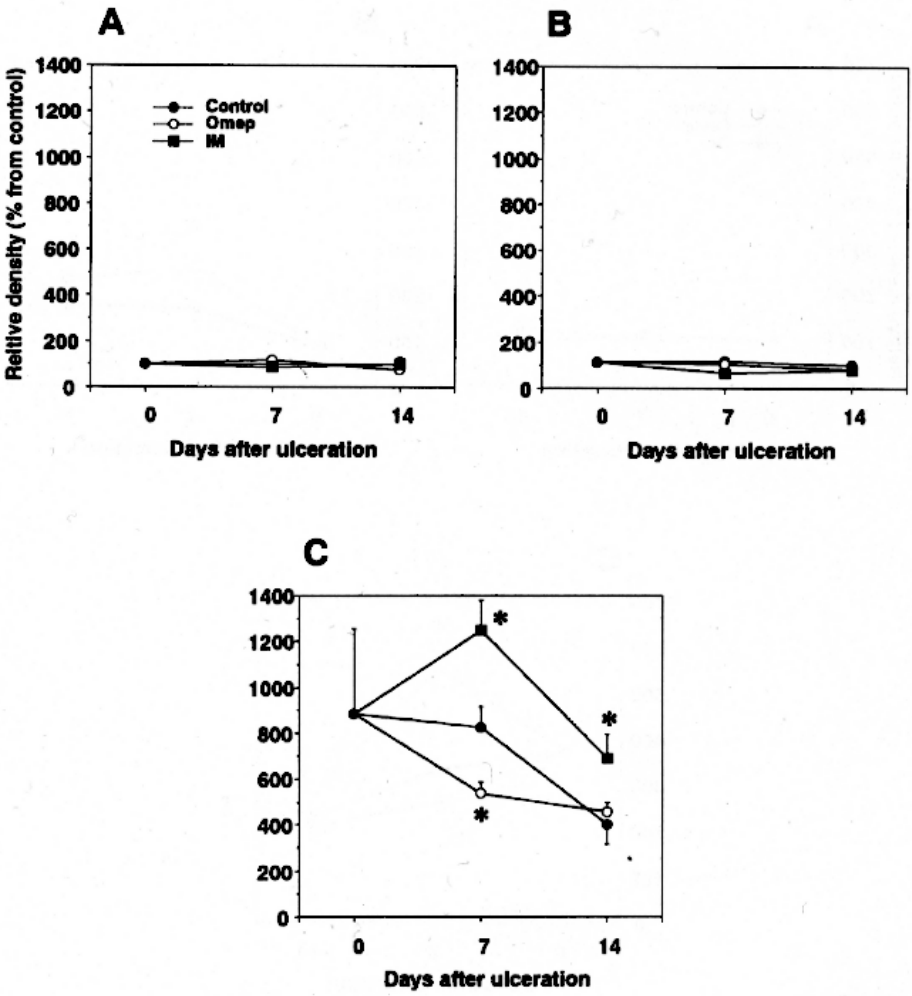

Fig. 7. Effects of omeprazole and indomethacin on HSP47 expression in the rat with ulcers. Omeprazole (Omep, 60 mg/kg, PO) or indomethacin (IM, 2 mg/kg, SC) was administered once daily for 1 or 2 weeks, starting from the time of ulcerations. HSP47 expression in the normal mucosa (A), the mucosa from the ulcer margin (B) and ulcer base (C) was detected by immunoblotting and quantified densitometorically. Data are presented as the means $\pm$ SEM for 5 animals, and expressed % changes from the value of corresponding normal mucosa of control animals. * Significantly different from corresponding normal mucosa, at $P < 0.05$.

The expression of HSP47 both in the normal mucosa and ulcer margin kept lower levels, and was not influenced by omeprazole and indomethacin (*Fig. 7*). In the ulcer base, omeprazole significantly reduced the expression of HSP47 at day 7, but not day 14, as compared with control. While, indomethacin significantly enhanced the expression of HSP47 in the ulcer base at day 7 and 14.

DISCUSSION

In the present study, we found that HSPs expression was dramatically changed during the healing process in the ulcer base and ulcer margin. It is well known that the healing of gastric ulcers comparises several phases that are based on the healing rate (16, 17). We have previously reported that the healing process of acetic acid ulcer produced by luminal application method was also divided into two phases: the first phase consists of the first 10 days from the acid application and is characterized by a rapid healing rate; the second phase is the following 10 days and is characterized by a slow healing rate (17). In that study, we suggested that different mechanism underlying ulcer healing were utilized in each healing phase. Since HSPs have various physiological functions, it is probable that HSPs are involved in each phase of the ulcer healing process through different mechanism.

In agreement with previous reports, we confirmed that omeprazole significantly accererated the healing of gastric ulcers with strong inhibition of gastric acid secretion, while indomethacin significantly delayed in ulcer healing. It was reported that non-steroidal anti-inflammatory drugs, such as indomethacin, increased gastric acid secretion by virtue of their ability to inhibit prostaglandin synthesis (18). Contrary to their report, we found that gastric acid secretion in the indomethacin treated animals showed tendency to decrease as compared with control group. Barnett *et al.* (19) recently reported that indomethacin significantly increased gastric acid secretion in inflammed stomach induced by iodeacetamide, while it had no effect on gastric acid secretion in the normal stomach. Therefore, it seems that the increase in gastric acid secretion by indomethacin was observed only in the special case such as inflammation. Although severe inflammation could be observed in the chronic gastric ulcers, the inflamed site was limited in the ulcer base and close to ulcers. Accordingly, different phenomenon in response to indomethacin might be observed, depending on the statement of the stomach. Furthermore, in our case, we determined gastric acid secretion in the animals with 14 days old ulcers, they were given indomethacin for 2 weeks. As the results treated with indomethacin had apparently larger size of ulcers, compared with control animals. Accordingly, the reduction of acid secretion in the indomethacin

treated animals might be due to the smaller area of oxyntic mucosa as compared with normal healing group.

HSC70 was expressed at a high level in the normal mucosa, ulcer margin and ulcer base with similar levels during ulcer development and healing. Furthermore, the expression of HSC70 did not change in the case of promoting the ulcer healing by omeprazole. Although HSC70 in the normal mucosa and ulcer margin was significantly increased after the repeated administration of indomethacin for 2 weeks, the increased levels was quite low as compared with changes in HSP70 and HSP47. These results suggested that HSC70 had little participation in healing of gastric ulcers. Considering that the cell composition of the normal mucosa, ulcer margins and ulcer base are quite different, it was speculated that HSC70 has basic function for maintenance of cellular activity in the different types of cells.

In regards to HSP70, the level expressed in normal mucosa was quite low. However, HSP70 was markedly expressed in the ulcer base during the time of ulcer development. Several stimuli besides heat, such as heavy metals, reactive oxygen, several cytokines etc. also induced expression of HSPs (2). It is well established that inflammatory responses occur in ulcerated tissue, that certain cytokines are produced, and that the degree of inflammation decrease upon ulcer healing (20). Consequently, HSP70 expression in the ulcer base appears to be induced by stressful conditions. Since we did not determine the histological localization of HSP70, which cell express HSP70 remains unknown. Additional studies are requisite to clarify this issue. Nonetheless, HSP70 expression in the ulcer margin gradually increased with ulcer healing. In our model, regenerated mucosa in the ulcer margin was observed in the late healing phase, but not in the early phase (17). Consequently, incresed expression of HSP70 in the ulcer margin is suggested to be involved in mucosal regeneration in the late phase of ulcer healing. Although we did not determine the histological localization, HSP70 appears to be expressed in growing cells in the ulcer margin. Indeed, a strong relationship has been reported between the expression of HSP70 and a marker for cell proliferation, such as proliferating cell nuclear antigen (PCNA) or silver-staining nucleolar organizer regions (AgNORs) as observed in human breast cancer biopsy samples (21). Based on these findings, it was suggested that HSP70 expressed in the proliferating cells and it was involved in the mucosal regeneration in the late phase of ulcer healing.

HSP70 expression was markedly changed by repeated administration of omeprazole. After the 1 week treatment with omeprazole, HSP70 expression in the ulcer margin and ulcer base was significantly increased, suggesting that overexpression of HSP70 might be related to ulcer healing promoting effect of omeprazole. The reason why HSP70 expression in the ulcer margin and ulcer base was increased by omeprazole treatment is unknown. Since omeprazole had little effect on HSP70 expression in the normal mucosa, it is considered

that direct action of omeprazole could be excluded from the mechanism of increased expression of HSP70 by omeprazole. One passible explanation is that overexpression of HSP70 in the ulcer margin and ulcer base resulted from the progress of the ulcer healing mechanism. In fact, we presiously reported that regeneration of ulcerated tissue and contraction of ulcer base was progressed by omeprazole treatment in the acetic acid ulcer model we used in the present study (14). It is well known that cell proliferation occurs in the regenerated mucosa in the ulcerated tissue. As mentioned above, HSP70 expression is related to cell proliferation, suggesting that large amount of HSP70 in the ulcer margin might be, necessary for mucosal regeneration. Furthermore, several growth factors, inducible type of cyclooxygenase and nitric oxide synthase are synthesized in the ulcer base (22—24). Accordingly, HSP70 might help to their synthesis in the ulcer base via molecular chaperon activity. Thus, a positive relationship between enhanced expression of HSP70 in the ulcer margin and ulcer base and healing promoting action of omeprazole. Contrary to omeprazole, indomethacin had little effect on HSP70 expression, suggesting that the change in HSP70 expression is unrelated to the delayed ulcer healing caused by indomethacin.

HSP47 is known to have a unique collagen binding ability, and is believed to essential for mature collagen biosynthesis (6—8). Since collagen biosynthesis in the granulation tissue in the ulcer base is an essential process for ulcer healing, it is possible that HSP47 plays an important role for ulcer healing. In the present study, we found that HSP47 was dramatically induced by ulcer development in the ulcer base. Furthermore, the increased HSP47 in the ulcer base gradually decreased with ulcer healing, but it kept higher levels at day 14. If HSP47 contributes to the granulation tissue formation through collagen biosynthesis, time course changes in HSP47 expression in the ulcer base was agreement with the fact that contraction of the granulation tissue contributes to ulcer healibg both in the early and late healing phases. Recently, Nagai *et al.* reported that HSP47 deficient mice was embryonic lethal due to the abnormal angiogenesis during development (25). Based on this finding, it was suggested that HSP47 are involved in the angiogenesis in the granulation tissue of ulcer base through collagen biosynthesis. Although the expression of HSP47 was detectable in the normal mucosa and ulcer margin, they were quite weak as compared with that in the ulcer base. Accordingly, HSP47 expressed in the normal mucosa and ulcer margin seems not to be involved in ulcer healing.

As well as in the case of normal healing, the expression of HSP47 in the normal mucosa and ulcer margin was not influenced by omeprazole and indomethacin, suggesting that HSP47 is not involved in ulcer healing in the normal mucosa and ulcer margin. In the omeprazole treated animals, the expression of HSP47 expression in the ulcer base was decreased in spite of healing promotion. It is well known that although omeprazole accelerated

healing of chronic gastric ulcers, the quality of ulcer healing is quite low. Accordingly, decrease in HSP47 expression in the ulcer base caused by omeprazole treatment might be related to low quality of ulcer base. Interestingly, we found that the expression of HSP47 in the ulcer base was markedly increased by indomethacin treatment. Generally, it is well known that severe fibrosis was observed in the ulcer base of indomethacin treated animals (26). Accordingly, it was suggested that overexpression of HSP47 in the ulcer base caused by indomethacin is involved in the fibrosis in the ulcer base, resulting in delayed healing of gastric ulcers. In fact, a positive relationship between HSP47 expression and fibrotic deseases such as chirosis, systemic sclerosis and nephropathy was reported (27—29). Furthermore, it was reported that antisense oligonucleotides against HSP47 suppress collagen accumulation in experimental glomerulonephritis (30).

From these findings, we conclude that the expression of HSP70 and HSP47 in the ulcerated tissue change during ulcer healing process. Furthermore, it was suggested that the enhanced expression of HSP70 is involved in drug-induced acceleration of ulcer healing, but overexpression of HSP47 is related to delay in ulcer healing.

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