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RELATIONSHIP BETWEEN VASCULAR ENDOTHELIAL GROWTH FACTOR AND ANGIOGENESIS IN SPONTANEOUS AND INDOMETHACIN-DELAYED HEALING OF ACETIC ACID-INDUCED GASTRIC ULCERS IN RATS

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Angiogenesis is an important event for gastric ulcer healing. Vascular endothelial growth factor (VEGF) is known to be a potent stimulator of angiogenesis. This study consequently examined VEGF production, VEGF mRNA expression and angiogenesis during the spontaneous and indomethacin-delayed healing of acetic acid-induced ulcers in rats. The production of VEGF, taking place in the normal mucosa, was significantly elevated by ulceration. The mRNA expression of three isoforms of VEGF (VEGF188, VEGF164 and VEGF120) was also detected. Following the increase in VEGF production, angiogenesis was significantly promoted in the ulcer base. VEGF-immunoreactivity was observed in granulocytes, fibroblasts and regenerated epithelial cells. Indomethacin markedly inhibited prostaglandin E_2 synthesis in the ulcer base, resulting in the prevention of ulcer healing. Angiogenesis was also significantly inhibited by indomethacin, but neither VEGF production nor VEGF mRNA expression was reduced. Such results suggest that VEGF might play a role in angiogenesis in the spontaneous healing of gastric ulcers in rats. However, the inhibition of angiogenesis in indomethacin-delayed ulcer healing is not explainable on VEGF expression.

Key words: *VEGF, gastric ulcer, angiogenesis, ulcer healing, indomethacin, prostaglandin*

INTRODUCTION

Gastric ulcer healing spontaneously proceeds through multiple steps, such as formation of granulation tissue, angiogenesis and epithelial regeneration. Tarnawski *et al.* (1, 2) proposed the hypothesis that angiogenesis is important for ulcer healing with high quality, because angiogenesis is essential for

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supplying oxygen and nutrients to the damaged area. Angiogenesis is largely regulated by growth factors, such as basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF). Among angiogenesis-promoting factors, VEGF is the most potent stimulator of endothelial cell growth (3) and migration *in vitro* (4), and remarkably induces angiogenesis *in vivo* (5). Szabo *et al.* (6, 7) first reported that exogenous VEGF accelerated the healing of duodenal ulcers in rats. In addition, Tarnawski *et al.* (8) recently demonstrated that exogenous VEGF enhanced angiogenesis in rat gastric mucosa damaged by ethanol and accelerated the healing of the damage, thus suggesting a causal relationship. Accordingly, we speculated that VEGF might play a central role in angiogenesis in gastric ulcer healing. Takahashi *et al.* (9) reported that VEGF is expressed in the ulcerated gastric tissue and the normal mucosa of humans. However, in their study, neither the level of VEGF nor angiogenesis was evaluated. To fully understand the role of VEGF in angiogenesis in ulcer healing, we examined the production of VEGF protein, VEGF mRNA expression and angiogenesis during the spontaneous healing of acetic acid-induced gastric ulcers in rats.

On the other hand, it is known that gastric ulcer healing is markedly impaired by indomethacin, in association with inhibition of angiogenesis in rats (2, 10, 11). It was reported that prostaglandin (PG) E_2 induces VEGF expression in osteoblasts (12) and fibroblasts (13). In addition, Majima *et al.* demonstrated that endogenous PGs promote angiogenesis in sponge implants in rats (14), partly through the induction of VEGF expression (15). It is possible that indomethacin interfere with the generation of VEGF, leading to inhibition of angiogenesis and results in delayed healing. Consequently, this study further examined whether or not VEGF expression in the ulcerated tissue is inhibited by indomethacin.

MATERIALS AND METHODS

Animals

Male Donryu rats (300—350 g; Nihon SLC, Shizuoka) were used. Prior to the experiments, all animals were deprived of food for 18 hr and water for 2 hr. The animals were kept in mesh-bottom cages to prevent corprophagy.

Induction of gastric ulcers

Gastric ulcers were induced by a previously described method (16). In brief, under ether anesthesia, the abdomens of the animals were incised and the stomachs exposed. The anterior and posterior walls of the gastric corpus were then clamped together by forceps with a round ring (ID, 9 mm) at the end of each arm. 60% acetic acid (0.2 ml) was injected with a needle (gauge 18) into

the clamped lumen through the antrum approximately 2 mm proximal from the pylorus. After 45 sec, the acid solution was removed and the abdomen was closed. The animals were normally fed thereafter. Clearly-defined, deep ulcers consistently developed in the corpus of both the anterior and posterior walls 3 days after the acid application. The third day after the acid application was thus designated as the day of ulceration (day 0). Following indomethacin treatment, the animals were killed under ether anesthesia, and the stomachs removed. The stomachs were then opened along their greater curvature and flattened with pins. The ulcerated areas in both the anterior and posterior walls were determined (mm^2) under a dissecting microscope ($\times$ 10; Olympus, Tokyo) with a square grid. The areas are presented as the sum of two ulcers. The author (S.O..) determining the ulcer areas was unaware of which treatment had been given to the animals.

Evaluation of angiogenesis in the ulcer base

Gastric specimens were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2). Following fixation, 10-μm frozen sections were prepared. The sections were incubated with the antibody against von Willebrand factor (factor VIII-related endothelial antigen) (DAKO, Glostrop, Denmark), after deactivation of endogenous peroxidase with 0.3% H_2O_2 and blockage of nonspecific binding sites. Microvessels were visualized by the avidin-biotin-peroxidase complex method using a Vectastain ABC-peroxydase kit (Vector Laboratories, Burlingame, CA) and 3,3-diaminobenzidine tetrahydrochloride (Dojindo Laboratories, Kumamoto). The sections were successively stained with hematoxylin. The number of microvessels in the granulation tissue of the ulcer base was determined in three randomly chosen 1 mm^2 fields. The microvessel density was expressed as the number of microvessels per mm^2 of the ulcer base.

Determination of PGE_2 synthesis in gastric tissues

PFE_2 synthesis was assayed according to the method of Lee and Feldman (17). Three hours after administration of indomethacin, gastric tissue were taken from both ulcerated tissue and the mucosa lacking ulcers. The specimens were placed in 50 mM Tris-HCl (pH 8.4) buffer and then finely minced. Following washing and resuspending in 1 ml of buffer, the tissues were subjected to vortex mixing at room temperature for 1 min to stimulate PGE_2 synthesis, followed by centrifugation at 10,000 $\times$ g for 15 sec. The amounts of PGE_2 in the resulting supernatants were determined by enzymeimmunoassaying (PGE_2 EIA kit; Cayman Chemicals, Ann Arbor, MI); PGE_2 synthesis was expressed as pg PGE_2/mg tissue/min.

VEGF mRNA analysis by RT-PCR

Total cellular RNAs were extracted from both the mucosa with or without ulcers by means of the acid-guanidinium thiocyanate-phenol-chloroform method (11) using TRIZOL (GIBCO BRL, Gaithersburg, MD). First strand cDNAs were prepared from 5 μg of total RNAs with Moloney murine leukemia virus reverse transcriptase (RT; GUBCO BRL) according to the manufacturer's procedure. PCR was performed with a PCR thermal cycler (TP3000; Takara Biochemical, Kyoto). The primers used were as follows: rat VEGF (sense: 5′-GCTCTCTTGGGTGCACTGGA-3′; antisense: 5′-TGTGACAAGCCAAGGCGGTG-3′) (18) and rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (sense: 5′-CCAGTATGATTCTACCCA CGGCAA-3′; antisense: 5′-ATACTTGGCAGGTTTCTC CAGGCG-3′) (19). After denaturation at 94°C for 5 min, PCR was performed for 30 cycles, which consisted of denaturation at 94°C for 1 min, annealing at 65°C for 1 min, and extension at 72°C for 1 min. The

amplification was terminated after a 15-min final extension step at 72°C. These procedures yielded cDNA products of 431 base pairs (bp) for VEGF120, 563 bp for VEGF164, 635 bp for VEGF188 and 625 bp for GAPDH. After aliquots of the reactants were subjected to agarose (1.5%) gel electrophoresis, the PCR products were visualized by ethidium bromide staining.

Immunohistochemical detection of VEGF protein in gastric tissues

Gastric specimens were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (ph 7.2). Following fixation, 10-μm frozen sections were prepared. The sections were incubated with anti-VEGF (antibody (Santa Cruz, Santa Cruz, CA) following deactivation of endogenous peroxidase with 0.3% H_2O_2 and blockage of nonspecific binding sites. The antibody recognizes all isoforms of rat VEGF. The VEGF protein was visualized by the avidin-biotin-peroxidase complex method, as described above.

Determination of VEGF production in gastric tissues

Three hours after administration of indomethacin, gastric specimens were taken from both ulcer area and the mucosa lacking ulcers. The specimens were then minced in 1 ml of 50 mM Tris-HCl buffer (pH 7.6) containing 1.6 M NaCl, 2 mM EDTA, 1 mM p-APMSF (Wako, Osaka) and 0.1% Tween 80. The specimens were homogenized in ice with a Potter-Elvehjem homogenizer. The homogenates were centrifuged at 10,000 × g for 15 min and the supernatants were stored at −40°C until the assay. The quantity of VEGF were determined by enzyme-linked immunosorbent assays (mouse VEGF ELISA kit; R&D System, Minneapolis, MN). The VEGF ELISA kit was applicable to rat samples, and all isoforms of VEGF were measured as VEGF content. The quantity of VEGF was expressed as pg VEGF/mg tissue.

Drug

Indomethacin (Sigma, St. Louis, MO) was suspended in a trace of Tween 80 and saline. The drug at 1 mg/kg was administered subcutaneously twice daily for 14 days at a volume of 5 ml/kg body weight beginning on day 0. Control animals received the vehicle alone.

Statistical analysis

The data are presented as means ± S.E.M. Statistical differences were evaluated by Student's t-test. P value of <0.05 was regarded as significant.

RESULTS

Angiogenesis during the spontaneous and indomethacin-delayed healing of gastric ulcers

By intramucosal application of acetic acid solution, two round and deep ulcers were consistently induced in the fundus three days after operation,

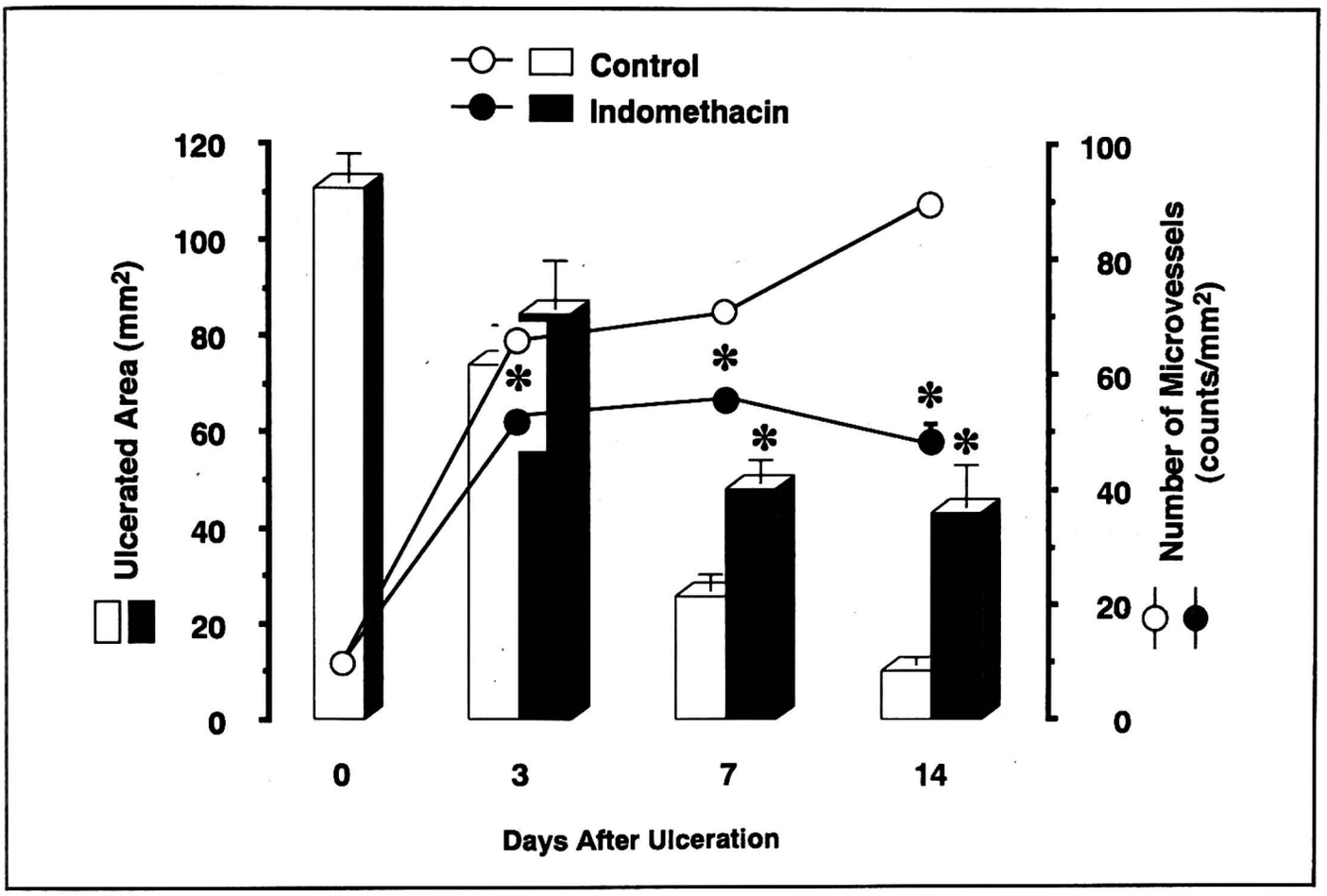

Fig. 1. Angiogenesis during the spontaneous and indomethacin-delayed healing of gastric ulcers in rats. Indomethacin (1 mg/kg) or vehicle was administered subcutaneously twice daily for 3, 7 and 14 days after ulceration. Data are presented as means ± SEM for 7—8 rats. * Significantly different from the control, $P < 0.05$.

the total ulcerated area being 110.5 ± 5.8 mm^2 ($n = 8$) (*Fig. 1*). The ulcers gradually healed with time. Treatment with indomethacin for 3 days exhibited little or no effect on ulcer healing. Nonetheless, treatment with the drug for 7 and 14 days significantly delayed ulcer healing; i.e., 25.6 ± 3.3 mm^2 vs. 48.2 ± 4.9 mm^2 in the control and 10.1 ± 1.5 mm^2 vs. 43.2 ± 8.8 mm^2 in control, respectively.

Immediately after ulceration, the density of microvessels in the granulation tissue of the ulcer base was observed to be 8.1 ± 0.3 counts/mm^2 (*Fig. 1*). The density of vessels profoundly increased in parallel with ulcer healing; i.e., 64.7 ± 1.1 counts/mm^2 (day 3), 69.8 ± 0.8 counts/mm^2 (day 7) and 87.8 ± 2.2 counts/mm^2 (day 14) (*Fig. 2A*). Upon treatment with indomethacin, the density of microvessels was significantly suppressed in comparison to the control group, i.e., the density being 52.2 ± 1.3 counts/mm^2 (day 3), 55.0 ± 1.6 conts/mm^2 (day 7) and 47.1 ± 1.1 counts/mm^2 (day 14) (*Fig. 2B*).

In the normal gastric mucosa (without ulcers), PGE_2 synthesis was found to be $92.1 + 16.2$ pg/min/mg tissue ($n = 7$) (*Fig. 3*). On Day 0, PGE_2 synthesis in the ulcer area was significantly elevated to 1,100 pg/min/mg tissue (about 11 times

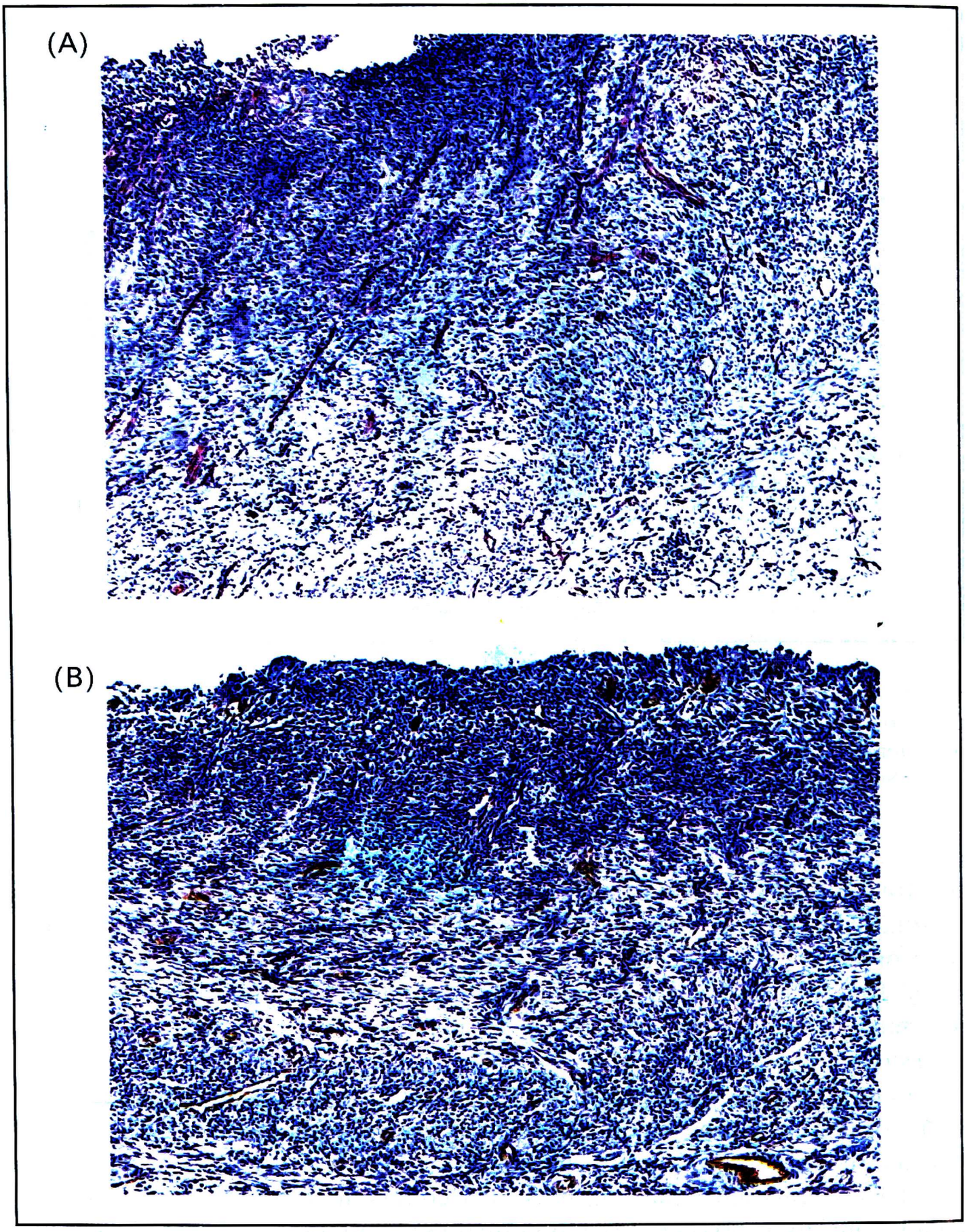

Fig. 2. Immunohistochemical detection of angiogenesis in the gastric tissues of rats. (A) control (X 25), (B) indomethacin on day 14 (X 25).

the control value) (*Fig. 3*). This increased synthesis of PGE_2 was maintained for 3 days after ulceration. The synthesis gradually declined with time; i.e., 500 and 300 pg/mg tissue/min at 7 and 14 days after ulceration, respectively. It should

be noted that continuous treatment with indomethacin significantly inhibited PGE_2 synthesis throughout the experimental period.

VEGF production during the spontaneous and indomethacin-delayed healing of gastric ulcers

In the extract prepared from the normal mucosa (without ulcers), the amount of VEGF protein was 1.7 ± 0.1 pg/mg tissue (n = 8) (*Fig. 4*). VEGF production was significantly elevated by gastric ulceration to 4.4 ± 0.3 pg/mg (approximately 2.6-fold increase compared with that in the normal mucosa). VEGF protein level in the ulcer base subsequently reduced to 2.9 ± 0.1 pg/mg tissue, 2.8 ± 0.1 pg/mg tissue and 2.3 ± 0.2 pg/mg tissue at 3, 7 and 14 days after ulceration, respectively. Indomethacin did not affect VEGF production in the ulcer base 3, 7 or 14 days after ulceration.

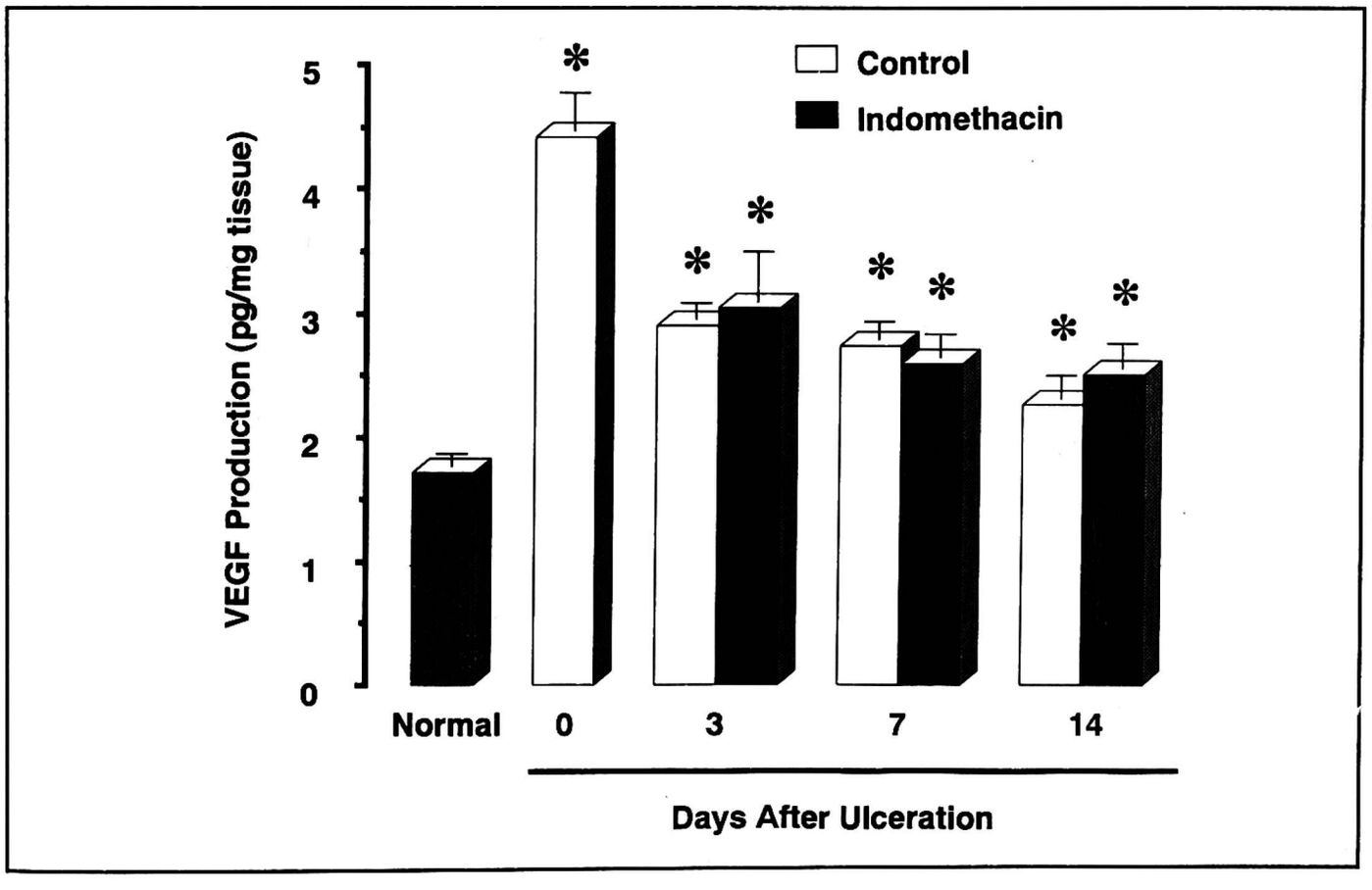

Fig. 3. Effect of indomethacin on PGE_2 synthesis during the spontaneous and indomethacin--delayed healing of gastric ulcers in rats. Indomethacin (1 mg/kg) or vehicle was administered subcutaneously twice daily for 3, 7 and 14 days after ulceration. Data are presented as means $\pm$ SEM for 7—8 rats. * Significantly different from the control, $P < 0.05$.

VEGF mRNA expression during the spontaneous and indomethacin-delayed healing of gastric ulcers

In the normal mucosa (without ulcers), there was apparent expression of VEGF mRNAs (data not shown). Three isoforms of VEGF(VEGF188,

VEGF164 and VEGF120) were all detected. Even after ulceration, VEGF mRNA expression in the ulcer area was found to be much the same as that in the normal mucosa, and the expression persisted during ulcer healing (*Fig. 5*). Although angiogenesis in the ulcer base was inhibited by indomethacin, VEGF mRNAs were similarly detected during indomethacin treatment.

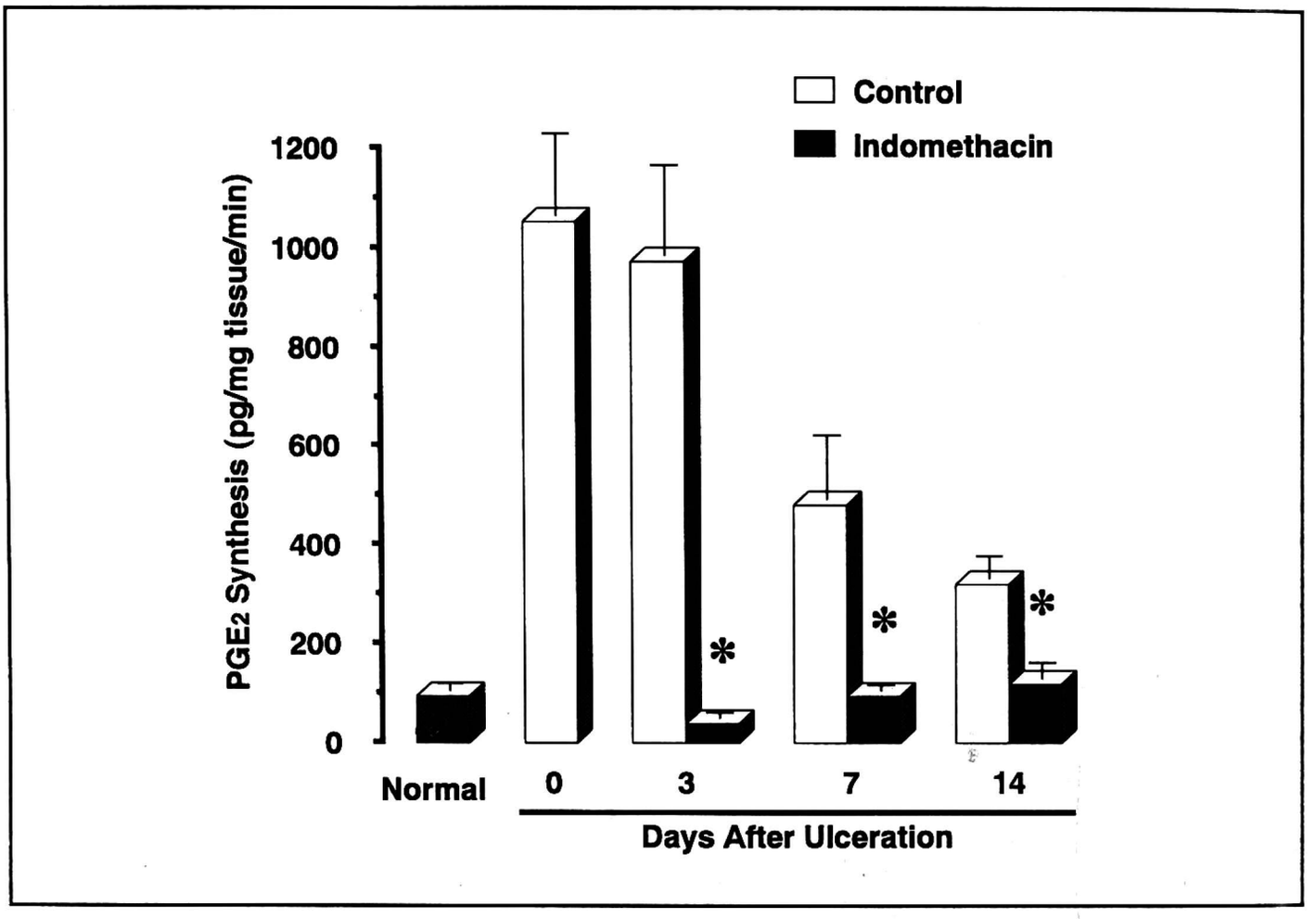

Fig. 4. VEGF production during the spontaneous and indomethacin-delayed healing of gastric ulcers in rats. Indomethacin (1 mg/kg) or vehicle was administered subcutaneously twice daily for 3, 7 and 14 days after ulceration. Data are presented as means ± 1SEM for 8—9 rats. * Significantly different from the normal, $P < 0.05$.

Localization of VEGF protein in gastric tissues

In the normal gastric mucosa (without ulcers), VEGF protein was detected in the cell proliferative zone (*Fig. 6A*). In the ulcer area, VEGF, immunoreactivity was abundant in the upper portion of the base (*Fig. 6B, C*). Strong immunoreactive signals were found in polymorphonuclear cells and spindle-shaped cells. In addition, VEGF protein was also detected in regenerated epithelial cells. The numbers of VEGF-positive cells was similar in both the indomethacin-treated and control groups. Upon application of inactivated anti-VEGF antibody to the sections, no immunoreactive signals were observed.

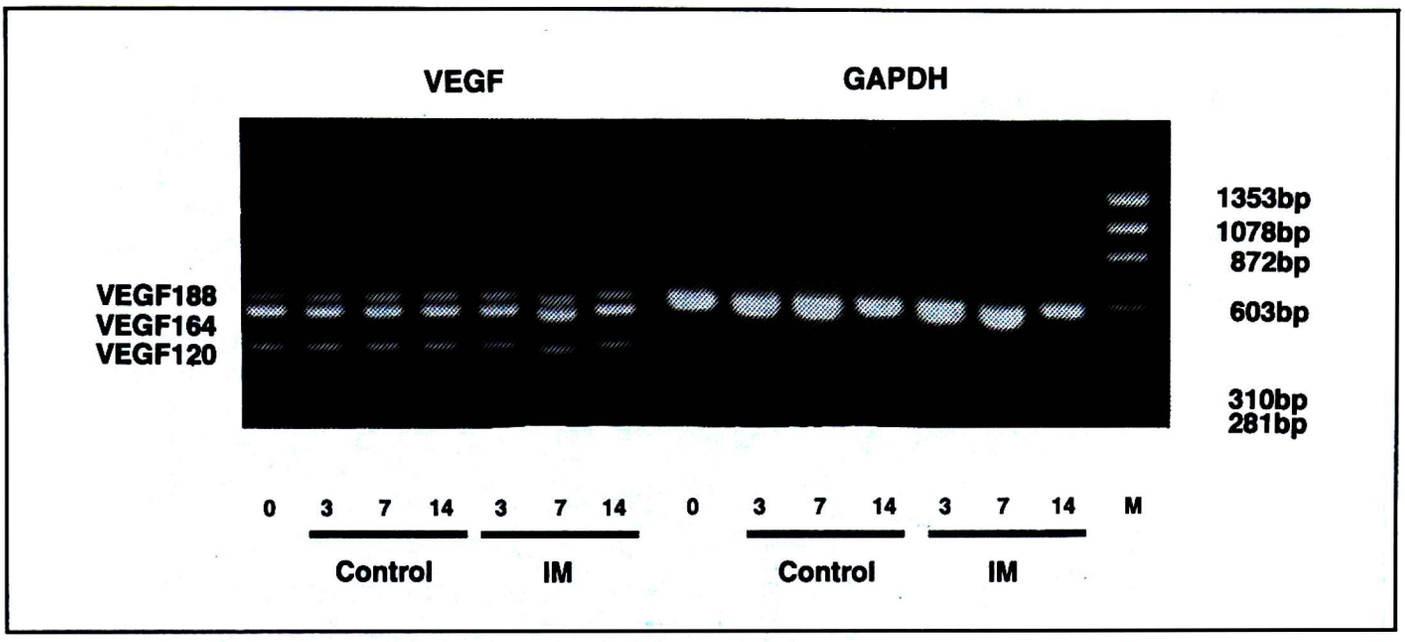

Fig. 5. mRNA expression of VEGF during the spontaneous and indomethacin-delayed healing of gastric ulcers in rats. Indomethacin (1 mg/kg) was administered subcutaneously twice daily for 3, 7 and 14 days after ulceration. RT-PCR amplification of total RNA with VEGF primers generated three products: VEGF188 (635 bp), VEGF164 (563 bp) and VEGF120 (431 bp). GAPDH cDNA (625 bp) was also amplified as an internal standard.

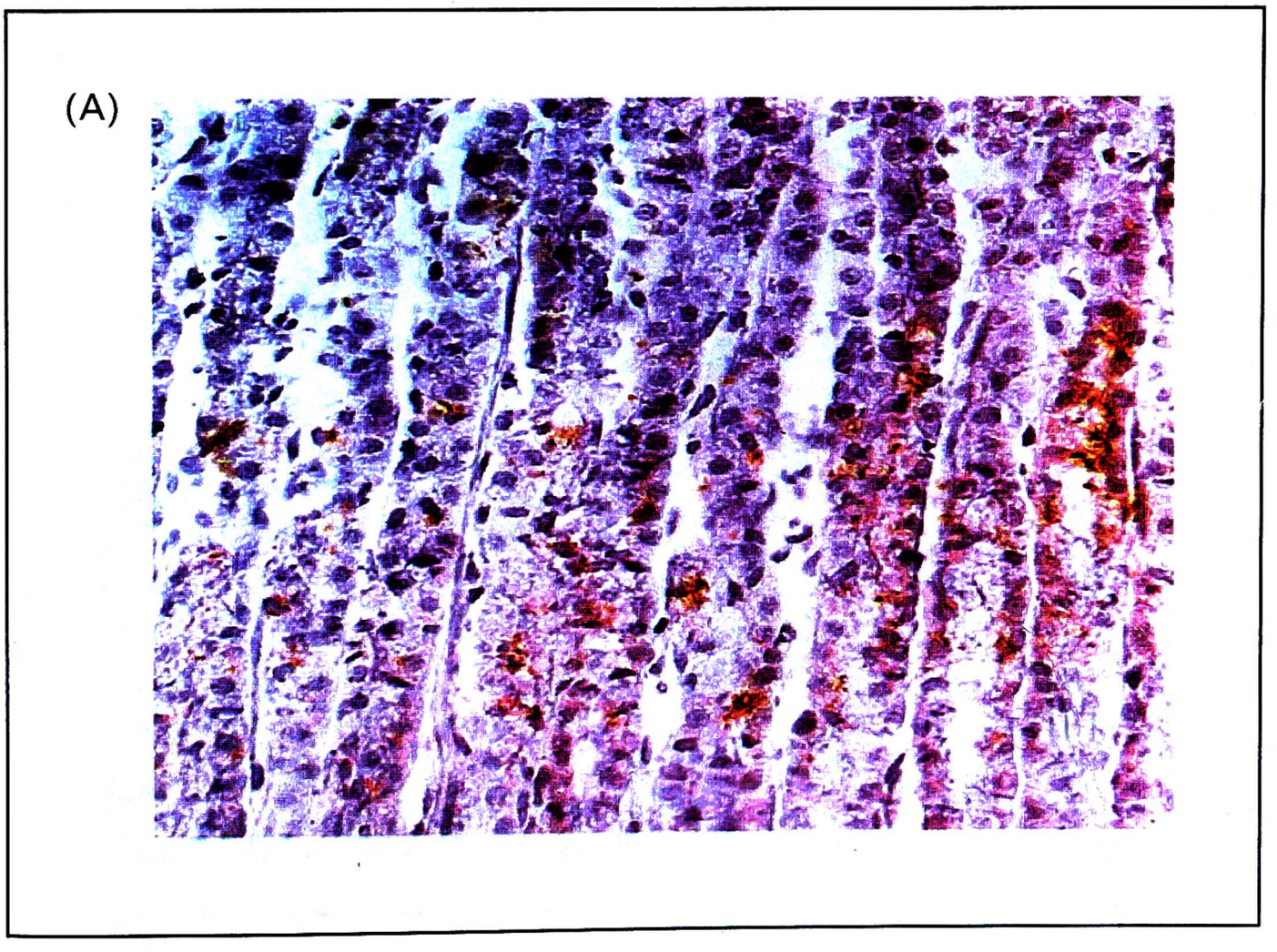

Fig. 6A

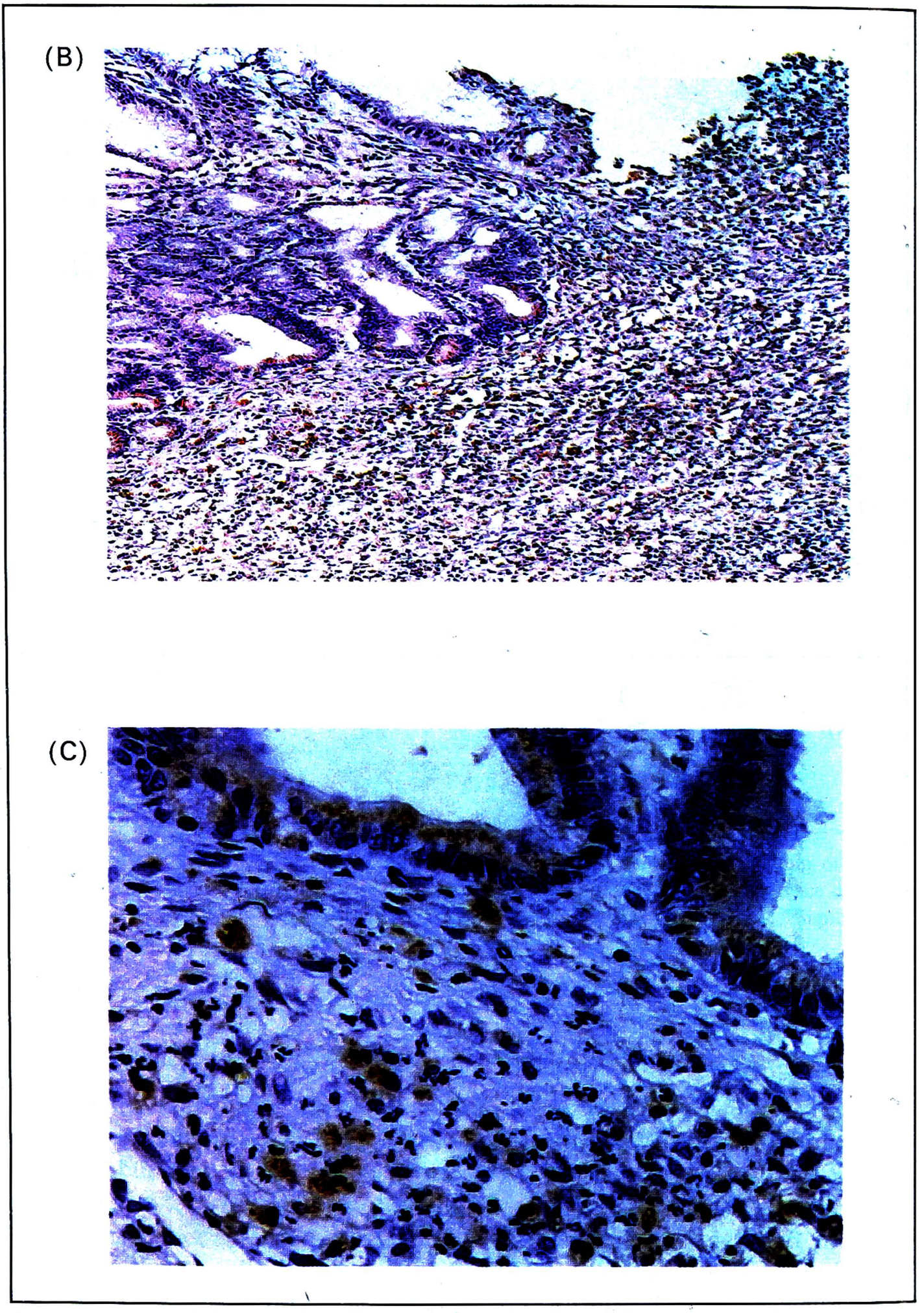

Fig. 6. Immunohistochemical detection of VEGF protein in the gastric tissues of rats. (A) normal mucosa (× 100), (B) ulcer base and margin on day 3 (× 25), (C) ulcer base and margin on day 3 (× 100).

DISCUSSION

It was found that VEGF production in the ulcer base of rats is significantly elevated by gastric ulceration, compared with production in the normal mucosa. In addition, the study also confirmed that the number of microvessels in the ulcer base increases following the rise in VEGF production. Upon RT-PCR analysis, mRNA expression of at least three isoforms of VEGF (VEGF188, VEGF164 and VEGF120) was detected. These results suggest that VEGF might be involved with angiogenesis in the spontaneous healing of gastric ulcers in rats, although it remains unclear which isoform (s) might exert a predominant effect on angiogenesis. Takahashi *et al.* (9) also reported that these VEGF isoforms are expressed in gastric ulcers in humans.

Immunohistochemical study revealed that VEGF-positive cells are polymorphonuclear cells and spindle-shaped cells in the ulcer base. Given their presence in granulation tissue, these cell types are morphologically identified to be granulocytes and fibroblasts. Regenerated epithelial cells in the ulcer margin were also immunoreactive to anti-VEGF antibody. It is likely that infiltrated granulocytes, fibroblasts and regenerated epithelial cells are VEGF-producing cells. It has also been reported that human gastric fibroblasts produce VEGF (9).

We reconfirmed that indomethacin inhibits PG synthesis in rats, resulting in delay of ulcer healing (10, 20, 21). Unexpectedly, VEGF production remained unaffected by indomethacin, although angiogenesis was significantly suppressed by indomethacin. Since, in general, PGs have no direct effect on endothelial cell migration or proliferation, PGs may be essential for the action of VEGF on angiogenesis during gastric ulcer healing. In contrast, the previous reports that PGE_2 induces VEGF expression suggest that PGE_2 promotes angiogenesis, partly through the induction of VEGF expression (12, 13). In such studies, the relation between PGE_2 and VEGF was examined in osteblasts, synovial fibroblasts and sponge implants. It seems that the effects of PGs on VEGF expression and angiogenesis may be different between ulcerated gastric tissue and other tissues. Alternatively, other factors such as bFGF may be reduced by indomethacin, resulting in inhibition of angiogenesis during ulcer healing. Satoh *et al.* (22) reported that endogenous bFGF plays an important role in angiogenesis in the spontaneous healing of gastric ulcers in rats. Further study on bFGF expression during indomethacin-delayed ulcer healing is needed.

In the present study, both VEGF protein and mRNA were found in the normal gastric mucosa of rats. Similar results have also been obtained in human gastric mucosa (9). Since VEGF-immunoreactivity was observed in the cell proliferative zone, it is possible that VEGF may be involved in division and/or differentiation of gastric stem cells. Altnernatively, VEGF might be required for maintenance of blood vessels in the normal mucosa. However, the role of VEGF in the normal mucosa remains unknown.

It is conclude that VEGF might play a role in angiogenesis in the spontaneous healing of gastric ulcers in rats. The inhibition of angiogenesis in indomethacin-delayed ulcer healing, however, is not explainable by VEGF expression. Studies concerning the expression of VEGF receptors in the ulcerated tissue of indomethacin-treated rats will be our ongoing subject.

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