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Effect of Polaprezinc (*N*-(3-Aminopropionyl)-L-histidinato zinc), a Novel Antiulcer Agent Containing Zinc, on Cellular Proliferation: Role of Insulin-like Growth Factor I

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ABSTRACT. The effect of polaprezinc (*N*-(3-aminopropionyl)-L-histidinato zinc), a novel antiulcer drug containing zinc, on cellular proliferation was studied using cultured cells. In human umbilical vein endothelial cells (HUVEC) or human foreskin fibroblast cells, bromodeoxyuridine (BrdU) uptake and the number of cells were increased by polaprezinc under low serum conditions, but polaprezinc had no effect on guinea pig gastric mucosal epithelial cells. In addition, L-carnosine (a component of polaprezinc) had no effect on cultured HUVEC, while zinc sulfate, a representative zinc compound, increased BrdU uptake by about 2-fold at 10^{-9} M. However, the action of zinc sulfate was weaker than that of polaprezinc. The insulin-like growth factor I (IGF-I) mRNA level was increased in HUVEC by polaprezinc at 10^{-9} M $\sim$ 3×10^{-8} M concentrations, causing stimulation of BrdU uptake. When an anti-IGF-I antibody was added to cultures, the effects of polaprezinc on BrdU uptake was suppressed. These results suggest that although polaprezinc, a novel antiulcer agent, does not have proliferative effects on epithelial cells, it does promote the proliferation of non-parenchymal cells, and IGF-I is involved in this action. BIOCHEM PHARMACOL **58**;2:245–250, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. antiulcer drug; polaprezinc; wound healing; cellular proliferation; IGF-I; zinc

Polaprezinc (*N*-(3-aminopropionyl)-L-histidinato zinc), a novel antiulcer drug, is a chelate compound consisting of zinc ion, L-carnosine, dipeptide of beta-alanine, and L-histidine [1]. It is known that this drug promotes wound healing action [2], has an antioxidant effect [3], and has anti-*Helicobacter pylori* activity [4]. We have already reported that the administration of polaprezinc inhibits gastric mucosal damage in several experimental models [5] and that it also accelerates the healing of chronic gastric ulcers induced by acetic acid in rats [6], as well as skin incisions in guinea pigs [2]. However, little is known about the mechanism of its action on wound healing.

Zinc is an essential trace element for animals, and is known to be a component of enzymes and transcription factors with several biological functions, such as nucleotide synthesis, protein synthesis, and gene expression during cellular proliferation and differentiation [7]. Zinc deficiency retards the growth of animals [7] and the healing of wounds such as gastric ulcers [8, 9] or arterial and venous leg ulcers [10, 11]. Polaprezinc contains zinc and has been shown to reverse the delay of gastric ulcer healing produced by zinc deficiency in rats [9]. Thus, zinc seems to be very significant in wound healing.

IGF-I† is a polypeptide that is similar in structure to proinsulin [11] and that plays an important role in cellular proliferation and differentiation as well as metabolic processes such as glucose uptake [11–13]. It is known that IGF-I gene expression is increased by several factors such as growth hormone [14], estrogen [15], and cyclic AMP-responsive factor [16]. The serum concentration of IGF-I is decreased in zinc-deficient rats, and this change is reversed by administration of zinc [17]. In addition, the IGF-I mRNA level of granulation tissue in detected areas of porcine skin is increased by treatment with zinc oxide [18]. Therefore, IGF-I may have a relationship with zinc in terms of its function and its gene expression.

In the present study, we investigated the proliferative action of polaprezinc and the role of IGF-I in this action using cultured cells in order to clarify the proliferate effect of the drug on wound healing.

MATERIALS AND METHODS

Chemicals

The drugs used in this study were prepared immediately before use. Polaprezinc (Zeria Pharmaceuticals) was dis-

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† *Abbreviations:* HUVEC, human umbilical vein endothelial cells; BrdU, bromodeoxyuridine; IGF-I, insulin-like growth factor I; MEM, Eagle's minimum essential medium; and RT-PCR, reverse transcriptase-polymerase chain reaction.

solved in HCl at a 2-fold molar concentration. $ZnSO_4/7H_2O$ and L-carnosine (Zeria Pharmaceuticals) were dissolved in distilled water, and then diluted with Eagle's MEM medium. Collagenase was obtained from Wako Pure Chemicals. Coon's modified F-12 medium, antibiotic–antimycotic solution, and gentamycin were obtained from Sigma Chemical Co. Eagle's MEM was obtained from GIBCO BRL. Polyclonal antibody against human IGF-I was obtained from Austral Biologic Company. The other chemicals used were of reagent grade.

Cells and Cell Cultures

HUVEC were obtained from Kurashiki Boseki and were grown in E-GM UV medium (Kurashiki Bouseki) for several days. Foreskin fibroblasts were obtained from In-vitro-Cyte and grown in 106 medium (Invitro-Cyte) for several days. Gastric epithelial mucosal cells were prepared from guinea pigs by using digestion with collagenase as described by Berglindh [19] or Lews *et al.* [20]. The cells were grown in Coon's modified F-12 with 3 μg/mL insulin and 3 μg/mL hydrocortisone as described previously by Okayama *et al.* [21] for several days. These cultured cells were then used for the study.

Analysis of Cellular Proliferation

Each type of cell was cultured for several days. After the cells were washed using MEM without fetal bovine serum, polaprezinc or the other agents with or without 3% serum were added to the culture plates. The cells were then cultured for 21 hr to assess BrdU uptake or for 48 hr for cell counting. BrdU uptake was analyzed using a Cell Proliferation ELISA, BrdU calorimetric kit (Boehringer Mannheim). The cells were cultured for 21 hr and then incubated with BrdU for 3 hr, after which the medium was removed and the cells were fixed with ethanol. After the ethanol was removed, anti-BrdU antibody was added to each well and incubation was done for 1 hr. Following removal of the antibody solution, the cells were washed and incubated in substrate solution for 15 min. The reaction was stopped with 1 N H_2SO_4, the absorbance of the medium was measured at 450 nm, and the cell count was determined using a hamacytometer.

To assess the time-course of BrdU uptake, cells were washed with MEM (without zinc or serum) and then cultured in MEM for 24 hr. Polaprezinc was added to the conditioned medium, and the cells were incubated for the period of time indicated in Fig. 2. BrdU uptake was analyzed after incubation for 3 hr.

In the experiment using anti-IGF-I antibody, cultured HUVEC were incubated in MEM (without serum or zinc) with or without polaprezinc or anti-IGF-I antibody for 21 hr, and then BrdU was added to the culture medium. After the cells had been incubated for 3 hr, BrdU uptake was determined. The anti-IGF-I antibody used is specific for human IGF-I and did not react with IGF-I of other species or with IGF-II.

Analysis of IGF-I Gene Expression

Cells were cultured in 75-cm^2 tissue culture flasks containing MEM (without zinc or serum) for 24 hr, polaprezinc was added to the culture medium, and the cells were incubated for the period of time indicated in Fig. 4. Then the culture medium was removed and the cells were washed with PBS (−). Total cellular RNA was isolated using the acid guanidinium thiocyanate-phenol-chloroform (AGPC) method, as described by Chomczynski *et al.* [22]. The IGF-I mRNA content was determined by a one-step PCR analysis using rTth DNA polymerase and Southern blotting. Total RNA was amplified under the following conditions. For IGF-I, reverse transcription was carried out for 30 min at 58°, followed by 35 PCR cycles (15 sec at 92°, 30 sec at 58°, and 30 sec at 58°) and a final extension for 7 min at 58°. For β-actin, reverse transcription was done for 30 min at 60°, followed by 24 PCR cycles (15 sec at 92°, 30 sec at 60°, and 30 sec at 60°) and extension for 7 min at 60°. The primers had the following sequences: for IGF-I, sense 5′-GTACTTCAGAAGCAATGGGA-3′, antisense 5′-GGTGCGCAATACATCTCCAG-3′; for β-actin, sense 5′-ATCGTGGGCCGCCCTAGGCA-3′, antisense 5′-TGGCCTTAGGGTTCAGAGGGG-3′. Part of the PCR products was analyzed by 3% agarose gel electrophoresis or 5% polyacrylamide gel electrophoresis. Following agarose gel electrophoresis, the products were transferred to a nylon membrane after the gel had been stained by ethidium bromide. After prehybridization, the nylon membrane was hybridized with fluorescein-labeled cDNA. Hybridization was performed for 2 days at 42° in the presence of 50% (v/v) formamide. The cDNA probes were prepared from a human stomach cDNA library. The region coded was 348 bp from the first exon to second exon. The probes used covered a region of the fragment amplified by our primer. Detection of signals on x-ray film was performed by an ECL detection kit using anti-fluorescein horseradish peroxidase-labeled antibody (Boehringer Manneim). The x-ray film was analyzed by a V10 image analyzer (Toyobo).

Statistical Analysis

All results are shown as means ± SE. The data were analyzed by Dunnett's test.

RESULTS

Effects of Polaprezinc on Cellular Proliferation

We first investigated whether polaprezinc had the ability to stimulate cellular proliferation. When polaprezinc (final concentration $3 \times 10^{-10} \sim 3 \times 10^{-8}$ M) with low serum medium was added to guinea pig gastric mucosal epithelial cells, neither BrdU uptake nor the number of cells was altered. In contrast, this drug gradually increased both

TABLE 1. Effects of polaprezinc on cellular proliferation in cultured cells

	Gastric mucosal cell		HUVEC		Foreskin fibroblast	
	Number of cells (cells/plate)	BrdU uptake (Abs., O.D. = 450 nm)	Number of cells (cells/plate)	BrdU uptake (Abs., O.D. = 450 nm)	Number of cells (cells/plate)	BrdU uptake (Abs., O.D. = 450 nm)
Control	3237 ± 351	0.259 ± 0.019	997 ± 133	0.526 ± 0.069	1493 ± 146	0.488 ± 0.045
0.3 nM	2968 ± 322	0.213 ± 0.029	1593 ± 129	0.611 ± 0.049	1792 ± 159	0.552 ± 0.015
1 nM	3068 ± 241	0.221 ± 0.028	2111 ± 196†	0.740 ± 0.048	2382 ± 149†	0.594 ± 0.021*
3 nM	2802 ± 244	0.186 ± 0.014	2326 ± 171†	0.825 ± 0.070†	2492 ± 153†	0.623 ± 0.019†
10 nM	3215 ± 223	0.250 ± 0.036	2652 ± 187†	1.108 ± 0.022†	2716 ± 252†	0.762 ± 0.025†
30 nM	3503 ± 232	0.278 ± 0.009	ND	0.932 ± 0.025†	2569 ± 223†	0.670 ± 0.083†

Mean ± SE, N = 5–6, *: $P < 0.05$, †: $P < 0.01$, versus control.

parameters in cultures of HUVEC and foreskin fibroblasts (Table 1). In cultures of rabbit gastric mucosal epithelial cells, MKN45 cells, and human gastric cancer cells, polaprezinc also did not affect cell growth (data not shown). To determine the components of polaprezinc that were important in enhancing cellular proliferation, we performed studies using L-carnosine or zinc sulfate. In HUVEC cultures, BrdU uptake was increased by zinc sulfate (10^{-8} M) under serum-free conditions, but was not changed by the addition of the zinc-free medium in the presence of L-carnosine (Fig. 1). However, the action of zinc sulfate was weaker than that of polaprezinc. We also determined the time-course of BrdU uptake after addition of polaprezinc. BrdU uptake did not change at 8 hr after addition of the drug to medium without zinc or serum, but subsequently increased at 16 hr as well as after the addition of serum (Fig. 2). These results suggested that polaprezinc enhanced the proliferation of non-parenchymal cells such as endothelial cells or fibroblasts, but had no effect on epithelial cells, and also suggested that zinc was significant in this action of the drug.

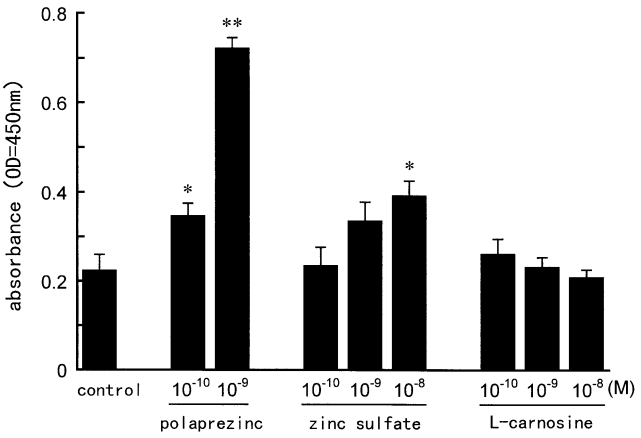

FIG. 1. Effects of polaprezinc, zinc sulfate, and L-carnosine on BrdU uptake in cultured HUVEC. Cultured HUVEC were incubated in MEM (without zinc or serum) with or without the indicated agents for 24 hr, and were finally incubated for 3 hr in the presence of BrdU. BrdU uptake was indicated by absorbance (O.D. = 450 nm) as in Materials and Methods. Each column represents the mean ± SE (N = 7). *: $P < 0.05$, **: $P < 0.01$ (versus control group).

Effect of Polaprezinc on IGF-I Gene Expression

To determine whether or not polaprezinc influences IGF-I gene expression, we performed RT-PCR or RT-PCR/Southern blot analysis. Before these experiments, we confirmed the optimum conditions for PCR analysis. In cultured HUVEC, the IGF-I mRNA content was increased by concentrations of polaprezinc that enhanced cellular proliferation (Figs. 3 and 4). The PCR products of IGF-I were shown to have two different lengths (345 bp and 420 bp) on PAGE (Fig. 3). Both products were recognized by Southern blotting using IGF-I cDNA (Fig. 5a). Moreover, the IGF-I mRNA content increased at 3 hr after addition of the drug, and then gradually decreased until 24 hr (Fig. 5a), but the β-actin mRNA content did not change (Fig. 5b). The mRNA levels of other growth factors, such as human growth factor, epidermal growth factor, and transforming growth factor beta, also did not change (data not shown).

Role of IGF-I in the Proliferative Effect of Polaprezinc

An anti-human IGF-I antibody was used to study the influence of IGF-I on the increase in BrdU uptake caused

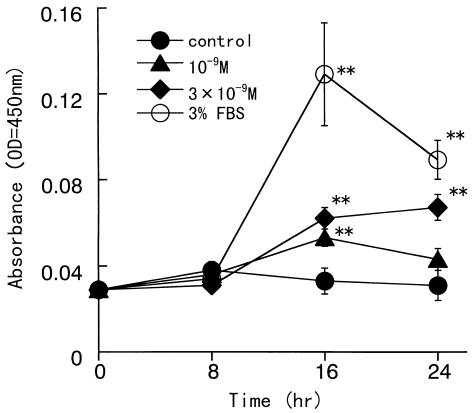

FIG. 2. Changes in time-course of BrdU uptake by polaprezinc in HUVEC. The cells incubated in MEM (without serum or zinc) for 24 hr were cultured for the indicated times in the absence (●) or presence of polaprezinc (10^{-9} M: ▲; 3×10^{-9} M: ◆) or 3% fetal bovine serum (FBS) (○). BrdU uptake was indicated by absorbance (O.D. = 450 nm) as in Materials and Methods. Each point represents the mean ± SE (N = 7). **: P 0.01 (versus 0 hr point).

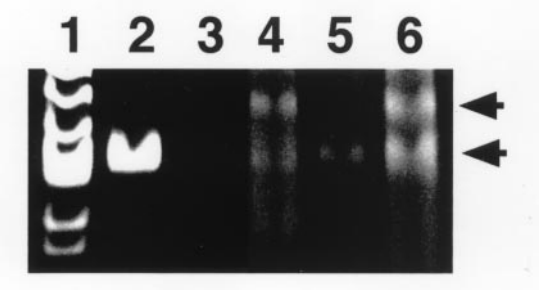

FIG. 3. RT-PCR analysis for changes in IGF-I gene expression by polaprezinc. Cells were treated with or without polaprezinc (final concentration 10^{-8} M) for 6 hr, and then total RNA was prepared from the cells. The products amplified by RT-PCR analysis were detected by PAGE/ethidium bromide staining. RT-PCR analysis was assessed using differential amounts of template RNA. Lanes 3 and 4 or lanes 5 and 6: 0.2 μg or 0.6 μg of the template RNA, lane 1: HincII-digested ØX174 DNA; lane 2: RT-RCR product from the human cDNA library, lanes 3 and 5: control, and lanes 4 and 6: polaprezinc treatment.

by polaprezinc. This antibody was specific to human IGF-I and did not recognize IGF-II. Figure 6 shows that the increase in BrdU uptake stimulated in cultured HUVEC by polaprezinc was suppressed by co-addition of the anti-IGF-I antibody. However, it was not affected by normal immunoglobulin G (data not shown).

DISCUSSION

In this study, we have shown that polaprezinc, a novel antiulcer agent containing zinc, enhanced the proliferation of non-epithelial cells such as endothelial cells or fibroblasts (Table 1). It was also suggested that this action of polaprezinc involves zinc, while L-carnosine, another constituent of polaprezinc, had no effect on BrdU uptake (Fig. 1). Interestingly, the influence of polaprezinc on BrdU uptake was greater than that of zinc sulfate (Fig. 1). We have already reported that the gastric mucosal adhesiveness or permeability of polaprezinc is higher than that of zinc sulfate [23, 24], and also that its antiulcer action is stronger than that of zinc sulfate [25]. It seems possible that the L-carnosine in polaprezinc may enhance the action or uptake of zinc, so that the difference in BrdU uptake may have been caused by differing levels of zinc uptake between polaprezinc and zinc sulfate. It is known that zinc is important for the growth of several animals, as well as being required by various enzymes or transcription factors involved in nucleotide synthesis, protein synthesis, and gene expression during biological events such as cellular proliferation [7]. It has already been reported that zinc deficiency delays gastric ulcer healing in rats, and that zinc compounds such as polaprezinc promote healing [8, 9]. It has also been reported that zinc compounds increase [^{3}H]-thymidine uptake by cultured cells [26], a finding consistent with our data. Therefore, it is suggested that the promotion of wound healing by polaprezinc may involve the stimulation of cellular proliferation caused by zinc. However, little is known about the details of the mechanism involved.

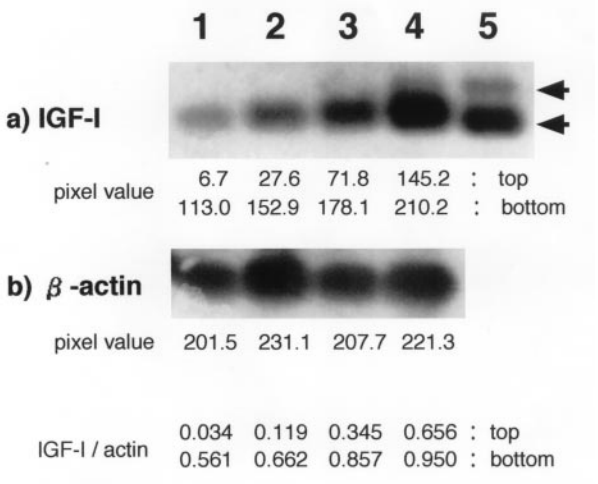

FIG. 4. Changes in IGF-I gene expression by polaprezinc as detected by RT-PCR/Southern blot. Cells were treated with or without polaprezinc for 6 hr, and total RNA was prepared from the cells. IGF-I (a) or β-actin (b) mRNA was detected by RT-PCR/Southern blot analysis. All pixel values were calculated by NIH image. Lane 1: control, lanes 2–4: polaprezinc 10^{-9} M, 3×10^{-9} M, and 10^{-8} M, and lane 5: RT-PCR products of IGF-I from the human cDNA library.

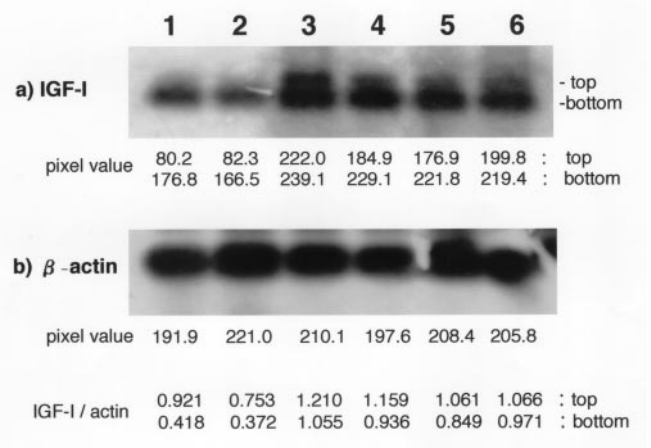

FIG. 5. Changes in time-course of IGF-I gene expression by polaprezinc as detected by RT-PCR/Southern blot. Cells were treated with or without polaprezinc and total RNA was prepared from the cells at the indicated times. IGF-I (a) or β-actin (b) mRNA was detected by RT-PCR/Southern blot analysis. All pixel values were calculated by NIH image. Lanes 1 and 2: 0 hr, 6 hr without polaprezinc, lanes 3–6: 3, 6, 12 hr, 24 hr with polaprezinc 3×10^{-9} M.

The present study indicated that polaprezinc caused increases in IGF-I gene expression (Figs. 3–5). Similarly, we previously reported that IGF-I mRNA was increased after stimulation of polaprezinc in cultured fibroblasts with polaprezinc [27]. As shown in Fig. 2, after IGF-I gene expression was stimulated by polaprezinc, BrdU uptake was increased, so the changes in the IGF-I mRNA content were correlated with those of the BrdU uptake. We also confirmed that zinc sulfate as well as polaprezinc increased the IGF-I mRNA content in cultured fibroblasts (data not

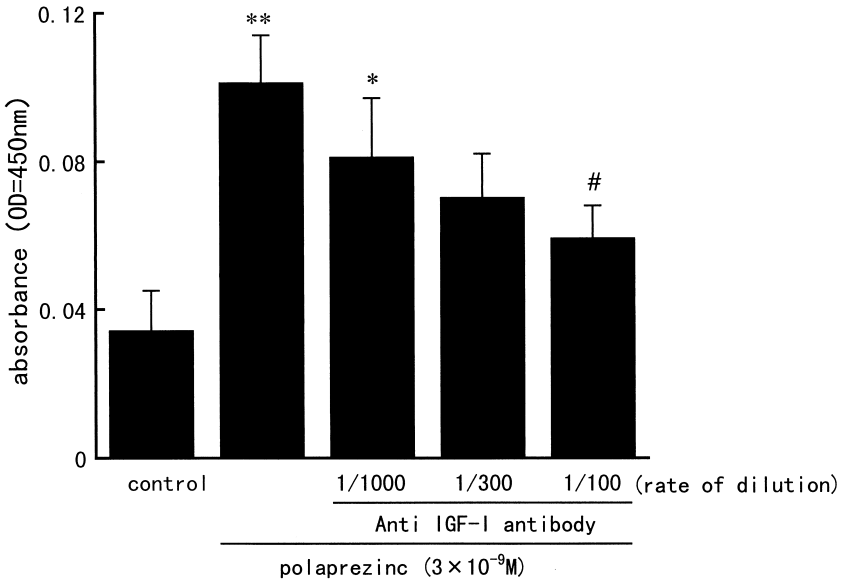

FIG. 6. Effects of IGF-I on BrdU uptake stimulated by polaprezinc in cultured HUVEC. Cells were incubated for 21 hr with or without anti-IGF-I antibody in the presence of polaprezinc, and were finally incubated for 3 hr with BrdU. BrdU uptake was indicated by absorbance (O.D. = 450 nm) as in Materials and Methods. Each column represents the mean ± SE (N = 7). *: $P < 0.05$, **: $P < 0.01$ (versus control group), #: $P < 0.05$ (versus polaprezinc without anti-IGF-I antibody).

shown). However, the mRNA content did not differ in the two zinc compounds, unlike the difference in BrdU uptake (data not shown). This suggests that the proliferative action of zinc may not only be mediated by IGF-I, but also by other factors. In this study, we identified two PCR products of IGF-I (Fig. 3). It is known that IGF-I expression is regulated by alternative splicing in several animals [28] and that the site of alternative splicing is within the region amplified by our primer. Therefore, alternative splicing may have produced the two PCR products that we found.

It is known that IGF-I gene expression is regulated by several factors, including growth hormone [14], estrogen [15], and cyclic AMP-responsive factor [16]. The serum concentration of IGF-I was shown to decrease in zinc-deficient rats, and this change was reversed by the administration of zinc [17]. In addition, the IGF-I mRNA content was increased by treatment with zinc oxide in the granulation tissue of porcine skin wounds [18]. These reports suggest that zinc might be involved in the function and gene expression of IGF-I, so it is interesting that we found that IGF-I mRNA was increased by treatment with zinc polaprezinc. Moreover, we confirmed that the stimulation of BrdU uptake by polaprezinc was suppressed by the co-addition of anti-IGF-I antibody, strongly suggesting that IGF-I was involved in the enhancement of cellular proliferation by polaprezinc.

In the present study, polaprezinc enhanced the proliferation of non-parenchymal cells such as endothelial cells and fibroblasts, but had no effect on epithelial cells. It has been reported that IGF-I is expressed by non-parenchymal cells [29], and our results indicated that these cells were capable of expressing IGF-I mRNA. Thus, IGF-I seems to be involved in the promotion of wound healing by polaprezinc. It is known that the IGF-I receptor is expressed by gastric epithelial cells [30] and that IGF-I promotes gastric epithelial cell proliferation [27, 31]. It was also previously reported that IGF-I and conditioned medium from fibroblasts cultured with polaprezinc accelerated healing in a wound repair model employing cultured gastric epithelial cells and that this action was suppressed by anti-IGF-I antibody [27]. Accordingly, polaprezinc may enhance the proliferation of epithelial cells via a paracrine action of IGF-I.

The present study suggested that the promotion of wound healing by polaprezinc, a novel antiulcer agent containing zinc, is related to a proliferative effect on non-parenchymal cells, with zinc and IGF-I being important for this action. These results are not only of interest with respect to the wound healing action of polaprezinc, but also with regard to the biological actions of zinc.

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