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Effect of Defibrotide on Rat Reflux Esophagitis

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This study was aimed at evaluating the effect of defibrotide on the development of the surgically induced reflux esophagitis, on gastric secretion, lipid peroxidation, polymorphonuclear leukocytes (PMNs) accumulation, polymorphonuclear leukocytes adherence, superoxide anion and hydrogen peroxide production in PMNs, scavenge of hydroxyl radical and hydrogen peroxide, cytokine (interleukin-1 β, tumor necrosis factor-α) production in blood, and intracellular calcium mobilization in PMNs. Defibrotide did not inhibit the gastric secretion and not change the gastric pH. Treatment of esophagitis rats with defibrotide inhibited lipid peroxidation, and myeloperoxidase (MPO) in the esophagus in comparison with untreated rats. Defibrotide significantly decreased the PMN adherence to superior mesenteric artery endothelium in a dose-dependent manner. Superoxide anion and hydrogen peroxide production in 1 μM formylmethionylleucylphenylalanine (fMLP)- or 0.1 μg/ml N-phorbol 12-myristate 13-acetate (PMA)-activated PMNs was inhibited by defibrotide in a dose-dependent fashion. Defibrotide effectively scavenged the hydrogen peroxide but did not scavenge the hydroxyl radical. Treatment of esophagitis rats with defibrotide inhibited interleukin-1 β production in the blood in comparison with untreated rats, but tumor necrosis factor-α production was not affected by defibrotide. The fMLP-induced elevation of intracellular calcium in PMNs was inhibited by defibrotide. The results of this study suggest that defibrotide may have partly beneficial protective effects against reflux esophagitis by the inhibition lipid peroxidation, PMNs accumulation, PMNs adherence to endothelium, reactive oxygen species production in PMNs, inflammatory cytokine production(i.e. interleukin-1 β), and intracellular calcium mobilization in PMNs in rats.

Key Words: Defibrotide, Polymorphonuclear leukocyte (PMN), Reflux esophagitis

INTRODUCTION

Esophageal reflux is a common condition that affects children and adults, and if untreated, may result in chronic esophagitis, aspiration pneumonia, esophageal strictures and Barrett's esophagus, a premalignant condition (Biancani et al, 1997). Reflux esophagitis is a multifactorial disease that may depend on transient lower esophageal sphincter (LES) relaxation, speed of esophageal clearance, mucosal resistance and other factors, and is often associated with LES pressure (Bell & Hunt, 1992).

Oxygen-derived free radicals have been known as mediator of acute gastric mucosal injury caused by ischemia-reperfusion (Stein et al, 1990), ethanol, non-steroidal anti-inflammatory drugs (Pihan et al, 1987) and Helicobacter pylori (Davies et al, 1994). Chronic free radical damage may also produce a carcinogenic effect by modulating the DNA information (Haegele et al, 1994). Recent studies have shown that oxygen-derived free radicals and superoxide anion are the main cause of free radical damage in reflux esophagitis in rats (Wetscher et al, 1995).

Neutrophils are implicated in the injury of tissue components in inflammatory diseases, esophagitis, rheumatoid arthritis and ulcerative colitis (Malech & Gallin, 1987). Stimulation of the respiratory burst in neutrophils produces superoxide anion, hydrogen peroxide and probably hydroxyl radicals. Iron-oxygen complex has also been proposed as the causative agent for the oxidative tissue damage (Minotti & Aust, 1987).

Defibrotide is a complex of single-stranded polydeoxyribonucleotides isolated by controlled depolymerization of porcine intestinal mucosa DNA and comprises a cluster of chains of different length and base sequences (Ma et al, 1991; Bianchi et al, 1993; Lanzarotti et al, 1993) with a molecular weight of 20~30 kDa. This substance either preserves or enhances the release of antiaggregatory eicosanoids (Niada et al, 1986) and exerts fibrinolytic activity by liberating tissue plasminogen activator and decreasing its inhibitor (Klcking, 1992). Recently, defibrotide has been found to exert cytoprotective actions in acute inflammation disorders probably by preserving nitric oxide (NO) release by the endothelium (Palmer & Goa, 1993). Interestingly, defibrotide has been shown to inhibit both activation and accumulation of inflammatory cellsin ischemia reperfusion-induced tissue injury (Hohlfeld et al, 1993; Palmer & Goa, 1993). Thus, the biological function

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ABBREVIATIONS: PMNs, polymorphonuclear leukocytes; MPO, myeloperoxidase; fMLP, formylmethionylleucylphenylalanine; PMA; N-phorbol 12-myristate 13-acetate.

of defibrotide in the reflux esophagitis has not been clarified. In addition, the action as an antioxidant in vivo is uncertain.

Therefore, the objectives of this investigation were to determine if defibrotide provided beneficial effects in a well-established rat model of reflux esophagitis, and to elucidate any mechanisms that may be involved.

METHODS

Compound used

Defibrotide (obtained from Crinos, Industria Farmacobiologica S.P.A., Villa Guardia (Como), Italy). N-phorbol 12-myristate 13-acetate (PMA), Formylmethionyl-leucyl-phenylalanine (fMLP), phorbol 12-myristate 13-acetate (PMA), sodium heparin, phenol red, horseradish peroxidase, hydrogen peroxide, type II oyster glycogen, cytochrome c, citrate phosphate dextrose, hexadecyltrimethyl ammonium bromide, and o-dianisidine, fura-2/AM were all purchased from Sigma Chemical Co. (St. Louis, MO, U.S. A.). The ELISA assay kit for tumor necrosis factor α (TNF α) and interleukin-1 β (IL-1 β) was bought from Amersham Pharmacia Biotech UK Ltd. (Little Chalfont, Buckinghamshire, England). Other chemicals were of analytical grade.

Animals

Male Sprague-Dawley rats (Chungang, Korea) weighing 200~250 g, were fasted for 24 h but allowed free access to water prior to the experiment. All animals were kept in raised mesh-bottom cages to prevent coprophagy. Five to seven rats were used in each group.

Esophagitis induction

The rats were anesthetized with optimal inhalation of ether. The abdomen was incised along the midline and then the limiting ridge (transitional region between the forestomach and corpus) was ligated very carefully, and continuously the pylorus portion was ligated. A longitudinal cardiomyotomy of about 1 cm length across the gastroesophageal junction was performed to enhance reflux from the stomach contents into the esophageal body. Immediately the incised regions were sutured and the animals were returned to their home cages, After 6 hr, the animals were sacrificed by cervical dislocation and the esophagus was harvested (Kil et al, 2000).

The total area (mm^2) of the lesions that had developed in the esophagus was determined under a dissecting microscope ($\times 10$) and graded as follows: 0, no visible lesions; 1, a few erosions; 2, total area of lesions ≤ 30 mm^2; 3, total area of lesions ≥ 30 mm^2; 4, total area of lesions ≥ 200 mm^2.

Rats were randomly assigned in one of three experimental groups; (a) five control rats with or without receiving defibrotide, (b) five esophagitis rats (c) five esophagitis rats with receiving defibrotide. Defibrotide (10 mg/kg rat body weight) suspended in saline were administered intravenous (i.v.) before 30 min abdominal surgery. The volume of the drug or vehicle was 0.1 ml/100 g of body weight.

Rat neutrophil isolation

Neutrophil donor rats (300~350 g) received an injection of 0.5 % 10 ml type II oyster glycogen. Eighteen hours later, the rats were anesthetized with ethyl ether and the PMNs were harvested by peritoneal lavage in PBS. The peritoneal lavage was centrifuged at 3,000 rpm and 4°C for 10 minutes as previously described (Lefer et al, 1997). Finally, the neutrophils were washed in Krebs buffer and counted using a microscope. These neutrophil preparations were >95% pure, and >95% viable using exclusion of 0.3% trypan blue as the criterion for viability. Furthermore, neutrophils obtained by this method have been found to respond normally in cell adhesion tests (Lefer et al, 1997).

Rat neutrophil labeling

Isolated rat neutrophils were then labeled with Zynaxis PKH-2 cell linker (Zynaxis Cell Science Inc., prepared for Sigma Immunochemical, Malvern, PA) based on a procedure of Yuan and Fleming (1990). One ml of diluents was added to a loose cell pellet containing, 20 million cells. After mixing the cell suspension together with 20 μl of PKH-2GL dye for 5 min by inversion, Two ml of PBS containing 10% rat plasma was added to stop the reaction, and another 8 ml of PBS was used to underlay the suspension. Cells were then centrifuged at 350 g for 10 min. The supernatant was removed, and the cells were resuspended in PBS, recounted and employed for adherence studies. This labeling procedure yields >95% of the cells possessing normal morphology and function (Yuan & Fleming, 1990).

Gastric secretory study

The gastric contents were collected. After centrifugation, the supernatant was measured for volume (ml/rat), pH (Toledo 320, Mettler, Swiss) and acidity (mEq/L). Total acidity was determined by automatic titration of the gastric juice against 0.1 N NaOH to pH 7.0 (665 Dosimat, Metrohm, Swiss). Acid output was expressed as μEq/h.

Measurement of lipid peroxidation

Lipid peroxidation, which is a marker of oxidative stress, was estimated from measuring malondialdehyde concentration by thiobarbituric acid method. Esophageal tissue was dissected and carefully rinsed in 0.9% NaCl. The sample was then homogenized in 10 volume Kreb's Henselite buffer by using Polytron (PCU-2) homogenizer. Homogenates were centrifuged at 12,500 g at 4°C for 30 minutes. The supernatant were collected. The supernatant of esophagus (1 mg/ml) were contained in the reaction mixture consisting of 150 mM KCl, 50 mM NaH_2PO_4, pH 7.4. After 30 min of incubation, the reaction was stopped by adding 1.0 ml of 1% TBA in 50 mM NaOH and 1.0 ml of 2.8% trichloroacetic acid. Boiling in a water bath for 10 min developed the chromophore. After cooling to the room temperature, the absorbance was measured at 532 nm (Gutteridge, 1981). The concentration of malondialdehyde was expressed as nmol/mg protein using the molar extinction coefficient of 1.52×10^5/M/cm (Placer et al, 1966). Protein assay was determined according to the Bradford method (1976).

Measurement of esophageal tissue myeloperoxidase

Esophageal tissue myeloperoxidase (MPO), an enzyme occurring virtually exclusively in PMNs (Mullane et al, 1985), and therefore, an increased esophageal MPO activity indicates a significant accumulation of PMNs in the esophagus. One unit of MPO is defined as that quantity of enzyme hydrolyzes 1 mmol of peroxide per minute at 25 °C. MPO was determined spectrophotometrically by the method of Bradley et al (Bradly et al, 1985) as modified by Mullane et al (Mullane et al, 1985). The assays were performed without knowledge of the group from which each sample originated.

PMN adherence to thrombin stimulated superior mesenteric artery (SMA) endothelium in vitro

Thrombin stimulation leads to rapid up-regulation of P-selectin (Lorant et al, 1991). In order to gain additional insight into the effects of defibrotide on PMN adherence, superior mesenteric artery segments obtained from additional anesthetized control rats were removed and placed into warmed Krebs-Henseleit (K-H) buffer consisting of (in mmol/L): NaCl 118, KCl 4.75, CaCl2 2.54, KH_2PO_4 1.19, $MgSO_4$ 1.19, $NaHCO_3$ 12.5, and glucose 10. The arteries were cut into 2~3 mm rings, opened, and placed into cell culture dishes filled with 3 ml of K-H buffer. These opened segments were incubated with 2 U/ml thrombin (Sigma immunochemical) for 10 min in order to stimulate the endothelial cells. After this 10 min incubation period, the opened segments were removed and placed in fresh K-H solution. Labeled inactivated rat PMNs (4×10^5 PMNs/ml) were added to thrombin-stimulated endothelium alone and in combination with increasing concentrations of defibrotide (0.1~0.5 mg/ml). After 20 min of incubation in a metabolic shaker bath at 37°C, the segments were washed in K-H buffer and placed endothelial side up on microscope slides, and adherent PMNs were counted using an epifluorescent microscope (Laborlux 12, Leitz, Germany). Five different fields each of endothelial surface were counted and the results were expressed as adherent PMNs/mm^2 of endothelial surface.

Measurement of superoxide anion production

The superoxide anion produced was assayed by superoxide dismutase-inhibitable reduction of ferricytochrome c. The reaction mixtures (200 μl) in 96 well microplate contained 3×10^5 PMNs, 75 μM ferricytochrome c, stimulating agent and DMEM, pH 7.4 and were placed at 5% CO_2 incubator, for 4 h at 37°C. The absorbance was measured in a microplate reader (Molecular Devices, Spectra MAX 340, Molecular Devices, Co., Sunnyvale, CA, U.S.A.). The amount of reduced ferricytochrome c was represented as nM using the extinction coefficient of 2.1×10^4 M^{-1} cm^{-1} at 550 nm (Pick & Mizell, 1981; Markert et al, 1984).

Measurement of hydrogen peroxide production

PMNs (3×10^5 cells/well) were incubated in 200 μl of DMEM containing 0.1 mg/ml phenol red and 0.2 mg/ml horseradish peroxidase for 4 h at 37°C. The reaction was terminated by adding 20 μl of 1 N NaOH, and absorbance was measured at 610 nm (Pick & Mizell, 1981). The concentration of hydrogen peroxide was calculated using hydrogen peroxide solution as the standard.

Measurement of 2-α deoxyribose degradation

Decomposing effect of defibrotide on hydroxyl radicals was determined by the assay of malondialdehyde chromogen formation due to 2-α deoxyribose degradation (Halliwell & Gutteridge, 1989; Aruoma, 1994). The reaction mixtures contained, in a final volume of 1.0 ml, 2 mM 2-α deoxyribose, 50 μM $FeCl_3$, 50 μM EDTA, 500 μM H_2O_2, 100 μM ascorbate, 150 mM KCl, and 50 mM NaH_2PO_4 buffer, pH 7.4 and other compounds (10 μg/ml defibrotide). After 30 min of incubation, adding 1 ml of 1% thiobarbituric acid in 50 mM NaOH and 1 ml of 2.8% trichloroacetic acid stopped the reaction. The absorbance was measured at 532 nm.

Measurement of H_2O_2 decomposition

The concentrationof hydrogen peroxide was measured by the method of Allen et al (1952). The reaction mixtures contained, in a final volume of 1 ml, 120 mM KCl, 0.1 mM H_2O_2, 10 μM sodium azide, 50 mM Tris-HCl, pH 7.4 and other compounds (10 μg/ml defibrotide). After reaction stopping solution (25 mg/ml of potassium biphthalate, 2.5 mg/ml NaOH, 82.5 mg/ml potassium iodide and 0.25 mg/ml ammonium molybdate) was added to the above mixture, absorbance change was read spectrophotometrically at 350 nm.

Measurement of cytokine production

The amounts of cytokines produced in blood were measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions with commercial kit for rat TNF-α and IL-1β (Amersham Pharmacia Biotech). Cytokine concentrations were calculated by interpolation of the regression curve of known amounts of recombinant cytokines (rIL-1β and rTNF-α from Amersham Pharmacia Biotech) and reported as pg/ml. using commercial kit for rat TNF-α and IL-1β (Amersham Pharmacia Biotech).

Assay of cytosolic free calcium

Fura-2 loading and fluorescence measurement were performed by the method of Luscinskas et al (1990). PMNs (approximately 5×10^7 cells/ml) were loaded with 2 mM fura-2/AM to 1 μM/10^7 cells at 37°C for 10 min in the reaction mixtures contained Hanks' balanced salt solution (HBSS) buffer without calcium and magnesium (HBSS-CMF) and 20 mM HEPES-tris, pH 7.4. The suspension was then diluted 5 fold with 0.5% bovine serum albumin containing HBSS-CMF and further incubated at 37°C for 15 min. After loading, the suspension was centrifuged at 200 g for 10 min, and PMNs were resuspended in 0.1% bovine serum albumin containing HBSS-CMF. This procedure was performed twice. PMNs were finally suspended in bovine serum albumin free, HBSS-CMF as approximately 5×10^7 cells/ml. Fluorescence measurement was done with Aminco-Bowman Series 2 spectrometer (SLM Instrument Inc. U.S.A.). Preloaded PMNs (4×10^6) were suspended in 1.23 mM calcium and 1 mM magnesium containing HBSS in a final volume of 1 ml. After preincubation at 37°C for 5 min with defibrotide, the response was initiated by the addition of 1 μM fMLP. The fluorescence change was read at an excitation wavelength of 340 nm

and emission wavelength of 505 nm.

Statistical analysis

All values in the text and figures are presented as mean ±standard errors of the mean (SEM). Statistical analysis was performed using Student's *t*-test. Probability values of 0.05 or less were considered to be statistically significant.

RESULTS

The effect of defibrotide on gastric secretion

With the surgical procedure used in this study, reflux esophagitis developed 4 h after ligation. However, both the severity and incidence of esophagitis were increased when each esophagus was examined 6 h later. So we selected 6 h as the experimental time (Table 1). The administration of defibrotide had no significant effect on esophageal tissue in non-reflux esophagitis rats. Defibrotide (10 mg/kg rat bodyweight) suspended in saline were administered i.v. 30 min before abdominal surgery. Defibrotide did not change the gastric volume, gastric pH and acid output (Table 2).

The effect of defibrotide on lipid peroxidation and MPO activity in esophageal tissue

We measured lipid peroxidation of esophagus as an index of oxidant-induced injured tissue, which results from the production of reactive oxygen species (i.e. superoxide anion, hydroxyl radical, hydrogen peroxide). The lipid peroxidation was low in control rats. However, the surgically induced esophagitis rats resulted in about 10.5 fold increase in lipid peroxidation. Treatment of esophagitis rats with defibrotide inhibited significantly ($p<0.01$) lipid peroxidation of esophagus in comparison to untreated rats (Fig. 1).

Accumulation of PMNs in the surgically induced reflux esophageal tissue is considered one of the primary contributory mechanisms to esophageal injury. The MPO activity in the esophageal tissue was measured as a marker for PMN accumulation (Fig. 2). The MPO activity was low in control rats. However, the surgically induced esophagitis rats resulted in high increase in esophageal MPO activity. Treatment of esophagitis rats with defibrotide significantly ($p<0.01$) attenuated esophageal MPO activity in comparison to untreated rats, indicating that defibrotide retarded the accumulation of PMNs in the esophagitis.

Table 1. Time response of the reflux esophagitis induced surgically in rats

Esophagits index	3 h	4 h	6 h
0	●●●●●		
1	●	●●●	
2		●	
3		●●	●●
4		●	●●●●

● Number of rats

Table 2. Gastric secretion volume, pH and acid output in esophagitis in rats

Group (ml)	Gastric volume (mEq h-1)	Gastric pH	Acid output
Esophagitis	1.48±0.25	2.06±0.38	93.12±16.54
Defibrotide (10 mg/kg)	1.39±0.28	2.14±0.47	89.45±15.20

Effect of defibrotide on PMN adherence to thrombin-stimulated superior mesenteric artery endothelium in vitro

We also examined the effect of defibrotide on rat PMN

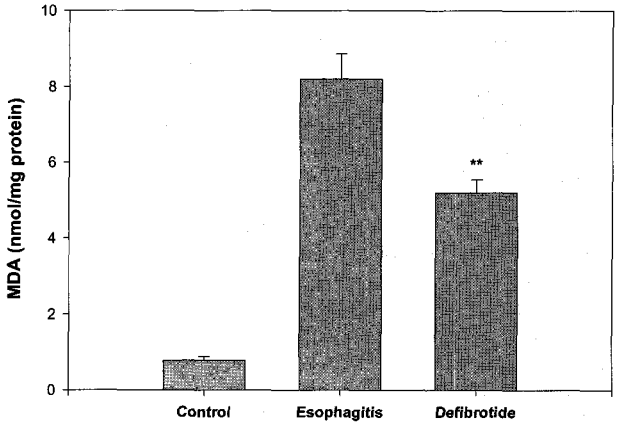

Fig. 1. Effect of defibrotide on esophageal lipid peroxidation. Malondialdehyde content, the end product of lipid peroxidation, increased significantly in the esophagus after the induction of reflux esophagitis. The dose of defibrotide was 10 mg/kg rat body weights. Data are expressed as malondialdehyde nmol/mg protein using the molar extinction coefficient of 1.52×10^6/M/cm. Data are mean±standard error of mean (SEM). $^{**}p<0.01$, vs esophagitis.

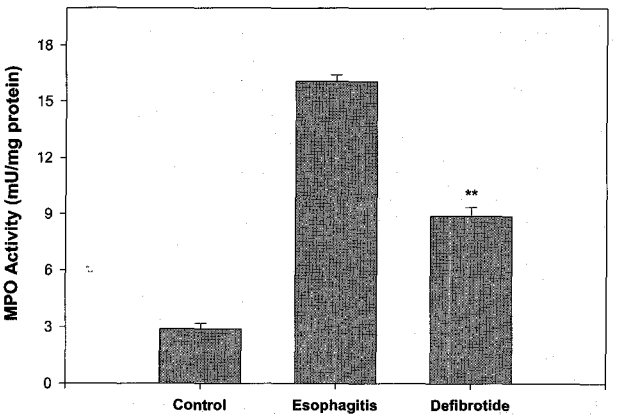

Fig. 2. Effect of defibrotide on esophageal myeloperoxidase (MPO) activity. MPO activity, an enzyme occurring virtually exclusively in neutrophils, increased significantly in the esophagus after the induction of reflux esophagitis. The dose of defibrotide was 10 mg/kg rat body weights. Data are expressed as mU/100 mg protein. Data are mean±standard error of mean (SEM). $^{**}p<0.01$, vs esophagitis.

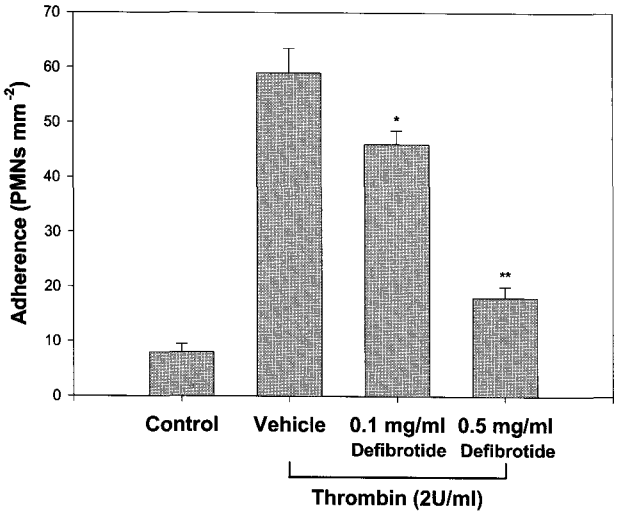

Fig. 3. In vitro effect of defibrotide on PMN adherence to thrombin stimulated (2 U/ml) rat superior mesenteric artery. Data are expressed as number of PMNs/mm^2. Bar heights represent means and vertical bars indicate SEM, n=5. *$p<0.05$, **$p<0.01$, vs vehicle.

adherence to rat superior mesenteric artery endothelial cells. Only a few of unstimulated rat PMNs adhered to unstimulated superior mesenteric artery endothelium, suggesting that the isolation procedure did not activate either cell type. In contrast, activation of endothelial cells with thrombin (2 U/ml), which up-regulates P-selectin, resulted in a dramatic increase in PMN adherence ($p<$ 0.001), indicating that the endothelium was not injured by the isolation procedures (Fig. 3). However, defibrotide significantly diminished the PMN adherence in a dose-dependent manner. At a concentration of 500 g/ml, defibrotide almost completely inhibited PMN adherence ($p<0.01$).

The effects of defibrotide on superoxide and hydrogen peroxide generation in PMNs

Out data obtained in the present study indicate significantly beneficial esophageal protective effects of defibrotide in surgically induced reflux esophagitis. In this connection, we were to investigate whether defibrotide had a direct effect of superoxide and hydrogen peroxide generation in PMNs. PMNs in response to fMLP and PMA produce superoxide and hydrogen peroxide.

FMLP and PMA have been shown to stimulate superoxide and hydrogen peroxide production significantly (Han et al, 1997). The effects of defibrotide on superoxide and hydrogen peroxide production in fMLP- and PMA-stimulated PMNs were examined. One μM fMLP- and 0.1 μg/ml PMA-stimulated PMNs produced 9.84$\pm$0.26 (n=5) and 12.85$\pm$0.52 (n=5) nmol/3$\times 10^5$ cells of superoxide anion, respectively. Superoxide production in 1 μM fMLP- or 0.1 μg/ml PMA-activated PMNs was inhibited by defibrotide in a dose dependent fashion (Figs. 4 and 5). One μM fMLP- and 0.1 μg/ml PMA-stimulated PMNs produced 17.43$\pm$0.52 (n=5) and 26.2$\pm$0.55 (n=5) nmol/3$\times 10^5$ cells of hydrogen peroxide, respectively. Hydrogen peroxide production in 1 μM fMLP- or 0.1 μg/ml PMA-activated PMNs was also inhibited by defibrotide in a dose dependent fashion (Figs. 6 and 7).

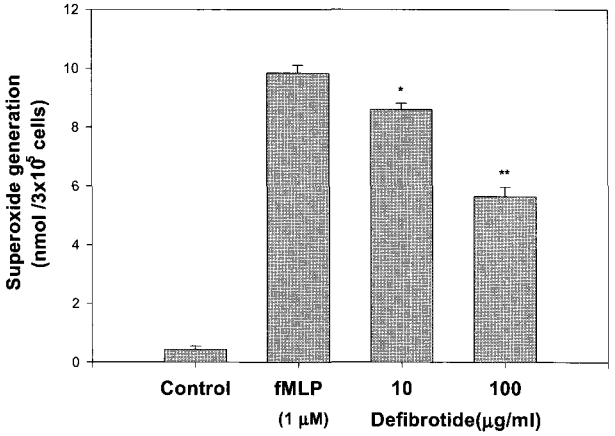

Fig. 4. Effect of defibrotide on fMLP stimulated superoxide generation in PMNs. PMNs was stimulated with 1 μM fMLP in the presence of defibrotide. Defibrotide significantly decreased the superoxide production in dose-dependent manners. Data are expressed as nmol/3$\times 10^5$ cells. Data are mean$\pm$standard error of mean (SEM). *$p<0.05$, **$p<0.01$ vs. fMLP treatment alone.

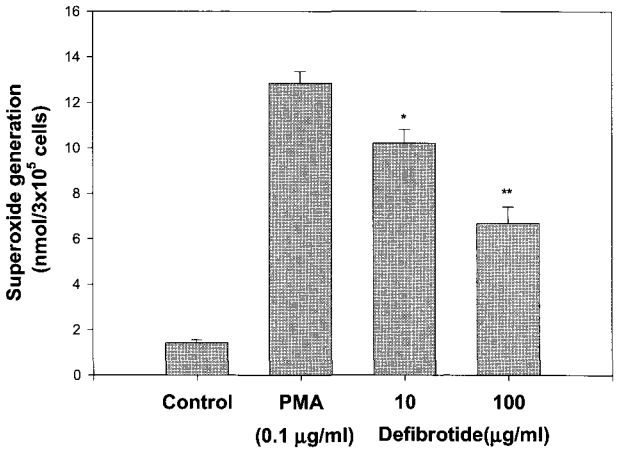

Fig. 5. Effect of defibrotide on PMA stimulated superoxide generation in PMNs. PMNs was stimulated with 1 μg/ml PMA in the presence of defibrotide. Defibrotide significantly decreased the superoxide production in dose-dependent manners. Data are expressed as nmol/3$\times 10^5$ cells. Data are mean$\pm$standard error of mean (SEM). *$p<0.05$, **$p<0.01$ vs. PMA treatment alone.

Scavenging action of defibrotide on hydroxyl radical and hydrogen peroxide

In biological systems, hydroxyl radical and hydrogen peroxide has been implicated as a precursor for more reactive oxygen species and cal also form complexes with metal ions.

Autooxidation of iron liberates reactive oxygen species, and iron causes formation of hydroxyl radical and iron-oxygen complexes. Hydroxyl radical produced was measured with TBA reactivity of 2-α deoxyribose. As can be seen in Fig. 8, 1, 10, and 100 μg/ml defibrotide did not

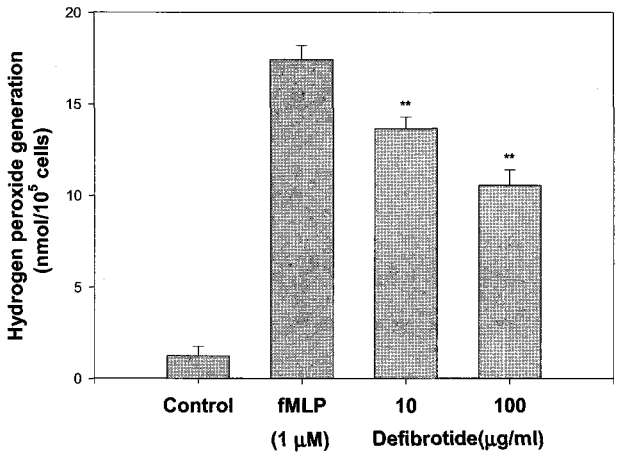

Fig. 6. Effect of defibrotide on fMLP-stimulated hydrogen peroxide generation in PMNs. PMNs was stimulated with 1 μM fMLP in the presence of defibrotide. Defibrotide significantly decreased the hydrogen peroxide production in dose-dependent manners. Data are expressed as nmol/3$\times 10^5$ cells. Data are mean$\pm$standard error of mean (SEM). **$p<0.01$ vs. fMLP treatment alone.

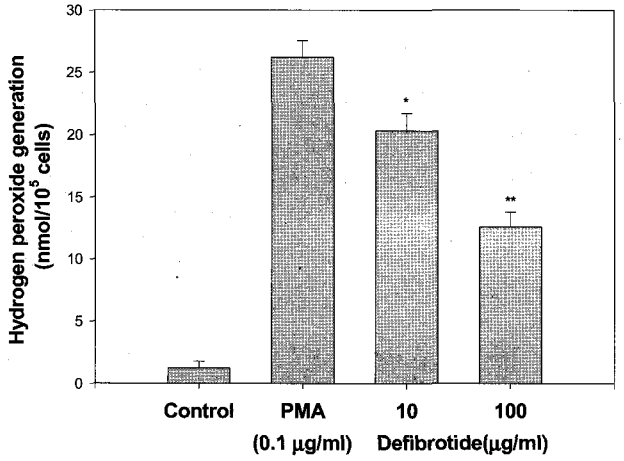

Fig. 7. Effect of defibrotide on PMA stimulated hydrogen peroxide generation in PMNs. PMNs was stimulated with 1 μg/ml PMA in the presence of defibrotide. Defibrotide significantly decreased the hydrogen peroxide production in dose-dependent manners. Data are expressed as nmol/3$\times 10^5$ cells. Data are mean$\pm$standard error of mean (SEM). *$p<0.05$, **$p<0.01$ vs. PMA treatment alone.

decrease the increased TBA reactivity of 2-α deoxyribose in the presence of defibrotide, respectively.

Fig. 9 shows that 100 μM H_2O_2 was significantly decomposed by 10 μg/ml of catalase and was also decomposed by 100 μg/ml defibrotide.

Effects of defibrotide on cytokine production in surgically induced reflux esophagitis

To exam the effect of defibrotide in surgically induced reflux esophagitis, we were to investigate whether defibrotide had a direct effect of TNF-α and IL-1β production. The amounts of cytokines produced in blood were measured by enzyme-linked immunosorbent assay (ELISA) according

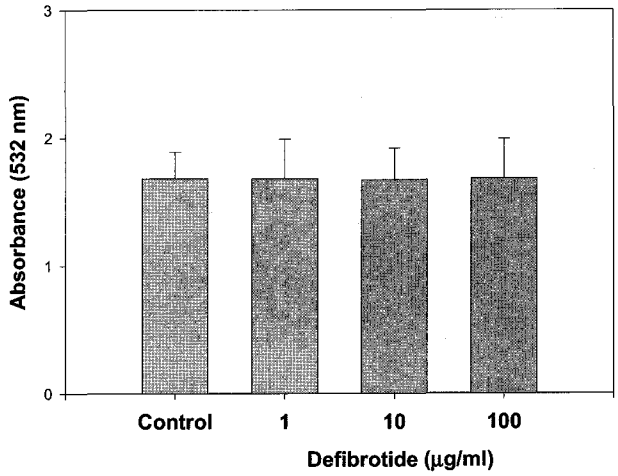

Fig. 8. Effect of defibrotide on a-deoxyribose oxidation. 2-Deoxy-D-ribose (2 mM) was treated with 50 μM $FeCl_3$, 50 μM EDTA, 500 μM H_2O_2, and 100 μM ascorbate for 30 min. Data are expressed as change in absorbance.

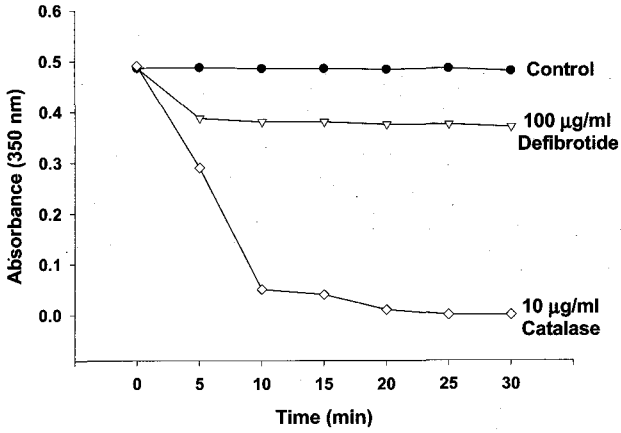

Fig. 9. Effect of defibrotide on hydrogen peroxide decomposition. Scavenging actions of defibrotide (100 μg/ml) and catalase (10 μg/ml) on 100 μM H_2O_2 were measured. Data are expressed as change in absorbance.

to the manufacturer's instructions with commercial kit for rat TNF-α and IL-1β (Amersham Pharmacia Biotech).

TNF-α and IL-1β was 15.0$\pm$0.97 pg/ml, 25.7$\pm$0.91 pg/ml in control rats, respectively (Figs. 10 and 11). However, the surgically induced esophagitis rats resulted in about 2-fold increase in TNF-α and IL-1β production. Treatment of esophagitis rats with defibrotide inhibited significantly ($p<0.01$) IL-1β production of esophagus in comparison to untreated rats (Fig. 11), but TNF-α production was not affected by defibrotide (Fig. 10).

Effect of defibrotide on calcium mobilization

The cytosolic calcium level was assayed by measuring fluorescence change of fura-2 due to the complex formation of fura-2 and calcium. One mM fMLP elicited an increase of intracellular calcium ([Ca^{2+}]i) in PMNs. The maximum

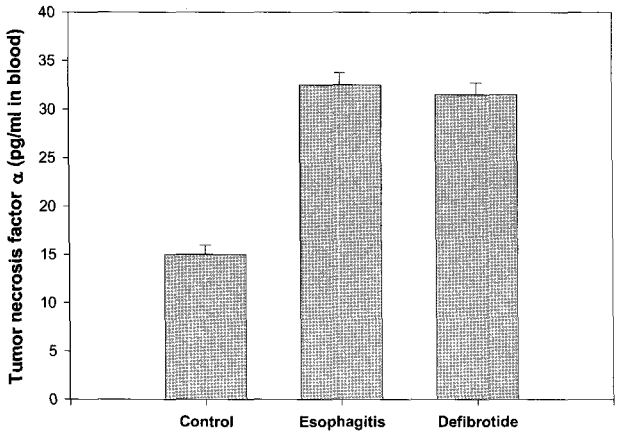

Fig. 10. Effect of defibrotide on tumor necrosis factor a (TNF-α) production. TNF-α, a cytokine occurring in inflammation, increased significantly in the esophagus after the induction of reflux esophagitis. The dose of defibrotide was 10 mg/kg rat body weight. Data are expressed as pg/ml blood. Data are mean±standard error of mean (SEM).

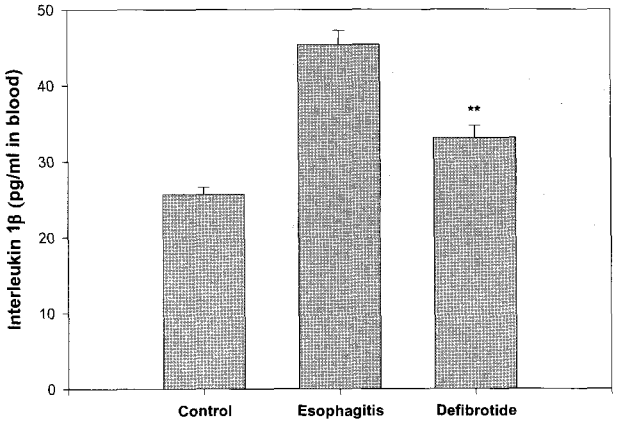

Fig. 11. Effect of defibrotide on interleukin-1β (IL-1β) production. IL-1β, an cytokine occurring in inflammation, increased significantly in the esophagus after the induction of reflux esophagitis. The dose of defibrotide 10 mg/kg rat body weights. Data are expressed as pg/ml blood. Data are mean±standard error of mean (SEM). $^{**}p<0.01$ vs esophagitis.

mobilization of $[Ca^{2+}]i$ occurredwithin 0.1~0.2 sec post addition, and then the level of $[Ca^{2+}]i$ was gradually decreased. Role of defibrotide in fMLP-induced intracellular calcium mobilization was examined. Fig. 12 shows that fMLP-induced elevation of $[Ca^{2+}]i$ was inhibited by 100 μg/ml defibrotide and 500 μg/ml defibrotide completely inhibited fMLP-induced elevation of $[Ca^{2+}]i$.

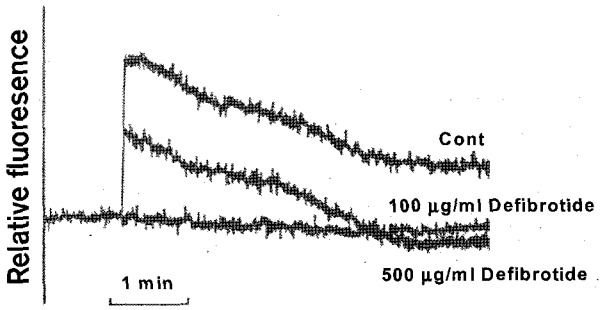

Fig. 12. Effect of defibrotide on intracellular calcium mobilization in PMNs. Fura-2 loaded PMNs (4×10^6 cells/ml) were preincubated with defibrotide or not for 5 min, and then the response was initiated by 1 μM fMLP. The traces are representative of three experiments. Cont, control.

DISCUSSION

The results of this study demonstrate that defibrotide have beneficial protective effects of reflux esophagitis, the attenuation of lipid peroxidation and MPO activity, the inhibition of PMN adherence to superior mesenteric artery, the inhibition of superoxide and hydrogen peroxide generation in PMNs, the scavenge of hydrogen peroxide, the inhibition of inflammatory cytokine (i.e., IL-1β), and the inhibition of intracellular calcium mobilization in PMNs.

It has been known that reflux esophagitis is induced by the regurgitation of digestive juice due to the insufficiency or destruction of the lower esophageal sphincter structure. As major factors relating to the pathogenesis of the disease, gastric juice contents such as hydrochloric acid and pepsin and duodenal contents such as trypsin and bile acids can be cited. This pylorus ligation method is very convenient for studying the etiology of esophagitis because various factors can be easily combined in the applied solution. It has adventage in the simultaneous evaluation of drug effects not only on esophageal ulceration but also on forestomach ulceration. In the present study, we succeeded in inducing esophageal ulcer in a high percentage and with great regularity within a very short time, i.e., in 60% of rats in 4 hr after the operation and in 100% by 6 hr afterwards. However, both the severity and incidence of esophagitis were increased when each esophagus was examined 10 h later. So we selected 10 h as the experimental time. The esophageal ulcer obtained was generally serious and hemorrhagic. Easily aggravating to perforation, and it may be the same that Shay et al (1945) and Selye (1938) called peptic hemorrhagic esophagitis in pylorus- ligated rats.

It has been reported that the pH-dependent pattern of mucosal injury is best explained by the very low acidity of gastric pepsin over pH 4.0 (Ito et al, 1998). It has also been reported that the effect of antisecretory therapy on reflux esophagitis can be predicted from the duration of suppression of intragastric acidity above pH 4.0 achieved by each drug regimen (Bell et al, 1992). However, defibrotide did not increase the pH of gastric content, and did not inhibit the gastric acid output.

In order to find the another protective mechanism of defibrotide in reflux esophagitis, we measured lipid peroxidation of esophagus as an index of oxidative stress, which results from the production of reactive oxygen species (i.e. superoxide anion, hydroxyl radical, hydrogen peroxide). Defibrotide significantly reduced the oxidative stress of esophagus in comparison to untreated rats. Central to the onset of surgically induced reflux esophagitis is the accumulation of inflammatory cells, which release significant quantities of reactive oxygen metabolites, proteases and arachidonic acid metabolites (i.e. leukotriene B4,

platelet activating factor), mediator which injure tissues. For inflammatory cells to gain access to an inflammatory site, they must leave the mainstream of blood. Extravasation of inflammatory cells in postcapillary venules is mediated by at least three sequential steps: (a) initial rolling of PMNs along the endothelium; (b) PMN activation, strengthening or PMN adhesion; and (c) transendothelial migration. Recruitment of PMNs to inflammatory tissue consists of a series of sequential processes, which are mediated by different cell adhesion molecules located either on the PMNs or the endothelium (Butcher, 1991). Among the well- known cell adhesion molecules, P-selectin plays a key role in initiating the first essential step in leukocyte-endothelial cell interactions, namely PMN rolling along the vascular endothelium. In this regard, Weyrich et al (1993) observed significant protection of the coronary endothelium and myocardium by a neutralizing antibody directed against P-selectin. P-selectin promotes rolling of leukocytes, thus facilitating PMNs activation and adherence (Lorant et al, 1993). Once activated, many leukocytes adhere to the endothelium and some undergo transmigration, thus potentiating endothelial dysfunction and tissue injury. Defibrotide significantly ($p<0.01$) diminished the PMN adherence to superior mesenteric artery in a dose- dependent manner. This finding suggests that the protective effect of defibrotide on reflux esophageal damage may be involved in inhibition of PMN adherence to superior mesenteric artery endothelium. The PMNs are major role in the surgically induced reflux esophagitis. Esophageal injury shows to be related to PMNs infiltrating into inflammed tissue. The MPO activity in the esophageal tissue was measured as a marker for PMN accumulation. Treatment of esophagitis rats with defibrotide significantly ($p<0.01$) attenuated esophageal MPO activity in comparison to untreated rats, indicating that defibrotide retarded the accumulation of PMNs in the esophagitis. PMNs are implicated in the injury of tissue components in inflammatory diseases (e.g. esophagitis, rheumatoid arthritis and ulcerative colitis) (Malech & Gallin, 1987). Superoxide anions produced by inflammatory cells (i.e. PMNs, macrophages and monocytes) play an important part in the pathogenesis of acid and pepsin induced esophagitis in rabbits (Naya et al, 1997). Stimulation of therespiratory burst produces superoxide anion, hydrogen peroxide and probably hydroxyl radicals. We were to investigate whether defibrotide had a direct effect of superoxide and hydrogen peroxide generation in PMNs. Defibrotide inhibited superoxide anion and hydrogen peroxide produced by PMNs in response to fMLP and PMA. Defibrotide did not scavenge the hydroxyl radical but did effectively scavenge the hydrogen peroxide. These effects may suggest that defibrotide have an inhibitory effect in inflammatory cells (i.e. PMNs) and reactive oxygen species.

Cytokines (i.e. IL-1 and TNF-α) produced by activated mononuclear phagocytes, lymphocytes, and other differentiated cell types in response to an inflammatory stimulus. Cytokines act locally and systemically to recruit and activate target cells including some that produce additional cytokines. The biological properties of IL-1 share remarkable similarities to those of TNF-α, most notably the induction of fever, inflammation, and hemodynamic shock (Akira et al, 1990; Dinarello, 1991). IL-1 induces gene expression of neutrophil and monocyte chemotactic cytokines, such as IL-8, IL-9, and macrophage inflammatory proteins, which, in turn, stimulate neutrophil migration and degranulation *in vivo* (Oppenheim et al, 1989). In this respect, to exam the effects of defibrotide in surgically induced reflux esophagitis, we were to investigate whether defibrotide had a direct effect of TNF-α and IL-1β production in blood. The surgically induced esophagitis rats resulted in about 2-fold increase in TNF-α and IL-1β production. Treatment of esophagitis rats with defibrotide inhibited significantly ($p<0.01$) IL-1β production of esophagus in comparison to untreated rats, but TNF-α production was not affected by defibrotide. Theses finding suggest that the protective effect of defibrotide on reflux esophageal damage may be involved in inhibition of IL-1β production in blood.

Surface stimulation by particulate or soluble agents leads to the elevation of $[Ca^{2+}]i$ in neutrophils (Goldstein et al, 1975). A rise in cytosolic calcium level is thought to play an important role in the activation of PMNs responses. The elevation of $[Ca^{2+}]i$ is attained by both release of calcium from the intracellular stores and calcium influx for the extracellular medium (Westwich & Poll, 1986). InsP3 (inositol triphosphate) mediates the release of calcium from the intracellular stores. The InsP3 activates specific calcium channels localized in the membrane of intracellular stores (Berridge, 1993). The channels appear to be regulated by calcium, ATP and probably protein kinases (Zhang et al, 1993). Intracellular calcium level of PMNs was significantly increased by the addition of fMLP. Role of defibrotide in the elevation of $[Ca^{2+}]i$ in PMNs activated by fMLP was investigated. Defibrotide inhibited the elevation of $[Ca^{2+}]i$ by fMLP. Thus, fMLP-induced elevation of $[Ca^{2+}]i$ may be regulated by defibrotide.

In summary, out results are the first to show an esophgeal protective effect of defibrotide on surgically induced reflux esophagitis. These esophageal protective effects appear partly to be related to the attenuation of lipid peroxidation and MPO activity, the inhibition of PMN adherence to superior mesenteric artery, the inhibition of superoxide and hydrogen peroxide generation in PMNs, the scavenge of hydrogen peroxide, the inhibition of inflammatory cytokine (i.e., IL-1β), and the inhibition of intracellular calcium mobilization in PMNs.

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