Role of modulation of vascular endothelial growth factor and tumor necrosis factor-alpha in gastric ulcer healing in diabetic rats

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ABSTRACT

The aim of the present study was to assess the effect of drugs that increase gastric vascular endothelial growth factor (VEGF) and suppress gastric tumor necrosis factor-alpha (TNF-α) in gastric ulcer healing in streptozotocin-induced diabetic rats. Sixty male albino rats were made diabetic by intraperitoneal (i.p.) streptozotocin injection and ten rats were injected i.p. by a single dose of saline. Six weeks following streptozotocin or saline injection, gastric ulcers were induced by serosal application of acetic acid. Three days after acetic acid application, rats were divided into: group I (non-diabetic control), group II (streptozotocin-injected), groups III–VII (streptozotocin-injected rats treated with insulin, insulin and pentoxifylline, insulin and simvastatin, pentoxifylline as well as simvastatin, respectively, for 7 days following acetic acid application. The use of insulin, combinations of insulin and pentoxifylline or simvastatin resulted in a significant decrease in gastric ulcer area, significant increase in epithelial regeneration assessed histologically, significant increase in gastric VEGF concentration, and gastric von Willebrand factor (vWF) as well as significant decrease in gastric TNF-α. A significant difference in gastric ulcer area as well as in gastric TNF-α, VEGF and vWF levels could be observed between rats that received combinations of insulin and pentoxifylline or simvastatin compared to rats that received either drug alone. Our results suggest the feasibility of a novel treatment strategy, namely pentoxifylline and simvastatin, for patients in whom impairment of ulcer healing constitutes a secondary complication of diabetes mellitus.

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1. Introduction

Ulcer healing is a complex process that seems to be modulated by several growth factors [1]. Angiogenesis is a critical component of the ulcer healing process, because it enables delivery of oxygen and nutrients to the healing site and is regulated by proangiogenic factors, including vascular endothelial growth factor (VEGF), and by antiangiogenic factors, such as endostatin. An imbalance in the production of antiangiogenic vs. proangiogenic factors could result in impaired angiogenesis and wound healing, as has been suggested to occur in diabetes mellitus [2]. On the other hand, a shift in the production of angiogenic factors in favor of those that promote angiogenesis could result in accelerated ulcer healing [3].

It has been reported that peptic ulcers occurring in the course of diabetic state are more severe and often associated with complications such as gastrointestinal bleeding [4]. The mechanism underlying the increased susceptibility of gastric mucosa in diabetic animals to damage is multifactorial and includes the attenuation of angiogenesis as well as the increased production of

proinflammatory cytokines, e.g. necrosis factor-alpha (TNF-α) as well as interleukin-1 beta (IL-1β) [5].

Studies showed that diabetes delays ulcer healing due to the significant reduction in the gastric microcirculation around the ulcer, possibly involving the increased release of proinflammatory cytokines such as TNF-α [5,6]. In addition, the hyperglycemia and increased production of proinflammatory cytokines such, as IL-1β and TNF-α result in sustained inflammatory reaction and thus delaying healing process at the ulcer area [5].

There are also reports of attenuation of VEGF in diabetes mellitus [7]. VEGF is the most important angiogenic factor that is known to play a major role in many repair processes such as healing of gastric ulcers [8].

A number of drugs have been reported to modulate the expression of TNF-α and VEGF and thus might be expected to affect the healing process of gastric ulcers in diabetes mellitus. Among the drugs reported to decrease TNF-α is pentoxifylline, which is a phosphodiesterase inhibitor that increases intracellular cyclic adenosine monophosphate (cAMP), through inhibiting its enzymatic degradation by phosphodiesterase enzyme [9]. Several observations indicate that cAMP acts on multiple targets in inflammatory cells signaling pathway and thereby regulates the production of various cytokines [10].

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Hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, also called statins, are recently reported to exert an angiogenic effect in ischemic tissues [11]. It has been reported that statins increase VEGF release and modulates VEGF receptor-2 expression [12]. Statins have been also reported to reduce TNF-α production [13].

Accordingly, the aim of the present study was to assess the changes in TNF-α and VEGF level in the gastric mucosa of streptozotocin-induced diabetes mellitus in rats in which gastric ulcers were induced using the acetic acid method. The later is a model that easily and reliably produces round, deep ulcers in the stomach, and highly resembles human ulcers in terms of both pathological features and healing process [14]. The role of pentoxifylline and simvastatin, an HMG-CoA reductase inhibitor, in the healing of gastric ulcers in streptozotocin-induced diabetes mellitus in rats was assessed. The effect of the studied drugs on healing of gastric ulcer (assessed by gastric ulcer area), angiogenesis (assessed by measuring Von Willebrand Factor [vWF] level) as well as levels of VEGF and TNF-α was determined.

2. Materials and methods

2.1. Animals

The present study was conducted on seventy male Wistar albino rats weighing from 150 to 200 g. The rats were obtained from Pharmacology Department-Faculty of Medicine-Alexandria University. The rats were housed under the same environmental conditions, fed normal laboratory diet and they had free access to water. All experiments were performed in accordance with national animal care guidelines and were pre-approved by Faculty of Medicine-Alexandria University Ethics Committee.

2.2. Chemicals

All chemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, Mo., USA).

2.3. Drugs and treatments

Ten rats were given saline intraperitoneally (i.p.) and served as control. Sixty rats were rendered diabetic by a single i.p. injection of streptozotocin dissolved in saline in a dose of 55 mg/kg b.wt [15], and 2 days later, successful induction of diabetes mellitus was confirmed by tail blood glucose (BG) measurement, using a reflectometer device (Lifescan, Milpitas, CA).

2.4. Induction of gastric ulcers

Six weeks after saline or streptozotocin injection, all rats were fasted for 18 h. Rats were anaesthetized, using pentobarbital, and gastric ulcers were induced using a modified acetic acid method originally proposed by Okabe et al. [16]. Briefly, the stomach was exposed and a round plastic mold (6 mm in diameter) was placed tightly on the anterior serosal surface of the stomach at the antro-oxyntic border and 75 μl of 100% acetic acid was poured into the mold and allowed to remain on the gastric wall for 25 s. Previous studies documented that these ulcers became chronic within 2–3 days and healed completely within 2–3 weeks [17]. After the application of acetic ulcers, the animals were allowed to recover from anesthesia and received only water at the day of operation.

2.5. Animal grouping

Beginning on day 3 and continuing for 7 days following acetic acid application, the rats were divided into 5 groups of 10 rats each:

Group I: (non-diabetic vehicle-treated control) rats injected by a single i.p. injection of normal saline and received daily subcutaneous (s.c.) injection of 1 ml saline and daily oral 1 ml 2%gum acacia, beginning on day 3 and continuing for 7 days following acetic acid administration.

Group II: (diabetic vehicle-treated control) rats that were rendered diabetic by a single i.p. injection of streptozotocin and received s.c. injection of 1 ml saline and daily oral 1 ml 2% gum acacia, beginning on day 3 and continuing for 7 days following acetic acid administration.

Group III: (insulin-treated) streptozotocin–diabetic rats treated daily with insulin (Insulatard HM, 100 UI/ml, Novo Nordisk, AIS, 2880 Bagsvaerd, Danemark) in a dose of 5 IU/kg s.c. [18], and received 1 ml gum acacia daily orally, beginning on day 3 and continuing for 7 days following acetic acid administration.

Group IV: (insulin- and pentoxifylline-treated) streptozotocin–diabetic rats treated daily with insulin in a dose of 5 IU/kg s.c. and pentoxifylline, suspended in 2% gum acacia, in a dose of 20 mg/kg daily orally [19] beginning on day 3 and continuing for 7 days following acetic acid administration.

Group V: (insulin- and simvastatin-treated) streptozotocin–diabetic rats treated daily with insulin in a dose of 5 IU/kg s.c. and simvastatin (Zocor–MSD-USA), suspended in 2% gum acacia, in a dose of 10 mg/kg daily orally [20] beginning on day 3 and continuing for 7 days following acetic acid administration.

Group VI: (pentoxifylline-treated) streptozotocin–diabetic rats treated with pentoxifylline, suspended in 2% gum acacia, in a dose of 20 mg/kg daily orally [19] beginning on day 3 and continuing for 7 days following acetic acid administration.

Group VII: (simvastatin-treated) streptozotocin–diabetic rats treated with simvastatin (Zocor–MSD-USA), suspended in 2% gum acacia, in a dose of 10 mg/kg daily orally [20] beginning on day 3 and continuing for 7 days following acetic acid administration.

2.6. Ulcer and biochemical measurements

Ten days following gastric ulcer induction, blood samples were collected from the retro-orbital venous plexus of the rat, in tubes containing EDTA. Tubes were immediately centrifuged at $3000 \times g$ for 8 min for separation of plasma that was stored at $-20\,^\circ$C for determination of plasma glucose [21] then rats were euthanized and the stomach was isolated from each rat and used for:

I-Evaluation of ulcer area by planimetry [17].
II-Histological evaluation of ulcer healing: Ulcer specimens were fixed in 10% buffered formalin and embedded in paraffin. Tissues were sectioned in 4 μm thickness for haematoxylin and eosin (HandE) staining.
III-Evaluation of angiogenesis in ulcer area by measuring gastric Von Willebrand Factor (vWF) level (as a reflection of angiogenesis) by Rat vWF enzyme linked immunosorbent assay (ELISA) Kit (Cusabio Biotech CO., LTD. Homogenates of gastric ulcer samples are added to the appropriate microtiter plate wells with a biotin-conjugated polyclonal antibody preparation specific for vWF and Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. Then a TMB (3,3′,5,5′ tetramethyl-benzidine) substrate solution is added to each well. Only those wells that contain vWF, biotin-conjugated antibody and enzyme-conjugated Avidin will exhibit a change in color. The enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450 ± 2 nm. The concentration of

Table 1

Area of gastric ulcers (mm^2), plasma glucose (mg/dl), gastric von Willibrand factor (vWF) (mg/g tissue wt), gastric tumor necrosis factor—alpha (TNF-α) (pg/mg protein) and gastric vascular endothelial growth factor (VEGF) (pg/mg tissue wt) concentrations (mean ± S.E.M.) of the studied groups, 10 days following gastric ulcer induction in rats.

Group	Group No	Area of gastric ulcers (mm^2)	Plasma glucose (mg/dl)	Gastric vWF (mg/g tissue wt)	Gastric TNF-α (pg/mg protein)	Gastric VEGF (pg/mg tissue wt)
Normal control $n = 10$	I	9.00 ± 1.60	102.56 ± 9.68	12.98 ± 1.92	4.91 ± 0.31	70.97 ± 2.87
Streptozotocin–diabetic $n = 8$	II	28.56 ± 4.93[a]	398.91 ± 23.86[a]	6.45 ± 0.45[a]	11.40 ± 0.81[a]	40.65 ± 6.39[a]
Streptozotocin–diabetic and insulin (5 U/kg) $n = 9$	III	19.12 ± 3.12[a,b]	197.45 ± 19.46[a,b]	8.85 ± 1.69[a,b]	9.05 ± 2.70[a,b]	51.16 ± 8.31[a,b]
Streptozotocin–diabetic and insulin (5 U/kg) pentoxifylline (40 mg/kg) $n = 9$	IV	10.05 ± 0.98[b,c]	201.81 ± 15.47[a,b]	11.98 ± 2.87[b,c]	6.91 ± 0.49[a,b,c]	62.67 ± 7.15[b,c]
Streptozotocin-diabetic and insulin (5 U/kg) simvastatin (10 mg/kg) $n = 9$	V	9.30 ± 1.20[b,c]	203.5 ± 27.15[a,b]	12.32 ± 2.29[b,c]	7.08 ± 0.66[a,b,c]	64.54 ± 8.29[b,c]
Streptozotocin–diabetic and pentoxyfylline (40 mg/kg) $n = 9$	VI	21.08 ± 3.10[a,b]	379.3 ± 37.32[a,c]	7.12 ± 1.29[a]	8.28 ± 1.10[a,b]	45.21 ± 7.19[a]
streptozotocin–diabetic and simvastatin (10 mg/kg) $n = 10$	VII	20.60 ± 5.43[a,b]	373.5 ± 49.21[a,c]	7.32 ± 2.29[a]	8.95 ± 0.96[a,b]	47.54 ± 9.19[a]
F value		46.23	57.32	52.31	38.53	27.31
P		0.001	0.001	0.001	0.001	0.001

SEM = standard error mean; n = number of rats in each group.

[a] Significant compared to normal control group (I).

[b] Significant compared to non-treated streptozotocin–diabetic group (II).

[c] Significant compared to insulin-treated group (III).

vWF in the samples is then determined by comparing the optical density of the samples to the standard curve [22].

IV-Evaluation of Gastric TNF-α concentration by ELISA kit (Genzyme Diagnostics, Cambridge, Mass., USA). Briefly, gastric ulcer samples were homogenized in 100 mg sample/0.9 ml PBS (pH 7.4) containing 0.75 μg/ml PMSF, 1 μg/ml leupeptin and 5 μg/ml aprotinin for 30 s. They were then centrifuged at 12,000 rpm for 20 min. 100 μl of the supernatant was added to the 96-well microtiter plate precoated with monoclonal anti-mouse TNFα antibody and incubated for 2 h at 37 °C. After thorough washing, the substrate solution was added. Color development was allowed for 10 min and the reaction was stopped by application of stop solution. Color absorbance was read in an MRX microplate reader (Dynex Technologies, Chantilly, VA, USA) at 450 nm. The amount of protein in the sample was determined using the Lowry method, and the mucosal TNFα level was expressed as picograms per milligram protein [23,24].

V- Evaluation of Gastric VEGF concentration: Part of the gastric ulcer margin was homogenized in 1% phosphate buffer saline (PBS) containing 50 μg/ml heparin using Mikro-Dismembrator(-Braun). The homogenates were centrifuged for 10 min, and supernatant fractions were collected. ELISA for VEGF protein in supernatant fluid was performed using Quantikine M-Mouse VEGF Immunoassay kit (RandD Systems, Minneapolis, MN) [25].

All previous parameters were determined in the gastric tissue of the ulcer base.

2.7. Statistical analysis

Data were fed to the microcomputer program Statistical Package for Social Science SPSS version 17.0. Results were expressed as a mean ± S.E.M. Tabulation and analysis of data was done using analysis of variance (ANOVA) test. Significance of differences between the groups studied was determined with U Mann Whitney test. Statistically significant differences were assumed at P less than or equal 0.05 [26].

3. Results

3.1. Mortality

No rats died in the normal control group or in drug-treated group (VII), whereas two rats died in the non-treated streptozotocin group. One rat died in each of the drug-treated groups (III–VI).

3.2. Gastric ulcer area

A significant increase in gastric ulcer area, in group II compared to group I could be observed. Whereas, a significant lower mean value in gastric ulcer area in drug-treated groups (III–VII) vs. non-treated diabetic control could be observed. There was a significant difference in gastric ulcer area between rats that received combinations of insulin and pentoxifylline or simvastatin compared to rats that received insulin alone (Table 1).

3.3. Histopathological results

Histological examination of the stomach showed enhanced mucosal regeneration and increased blood vessels in the acetic acid induced ulcers in drug-treated groups (III–VII) compared to non-treated diabetic control (Fig. 1).

3.4. Biochemical results

3.4.1. Plasma glucose level

Intraperitoneal injection of streptozotocin-induced diabetes in rats, where the plasma glucose level was significantly increased in group II compared to the normal control group I. Drug-treated groups: III, IV, and V showed a significant decrease in plasma glucose concentration (Table 1). Whereas, no significant change in plasma glucose level was observed in groups treated with pentoxifylline or simvastatin alone (Table 1).

3.4.2. Gastric vWF concentration

A significant decrease in gastric vWF concentration in group II compared to group I could be observed. A significant higher mean value in gastric vWF concentration in drug-treated groups (III, IV and V) vs. non-treated diabetic could be observed. Whereas, no significant change in gastric vWF concentration was observed in groups treated with pentoxifylline or simvastatin alone (VI and VII). A significant difference in gastric vWF concentration was also found between the groups that received combination of insulin and pentoxifylline or simvastatin compared to the group that received insulin only (Table 1).

3.4.3. Gastric TNF-α concentration

A significant increase in gastric TNF-α in group II compared to group I could be also observed. A significant lower mean value in gastric TNF-α concentration could be observed in drug-treated groups (III–VII). A significant difference in gastric TNF-α concentration could be observed between rats that received combinations
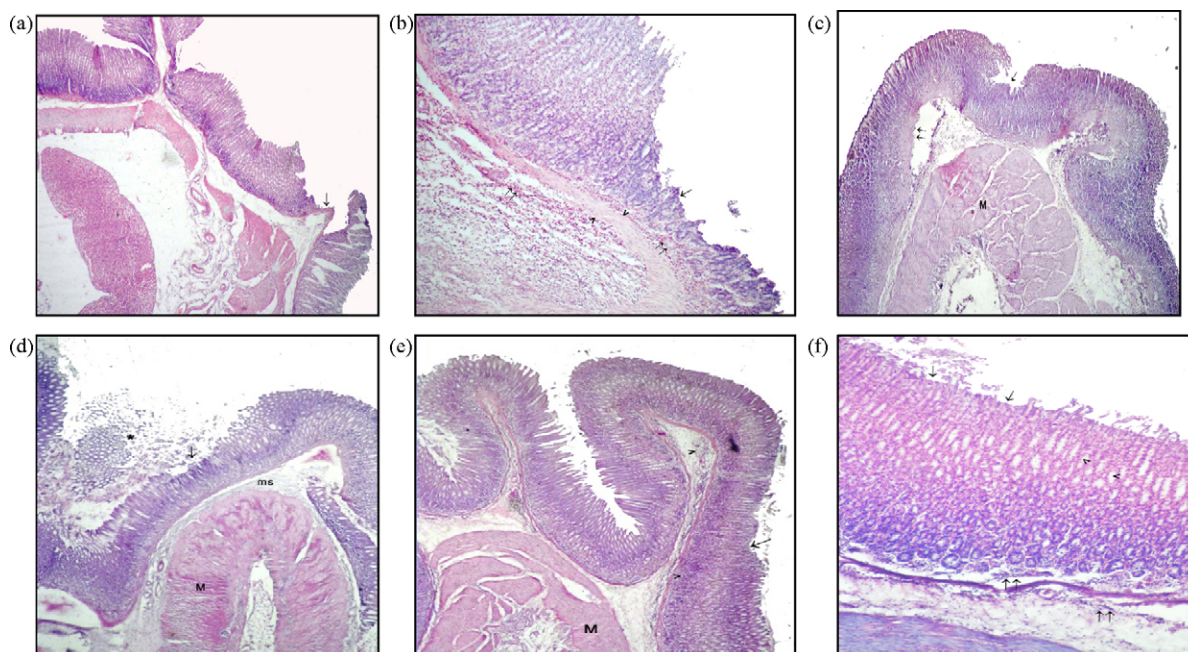
Fig. 1. Photomicrographs of acetic acid induced gastric ulcers in: (a) non-treated diabetic rats showing localized ulcer with loss of entire mucosa (↑) exposing the muscularis mucosa. (b) Insulin-treated diabetic rats showing increased epithelial regeneration (↑) and marked increase in blood vessels (↑↑) and increased thickness of muscularis mucosa (^). (c) Pentoxifylline-treated diabetic rats showing some epithelial regeneration (↑) with increased muscularis mucosal thickness (↑↑). (d) Simvastatin-treated diabetic rats showing increased epithelial regeneration (↑). (e) Insulin and pentoxifylline-treated diabetic rats showing increased epithelial regeneration (↑). (f) Insulin and simvastatin-treated diabetic rats showing increased epithelial regeneration (↑) and increased muscularis mucosal thickness (↑↑). HandE stain. Mic. mag.: 100×.

of insulin and pentoxifylline or simvastatin compared to rats that received insulin only (Table 1).

3.4.4. Gastric VEGF concentration

A significant decrease in gastric VEGF concentration in group II compared to group I could be observed. A significant higher mean value in gastric VEGF concentration in drug-treated groups (III, IV and V) vs. non-treated diabetic could be observed. Whereas, no significant change in gastric VEGF concentration was observed in groups treated with pentoxifylline or simvastatin alone (VI and VII). A significant higher mean in gastric VEGF concentration could be observed between rats that received combinations of insulin and pentoxifylline or simvastatin compared to rats that received insulin only (Table 1).

4. Discussion

Our present study showed that healing of gastric ulcers induced by acetic acid was delayed in streptozotocin-induced diabetic rats. This confirmed the earlier observations of increased propensity to ulceration in both experimental and clinical diabetes [27].

Diabetes mellitus being a chronic disease, may lead to a decrease in the mucosal defensive factors with a concomitant increase in propensity to ulceration in response to various factors. A significant increase in gastric TNF-α concentration was found in non-treated streptozotocin–diabetic compared to normal control. TNF-α is a proinflammatory cytokine that has been reported to be triggered by chemical injury of the gastroduodenal mucosa e.g. by acetic acid. This cytokine stimulates caspase-3 in epithelial and endothelial cells of gastric mucosa and thus contributes to apoptosis and subsequent damage [28].

The present study demonstrated a significant decrease in angiogenic response of gastric mucosa of streptozotocin–diabetic rats to chemical injury induced by acetic acid, evidenced by a significant decrease in gastric vWF level, accompanied by a significant decrease in gastric VEGF level, compared to normal control. These results are in accordance with a study reporting that gastric mucosal blood flow is decreased in streptozotocin–diabetic rats [29]. Similarly, it has been previously shown that diabetes mellitus is associated with attenuated cardiac VEGF expression, which may decrease capillary density in the myocardium [30].

Healing of mucosa damage in gastric ulcer occurs by at least two different mechanisms. Initially, the rapid process of mucosal restitution or reepithelization takes place by migration of surrounding epithelial cells from the ulcer margin to cover the denuded area. Secondary to that is the replacement of lost cell by cell proliferation. Other mechanism involved in the healing of gastric ulcer seems to be related to the increase in mucosal blood flow. Mucosal blood flow plays an important role in the protection of gastroduodenal mucosa against damage. In the stomach, numerous experimental studies have shown that exposure of gastric mucosa to potentially noxious environment results in little or no damage, as long as adequate blood flow is maintained; whereas reduction in mucosal blood flow leads to severe gastric injury. Protective effect of adequate blood flow depends on supplying the mucosa with oxygen, bicarbonate and nutritious substances, and removing carbon dioxide, hydrogen ions and toxic agents diffusing from the gastric lumen [31]. Thus induction of an angiogenic factor, e.g. VEGF that increases mucosal blood flow can accelerate healing of gastric ulcer.

During the gastric ulcer healing process, the proliferation and migration of gastric epithelial cells are regulated by cytokines, e.g. TNF-α [32].

In the current study, daily injections of insulin to the diabetic rats, while reducing blood glucose, counteracted in part, the delay in ulcer healing. Daily administration of pentoxifylline or simvastatin resulted in a significant decrease in ulcer area which could be attributed to a decrease in gastric TNF-α. Combining pentoxifylline or simvastatin with insulin enhanced the insulin-induced acceleration of ulcer healing and the accompanying rise in the gastric vWF and VEGF as well as further decreasing gastric TNF-α.

Insulin-induced normalization of elevated plasma glucose level could account, in part, for insulin-induced increase in gastric VEGF.

This is based on the reported studies that hyperglycemia suppresses VEGF [33,34]. Induction of VEGF expression by insulin has been also reported in several cell types, including vascular smooth muscle cells, epithelial cells, and fibroblasts [35]. It has been also reported that decreased expression of VEGF and its receptors in the myocardium can be normalized by insulin treatment [36].

The failure of pentoxifylline and simvastatin, when given alone, to result in any significant increase in gastric VEGF could be explained by their failure to decrease elevated plasma glucose level, these elevated blood glucose levels could abrogate any induction of VEGF exerted by either drug when given alone in absence of insulin. This is proven by the fact that giving either drug, in combination with insulin, enhanced the insulin-induced ulcer healing effect and this was accompanied by a significant increase in gastric VEGF, vWF as well as a significant decrease in gastric TNF-α.

Our results regarding the gastroprotective effect of either pentoxifylline or simvastatin are in accordance with other studies demonstrating gastroprotective effect of: pentoxifylline against induced gastric ulcers [37,38] and of simvastatin against indomethacin-induced gastric ulcers [39,40]. Indeed, results suggest that other TNF-α inhibitors play a critical role in the pathogenesis of NSAID-induced gastric injury. Thus, our results provide further evidence to support the protective effect of pentoxifylline and simvastatin on gastric ulcer, while proposing a new mechanism for how both drugs exert their function probably via induction of VEGF and suppression of TNF-α.

Pentoxifylline-induced significant decrease in gastric TNF-α as well as significant increase in gastric VEGF level could be explained by pentoxifylline-induced rise in cyclic cAMP. It has been reported that cAMP increases transcription of VEGF [41] and down regulates transcription of inflammatory cytokines, including TNF-α. A study conducted by Zhou et al. reported that pentoxifylline directly up-regulates the expression of VEGF mRNA by stabilization of its mRNA [42]. In addition, pentoxifylline regulates proinflammatory cytokine synthesis likely by attenuating p38 mitogen activated protein kinase (MAPK) activation [43].

Our results regarding simvastatin-induced acceleration of gastric ulcer healing in diabetes are in line with a study reporting that simvastatin enhances VEGF production and ameliorates impaired wound healing in experimental diabetes [44].

Multiple prior reports have provided evidence that statins induce VEGF release [45]. Data demonstrate that a cellular signal evoked by statins is transmitted to the nucleus to stimulate VEGF gene expression. Results also indicate that mevalonate depletion resulting from addition of statins, up-regulates VEGF transcription. Statins may also activate the VEGF gene through increased binding to specific elements of the VEGF promoter of activators for gene transcription such as hypoxia-inducible factor-2 [46].

The ability of simvastatin to inhibit the production of TNF-α could be explained, in part, by inhibiting de novo synthesis of TNF-α transcript [47].

In conclusion, the findings of the current study support the notion that impairment of healing of gastric ulcers in diabetes mellitus results from impairment of angiogenic response of the gastric mucosa to injury together with up regulation of gastric TNF-α. Our results suggest the feasibility of a novel treatment strategy, namely pentoxifylline and simvastatin, for patients in whom impairment of ulcer healing constitutes a secondary complication of diabetes mellitus. Findings of the current study may have implication for clinical use of drugs that induce VEGF or suppress TNF-α in patients with gastric ulcers complicating diabetes mellitus.

Acknowledgement

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