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Expression Pattern of Angiogenic Growth Factors at Early Stages of Experimental Gastric Ulcers Pathogenesis.

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ABSTRACT

Despite the fact that angiogenic pathways are involved in peptic ulcer development, their precise role in ulcers pathogenesis induced by different etiologic factors (e.g. stress, NSAIDs, alcohol) is poorly understood. The aim of present study was to investigate the changes in the expression of pro-angiogenic factors VEGF, bFGF and activation of Erk-1/2 kinase pathway during experimentally induced gastric ulcers of different genesis. Methods. Gastric ulcers were induced in male rats (180-230 g): 1) by immobilization stress combined with water-immersion for 20 min, 1 and 3 h duration; 2) by oral administration of ethanol (96%, 1ml) for 20 min and 1 h; 3) by oral administration of aspirin (10 mg/100g) for 20 min, 1 and 3 h duration. We found for the first time that pattern of VEGF and bFGF levels are differ among different experimental models of gastric ulcer. The common feature of stress, ethanol and aspirin-induced gastric lesions is early increase in VEGF levels. A gradual increase of bFGF levels were showed for stress and aspirin-induced gastric ulcers. Only stress-induced gastric ulcer development was associated with significant activation of Erk-1/2. Less profound changes were observed during aspirin-induced gastric lesions and no changes at early time points during ethanol-induced gastric ulcer.

Keywords: gastric ulcer, VEGF, bFGF, Erk1/2.

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INTRODUCTION

According to World Health Organization (WHO) peptic ulcer takes a leading place among the diseases of the gastrointestinal tract, and occur mostly in people of adult age [1, 2]. Poor diet, stress, alcohol or nonsteroidal anti-inflammatory drugs, infection with Helicobacter pylori, genetic predisposition are main etiologic factors of peptic ulcer development. Although there are many factors that can lead to ulceration of the stomach but not all people suffering from this pathology although it is known that in some patients, ulcers can self-heal [3]. Microvascular endothelial barrier is an important component of maintaining the mucosa integrity of the gastrointestinal tract through regulation the supply of nutrients, oxygenation and removal of toxic metabolites, obstacles to penetrations of circulating leukocytes and other inflammatory cells and proteins and the immune response through the synthesis of pro-inflammatory cytokines [4, 5]. Disturbance of the microvascular endothelial barrier which is observed in the pathogenesis of gastric and duodenal ulcers leads to changes in the regulation of cells transcription and as a result, expression of genes associated with the development of vascular pathology [6]. Healing of ulcers is a process of tissue regeneration, which involves cell migration, proliferation, re-epithelialization, gland reconstruction and angiogenesis.

These processes are regulated by hormones, cytokines, transcription and growth factors [7]. Vascular endothelial growth factor (VEGF) is the key pro-angiogenic growth factor which is important for the process of neovascularization. Increased expression of VEGF in patients with dyspepsia allowed to suggest its involvement in gastric ulcer development . On the other hand, VEGF expression is necessary for the healing of gastric ulcers caused by the NSAIDs [8]. Basic fibroblast growth factor (bFGF) is other angiogenic growth factor participated in ulcer healing [7].

Treatment with recombinant bFGF improved healing of NSAIDs - associated gastric ulcers [9]. Erk-1/2(extracellular signal-regulated kinase) has been activated during chronic ulcer disease while VEGF and bFGF expression is increased [10]. Dellinger et al [11] demonstrated that activation of Erk-1/2 lead to VEGF expression and as a result to proliferation and migration of lymphatic endothelium. Erk-1/2 also regulates expression of bFGF in Müller cells [12]. Despite the fact that angiogenic pathways are involved in peptic ulcer development, their precise role in ulcers pathogenesis induced by different etiologic factors (e.g. stress, NSAIDs, alcohol) is poorly understood.

The aim of present study was to investigate the changes in the expression of pro-angiogenic factors VEGF, bFGF and activation of Erk-1/2 kinase pathway during experimentally induced gastric ulcers of different genesis.

MATERIALS AND METHODS

Animals

Wistar male rats (180-230 g) (Animal Research Facility, KNU, Kyiv, Ukraine) were used for these experiments. These studies were approved by the Institutional Animal Care and Use Committee of Taras Shevchenko National University of Kyiv, Ukraine (protocol # 0109U005336). Animals were housed in the animal research facility under standard environmental conditions (12:12 h light-dark cycle starting at 6 a.m.; temperature 21-23ºC; humidity 30-35%). All animals had unlimited access to Purina chow and tap water. Rats were euthanized by CO2 inhalation with subsequent cervical dislocation according to the guidelines of the Institutional Animal Care and Use Committee.

Experimental models of gastric ulcer

Before modeling of the gastric ulcers, rats were fasted for 18 hr, but had unlimited access to water. The gastric ulcer by *immobilization stress combined with water-immersion* (IMO-WI) was performed according [13]. Rats (*n* = 5/group) were immobilized in metal tubes with continuous holes, adjusted to body size. Metal tubes were placed in upper-head position in plastic container filled with tap water (T=21-22 °C) by the level of rat's neck to prevent animal's drown. Rats were euthanized after 20 min, 1 or 3 hr of IMO-WI. Control group was not subjected to any manipulation. To model *aspirin-induced gastric ulcer*, rats (*n* = 5/group) were given acetylsalicylic acid ("Darnitsa", Ukraine) at a dose 10 mg/100 g dissolved in 0.2 N hydrochloric acid by gavage once and euthanized 20 min, 1 or 3 h later. The control rats were given 0.5 ml dH2O by gavage. To model

ethanol-induced gastric ulcer, rats (*n* = 5/group) were given either 1 ml of 96% ethanol or 1 ml dH2O by gavage once and euthanized 20 min or 1 h later.

At the autopsy, the stomach was removed, cut along the lesser curvature, thoroughly rinsed in cold PBS, gently wiped by paper towel and quickly estimated for macroscopic lesions. After that, stomach was flat by the mucosa side up on ice and gently scraped from the muscular layer using metal spatula. Scraped mucosa was weighted and dipped in liquid nitrogen.

Western blot

The concentration of total proteins extracted from gastric mucosa in a lysis buffer (0,1% SDS, 1% Triton x100, 2 µM PMSF) containing protease inhibitor cocktail (16.65 ml) and phosphatase inhibitor (1 µM sodium orthovanadate) (Sigma-Aldrich, Germany) was measured by the «Bio-Rad protein for analysis» based on the Bradford assay (Bio-Rad, USA). Separation and identification of proteins (50, 75 or 100 µg total protein/sample) were performed by Western blot in 12%, 10% or 8% SDS polyacrylamide gel, followed by transfer to nitrocellulose membrane (Hybond ECL™, Amershame) according to standard protocol of Bio-Rad. The primary antibodies were used against VEGF (1:1000), bFGF (1:1000), Erk-1 (1:1000), Erk-2 (1:1000), phospho-ErkTyr204 (1:1000), β-actin (1:1000) (Santa-Cruz Biotech., Santa Cruz, CA), followed by incubation with secondary anti-rabbit or anti-mouse antibodies conjugated with horseradish peroxidase (1:2500, Santa-Cruz Biotech., Santa Cruz, CA). Visualization was performed by Western blot ECL-reagent. Every Western blot was repeated at least two times.

Statistical analysis

Quantitative results are expressed as mean ± SD. The statistical significance was determined by the non-parametric Mann-Whitney U-test, or Student's t-test where appropriate, and p values of <0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Expression pattern of VEGF, bFGF and Erk1/2 activation at early stages of IMO-WI-induced gastric ulcers development

Development of IMO-WI-induced gastric lesions was accompanied by increase 2.7- and 1.8-fold (P<0.05) VEGF level in gastric mucosa after 20 min and 1 h respectively. After 3 h of IMO-WI stress, when macroscopic gastric lesions were identifiable, VEGF level returned to the control value (Fig 1A). The gastric level of bFGF gradually increased and reached a significant difference after 1 h (1.4-fold, P <0.01) and 3 h (1.6-fold, P <0.01) of IMO-WI stress (Fig. 1B). MAP kinases mediate the effects of VEGF and bFGF on proliferation and migration of endothelial cells [14]. IMO-WI stress for 20 min, 1 and 3 h increased 2.2-, 2.6- and 2.5-fold (P<0.05) levels of phosphorylated form of Erk1/2 (pErk1/2) in gastric mucosa, while levels of total proteins Erk1/2 were unchanged (Fig. 1C).

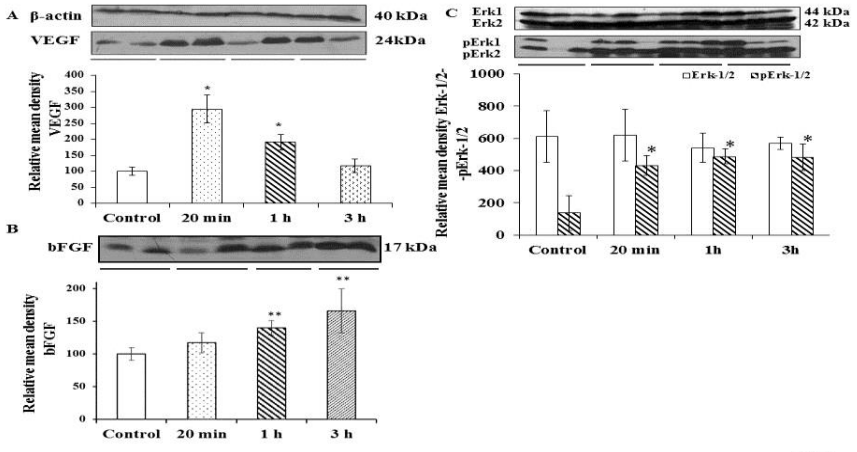

Fig 1

Fig 1 Changes in VEGF (A), bFGF (B) protein expression and altered activation of Erk1/2 (C) in rat gastric mucosa during immobilization stress combined with water-immersion. Expression was determined by Western blot. β-actin levels were used as loading controls. Assays were repeated 3 times with highly reproducible results using proteins from 3 different animals. Results are expressed as mean±SD. Statistical significance was calculated *vs*. control group of rats.

VEGF is a potent vascular permeability factor which is 50000 times stronger than histamine [15]. Szabo et al [16] found that increased vascular permeability and morphologically detectable vascular lesions consistently preceded the development of grossly visible hemorrhagic erosions during experimental gastric ulcers. They concluded that early increase vascular permeability at the site of injury plays the protective role by dilution noxious agent (e.g. ethanol). Rapid increase of VEGF level at early time points with its gradual downregulation with IMO-WI stress gastric ulcer development might be explained by its protective role as vascular permeability factor. The gradual increase in bFGF level which is associated with more severe gastric lesions during IMO-WI stress might be result of tissue remodeling and fibroblast activation which is main feature of inflammatory process. Szabo et al [17] demonstrated decreased expression of VEGF, bFGF and PDGF during cysteamine-induced duodenal ulcer. High levels of pErk-1/2 have been demonstrated during chronic gastric ulcers in mice [18]. Moreover, these pathway is involved in effects of VEGF and bFGF [19]. In present study, activation of Erk1/2 was associated with development of stress-induced gastric ulcers in rats and changes in VEGF and bFGF levels.

Expression pattern of VEGF, bFGF and Erk1/2 activation at early stages of ethanol-induced gastric ulcers development

Development of ethanol-induced gastric lesions was accompanied by rapid and sustained 1.5-fold ($P<0.05$) increase in VEGF level in gastric mucosa (Fig 2A). Similar data were showed by Jones et.al. [20] after 3, 6 and 24 h ethanol action.

Fig 2 Changes in VEGF (A), bFGF (B) protein expression and altered activation of Erk1/2 (C) in rat gastric mucosa during ethanol-induced gastric ulcer. Expression was determined by Western blot. β-actin levels were used as loading controls. Assays were repeated 3 times with highly reproducible results using proteins from 3 different animals. Results are expressed as mean±SD. Statistical significance was calculated *vs*. control group of rats.

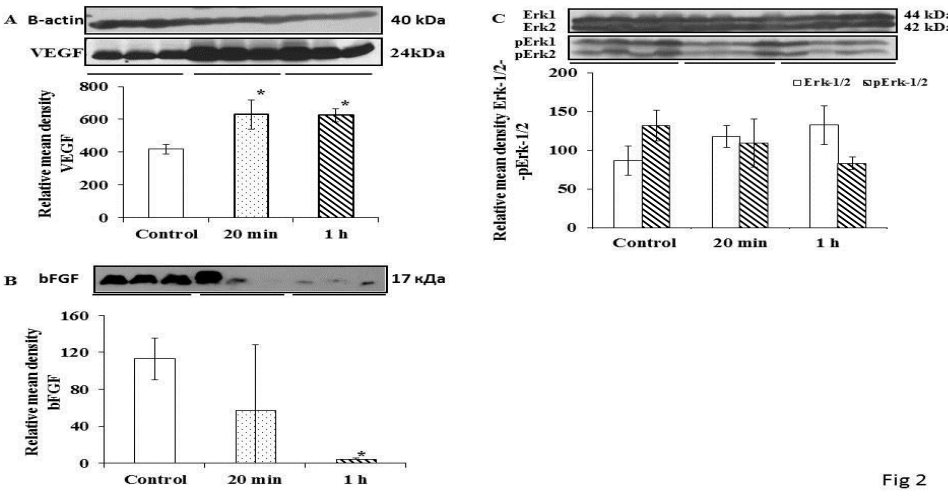

Fig 2

Unlike VEGF, levels of bFGF significantly decreased at early stages of ethanol- induced gastric ulcer. In 1 h after ethanol gavage, when 100% animals had gastric ulcers, levels of bFGF was 22.3-fold lower *vs.* control group ($P<0.05$) (Fig. 2B). Nakamura et al [21] demonstrated, that bFGF expression in rat gastric mucosa was decreased after 3 and 12 h ethanol action.

Development of ethanol-induced gastric ulcer didn't affect levels of total Erk1/2 as well as its phosphorylated form in rat gastric mucosa (Fig 2C). These data correlates with results of Luo et al [22]. They

found, that administration of 30% ethanol didn't change levels of Erk-1/2 phosphorylation in rats gastric mucosa at 1 h.

Expression pattern of VEGF, bFGF and Erk1/2 activation at early stages of aspirin-induced gastric ulcers development

Development of aspirin-induced gastric ulcers was associated with gradual increase in VEGF levels, with significant changes in 1 and 3 hr, 1.5- and 2-fold respectively (P<0.05) (Fig 3A). Similarly, Gyenge et al [23] showed changes in VEGF expression during indomethacin-induced gastric ulcer in rats.

Fig 3 Changes in VEGF (A), bFGF (B) protein expression and altered activation of Erk1/2 (C) in rat gastric mucosa during aspirin-induced gastric ulcer. Expression was determined by Western blot. β-actin levels were used as loading controls. Assays were repeated 3 times with highly reproducible results using proteins from 3 different animals. Results are expressed as mean±SD. Statistical significance was calculated *vs*. control group of rats.

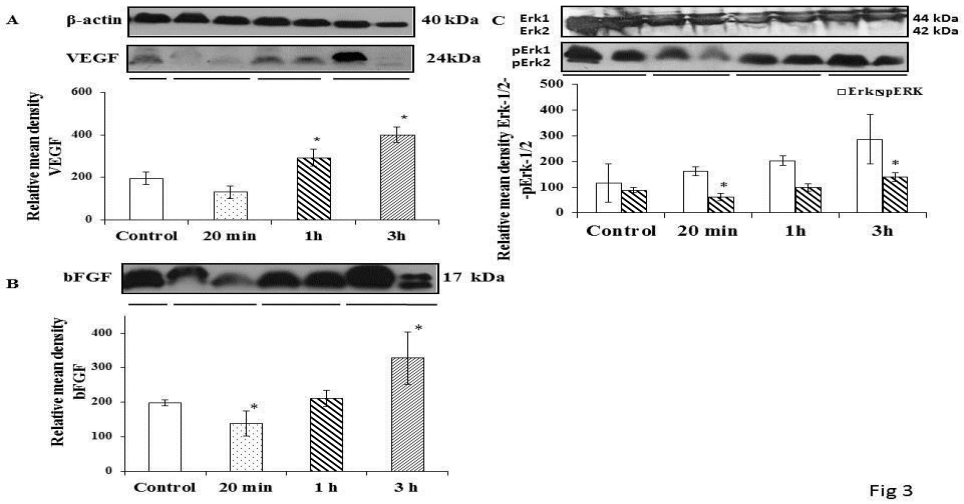

Fig 3

Levels of bFGF in rat gastric mucosa was lower by 1.4-fold *vs.* control group (P <0.05) in 20 min of aspirin administration. In 1 h, its level returned to the control value and was significantly increased 1.7-fold (P <0.05) in 3 h (Fig 3B). It has been demonstrated that oral or parenteral administration of bFGF led to accerilation of the healing of aspirin-induced gastric ulcers in rats [24].

The levels of total protein Erk-1/2 was unchanged in rat gastric mucosa during aspirin-induced gastric lesions. The levels of phosphorylated forms of Erk-1/2 were decreased by 1.4-fold (P<0.05) in 20 min after aspirin gavage, in 1 h - returned to the control value. In 3 h after aspirin gavage, levels of pERK-1/2 increased by 1.6-fold (P<0.05) (Fig 3C). Liu et al [25] showed decreased levels of total Erk-1/2 and its phosphorylated form in vascular endothelial cell after aspirin action. Unlike, Ganguly et al [18] demonstrated elevated Erk-1/2 phosphorylation during indomethacin-induced chronic gastric ulcers.

CONCLUSIONS

Development of experimental gastric ulcers are accompanied by significant changes in levels of angiogenic growth factors VEGF and bFGF. We found for the first time that pattern of VEGF and bFGF levels are differ among different experimental models of gastric ulcer. The common feature of stress, ethanol and aspirin-induced gastric lesions is early increase in VEGF levels. Since, VEGF is a potent vascular permeability factor, its upregulation at early time points might be the part of protection mechanism associated with acute inflammatory response. A slow increase of the bFGF levels was showed for stress and aspirin-induced gastric ulcers. It might be result of tissue remodeling and fibroblast activation which is the main feature of the inflammatory process. Opposite pattern was observed for ethanol-induced gastric ulcers. It might be explained by more rapidly developed, severe lesions with cells destruction after ethanol gavage. Effects of bFGF and

VEGF on endothelial cells might be mediated by Erk-1/2 MAP kinase. In present study, only stress-induced gastric ulcer development was associated with significant activation of Erk-1/2. Less profound changes were observed during aspirin-induced gastric lesions and no changes at early time points during ethanol-induced gastric ulcer. Thus, the involvement of Erk-1/2 MAP-kinase pathway in regulation of angiogenic factors effects depends on the nature of gastric ulcers. Investigation of bFGF, VEGF role and Erk-1/2 MAP-kinase activation will allow to understand the molecular mechanisms of development and healing of gastric ulcers with different genesis.

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