Angiogenesis and VEGF play a major role in many repair processes such as healing of gastric ulceration. The present study was undertaken to assess the dynamics of changes in VEGF expression and angiogenesis in stress-induced gastric ulcers in rats. Acute gastric ulceration was induced using a water-immersion and restraint stress method. The VEGF expression, angiogenesis, size of area and depth of ulcers in gastric specimens were evaluated. The study shows that as early as one day after the development of ulcers there is a significant increase in both the expression of the VEGF protein and the number of newly formed microvessels, while an abrupt decrease in VEGF expression, observed on the fifth day, results in a decreased intensity of angiogenesis. Moreover, it has been demonstrated that the increase in VEGF expression and angiogenesis is accompanied by a reduction in the size of area and depth of stress-induced ulcers in rats. Six days after ulcer development both VEGF expression and angiogenesis return to normal levels.

Key words: angiogenesis, VEGF gastric ulcer, stress

INTRODUCTION

The vascular system develops as a result of two processes: embryonic vasculogenesis and extraembryonic angiogenesis (1). Physiological neovascularization occurs during growth and development of the organism, e.g. in the course of pregnancy and lactation, tissue differentiation in embryogenesis or wound healing, as well as during tissue regeneration. The cause of capillary proliferation is the necessity to respond to increasing demands for oxygen (2).
Angiogenesis plays an important role in many diseases (3-5). Reduction in the number of capillaries occurs only in some pathological states with the growth of the connective tissue which is known to require small amounts of oxygen and nutrients, e.g. in cirrhosis of the liver, scleroderma, pulmonary fibrosis, and scarring (2). Endogenous regulation of angiogenesis is possible due to the balance between stimulatory (angiogenic) and inhibitory (angiostatic) factors.

Vascular endothelial growth factor (VEGF) is the most important and best recognized angiogenic factor. It is produced by endothelial cells, fibroblasts, macrophages, smooth muscle cells, and neoplastic cells, and is involved in both physiological and pathological regulation of angiogenesis (6). It presents all the angiogenic factor properties, i.e. it has specific receptors on endothelial cells and its presence enhances angiogenesis while its absence suppresses the process (7,8). Initially, VEGF was identified as a vascular permeability factor (VPF) (9,10). Factors which stimulate VEGF expression include hypoxia and ischemia, interleukin-1, endothelin-1, cAMP, CA++ ions, phorbol esters, cytokines, steroid hormones, and heavy metals (11). The most potent of these are hypoxia and ischemia which cause an increase in the expression of both the growth factor and its receptors (12). In contrast, actinomycin D, CO, and cGMP are among the factors which inhibit VEGF production (13).

Angiogenesis and VEGF play a major role in many repair processes such as healing of gastric ulceration resulting from a disturbed balance between factors which damage the gastric mucosa barrier and those which have a protective role.

Spontaneous gastric ulcer healing is a complicated, multistage process involving granulation, angiogenesis and endothelial regeneration. Studies in animals have demonstrated that angiogenesis guarantees a high quality of the process by providing the damaged area with oxygen and nutrients (14). Restoration of adequate blood supply to the area of ulceration is of particular importance since a correlation has been found between a reduced mucosal blood flow and the development of gastric ulcers (15-17). Carried studies also revealed that disturbances in blood perfusion of gastric mucosa may result in the formation of erosions and ulcers (18-20). Local ischemia of the gastric mucosa is regarded as one of the main pathomechanisms of stress-induced ulceration in this region.

The present study was undertaken to assess the dynamics of changes in VEGF expression and angiogenesis in stress-induced gastric ulcers in rats.

MATERIALS AND METHODS

Animals

Sixty-four adult male Wistar rats (225-250g) were used. The animals were kept under standard conditions, 12h light/darkness cycle, one animal per cage, with free access to standard diet and water. The experiment was approved by the University Bioethics Committee (No 13/03, April 30, 2003).
**Induction of gastric ulcers**

Acute gastric ulceration was induced using a water-immersion and restraint stress (WRS) method by Takagi et al. (21).

Twenty-four hours before the experiment, food, but not water, was withdrawn. The animals were randomly divided into eight groups (one control- K and seven experimental):

- **K** - control group,
- **Group 1** - animals killed and examined 1 day after termination of exposure to stress,
- **Group 2** - animals killed and examined 2 days after termination of exposure to stress,
- **Group 3** - animals killed and examined 3 days after termination of exposure to stress,
- **Group 4** - animals killed and examined 4 days after termination of exposure to stress,
- **Group 5** - animals killed and examined 5 days after termination of exposure to stress,
- **Group 6** - animals killed and examined 6 days after termination of exposure to stress,
- **Group 7** - animals killed and examined 7 days after termination of exposure to stress.

Acute gastric stress ulcers were induced by immobilization of the animals in cages and immersion them, up to the xiphoid process, in thermoregulated water bath at 23°C for 7h (7a.m.-2p.m.).

**Evaluation of VEGF expression**

VEGF protein was detected using monoclonal antibodies (VEGF[c-1] K: sc-7269K ImmunoCruz Staining System) against the protein. Mentioned antibodies are specific for VEGF and do not cross-react with other growth factors.

The number of cells with positive VEGF protein expression was assessed as 100 cells in three large visual fields.

**Evaluation of angiogenesis**

Measurements were made in paraffin sections. Angiogenic neovascularization was detected using antibodies against von Willebrand factor (DAKO Envision+System, Peroxidase (DAB)). Density of the newly formed vessels was expressed as the number of microvessels in 1 mm² of gastric area.

**Measurement of ulcer area**

The area of ulceration was measured with a planimetric method (22). For comparison between animals, an ulcer index was calculated based on the results obtained. It was expressed in per cent as the ulceration area / gastric glandular area ratio.

**Measurement of ulcer depth**

The depth of ulcers was assessed histopathologically.

**Statistical analysis**

The data were analyzed using Statistica v.6.0. Arithmetic means and standard deviations were calculated for each parameter. Kruskal-Wallis ANOVA test was used to determine the effect of time after stress termination on the parameters studied. Significance of differences between the groups studied was determined with U Mann Whitney test. In addition, correlations between the parameters were analyzed according to Spearman.
RESULTS

VEGF expression (Tab. 1, Fig. 1)

A rapid increase in VEGF expression was observed in Groups 1 and 2, i.e. one and two days after the development of ulceration. In Groups 3 and 4 (animals killed and examined respectively 3 and 4 days after ulceration), mean VEGF expression showed no statistically significant alterations and remained as high as that on the second day. On the fifth day (Group 5), there was an abrupt decrease in VEGF expression. Six days after ulceration, VEGF expression was similar to that in the control animals.

Tab. 1. VEGF expression (mean ± standard deviation) in gastric mucosa of control group of rats and rats exposed to 7.0 h of water immersion and restraint stress (WRS), killed and examined respectively 1,2,3,4,5,6 and 7 days after ulcer induction.

<table>
<thead>
<tr>
<th></th>
<th>K</th>
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<th>4</th>
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<tr>
<td>VEGF</td>
<td>3.53±2.10</td>
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<td>27.13±6.08</td>
<td>27.33±8.87</td>
<td>31.33±8.38</td>
<td>8.78±2.68</td>
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<td>2.06±1.48</td>
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<td>1</td>
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<td>4</td>
<td>&lt;0.01</td>
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<td>6</td>
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</tbody>
</table>

* p value at the intersection of rows corresponds to the comparison of the respective groups

● K - control group,
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● Group 2 - animals killed and examined 2 days after termination of exposure to stress,
● Group 3 - animals killed and examined 3 days after termination of exposure to stress,
● Group 4 - animals killed and examined 4 days after termination of exposure to stress,
● Group 5 - animals killed and examined 5 days after termination of exposure to stress,
● Group 6 - animals killed and examined 6 days after termination of exposure to stress,
● Group 7 - animals killed and examined 7 days after termination of exposure to stress.
Angiogenesis (Tab. 2, Fig. 2)

The number of blood vessels in 1 mm$^2$ of tissue increased until the third day of ulceration. In Group 4, the mean number of vessels remained the same as that in Group 3. A significant decrease was observed in Groups 5, 6, and 7 (animals killed and examined respectively on days 5, 6 and 7 following exposure to stress). Angiogenesis reached the control value six days after stress withdrawal.

Area and depth of ulceration

Ulcer depth (Fig. 3)

Stress-induced gastric ulcers developed in Groups 1-6. In animals killed seven days after exposure to stress, no ulceration was found either macroscopically or histopathologically.
The depth of ulcers (in mm) was the highest in Group 1 (killed and examined 1 day after termination of stress) (0.69±0.20) and decreased with time (Group 2 - 0.43±0.38, Group 3 - 0.36±0.23, Group 4 - 0.39±0.35, Group 5 - 0.27±0.14, Group 6 - 0.14±0.12).

Ulcer depth in relation to the thickness of mucosal lamina propria

Involvement of lamina propria was the highest in Group 1 (animals killed and examined 1 day after termination of stress) (0.81±0.14) and decreased with time (Group 2 - 0.50±0.44, Group 3 - 0.39±0.23, Group 4 - 0.46±0.32, Group 5 - 0.22±0.07, Group 6 - 0.15±0.12). Mean values in Group 4 (animals killed 4 days after ulceration) were higher than those in Group 3 (animals killed 3 days after

Tab. 2. Number of vessels (mean ± standard deviation) per 1 mm² in gastric mucosa of control group of rats and rats exposed to 7.0 h of water immersion and restraint stress (WRS), killed and examined respectively 1,2,3,4,5,6 and 7 days after ulcer induction.

<table>
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<tr>
<td>NV</td>
<td>19.94±3.73</td>
<td>27.24±5.31</td>
<td>37.58±15.49</td>
<td>59.58±12.56</td>
<td>58.71±14.20</td>
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- Group 4 - animals killed and examined 4 days after termination of exposure to stress,
- Group 5 - animals killed and examined 5 days after termination of exposure to stress,
- Group 6 - animals killed and examined 6 days after termination of exposure to stress,
- Group 7 - animals killed and examined 7 days after termination of exposure to stress.
Ulcer area (ulcer index) (Fig. 4)

Ulcer index was calculated for comparison of ulceration areas between the groups. The highest index values were obtained in Group 1 (0.92±0.31) and Group 2 (0.99±0.27), and the differences between these two groups were not statistically significant. Significantly lower index values were obtained in Group 3 (0.34±0.12) and Group 4 (0.33±0.21). Another significant decrease was observed in Group 5 (0.04±0.04) and Group 6 (0.03±0.03). In Group 7, no ulcers were detected either macroscopically or histopathologically.
Analysis of correlation between the parameters studied

The analysis showed a statistically significant positive correlation between VEGF expression and the number of blood vessels in 1 mm² of gastric mucosa in rats (Spearman correlation coefficient r=0.78).

DISCUSSION

The results of the present study confirm the role of VEGF and angiogenesis in the healing of stress-induced gastric ulcers in rats. The dynamics of changes in VEGF expression and angiogenesis indicates a close relationship between the area and depth of ulcers induced by water-immersion and restraint stress on the one hand and the expression of VEGF and the number of new microvessels on the other. Our study shows that as early as one day after the development of ulcers there is a significant increase in both the expression of the VEGF protein and the number of newly formed microvessels, while an abrupt decrease in VEGF expression,
observed on the fifth day after the development of stress-induced ulcers, results in a decreased intensity of angiogenesis. Moreover, it has been demonstrated that the increase in VEGF expression and angiogenesis is accompanied by a reduction in the area and depth of stress-induced ulcers in rats. The observed dynamics of changes in angiogenesis shows that the intensity of neovascularization is the highest on the third and fourth days following exposure to stress. Studies on the expression of angiopoetin-1 and angiopoetin-2 receptors have revealed that the receptors are most active on the third, fourth and fifth days after the development of stress-induced ulcers. Although in these studies acetic acid was used to induce ulceration, the findings also confirm a major role of angiogenesis in the early stage of acute gastric ulcer healing (23). Similarly, VEGF receptor expression was found to be the highest in the early phase of the healing process (24).

Studies on the role of VEGF and angiogenesis in the healing of gastric ulcers in rats have provided evidence that substances such as growth factors (25-27),
growth factor-coding plasmids (28), micromolecular heparin (29), and protamine sulphate (30) enhance and facilitate the process by promoting angiogenesis. Conversely, inhibition of angiogenesis with, for example, non-steroidal anti-inflammatory drugs (aspirin, indomethacin) (31-32), cyclooxygenase inhibitors (celecoxib, flurbiprofen) (33), steroids (dexamethasone) (34), or cigarette smoke (35) slows down the healing process (36).

Our study shows that the ulcer depth and the involvement of mucosal lamina propria are the greatest one day after ulcer development. The VEGF protein expression and angiogenesis are then already increased but reach the highest levels on days 2, 3, 4 (VEGF), and 3, 4 (angiogenesis) after ulceration, this being accompanied by a gradual decrease in both the depth of ulceration and the involvement of lamina propria. Similarly, there is a statistically significant decrease in the ulcer index on consecutive days. The results indicate a major role of VEGF and angiogenesis in reducing the area and depth of gastric ulceration. In the water-immersion and restraint stress model used, the ulcer index had the highest value one and two days after the development of ulceration. Similar index values were obtained when sensitivity to gastric mucosal damage caused by exposure to water-immersion and restraint stress was evaluated in different rat strains (37). In our study, macroscopic and histological examinations revealed no ulceration on the seventh day after stress withdrawal. Other investigators using the same stress model reported similar results in the majority of rats examined (38).

The present study has also revealed a strong positive correlation between VEGF expression and the number of blood vessels in 1 mm$^2$ of gastric mucosa in rats, which is in accordance with other reports from the literature. In addition, it has confirmed the key role of VEGF in initiating angiogenesis. Data from the literature and our present findings show angiogenesis to play a major role in growth and repair of the organism. Analysis of the dynamics of changes clearly indicates the time point of the highest VEGF expression and neovascularization. This knowledge could help develop a model of angiogenesis regulation. Proper stimulation or inhibition of angiogenesis could be of value in the treatment of gastric ulceration which is a frequent complication in patients with burns or after extensive operations, particularly those involving the central nervous system.

Administration of angiogenesis-inducing agents to enhance gastric ulcer healing should be undertaken with great caution, due to the risk of tumor development, and preceded by a large number of experimental studies and clinical trials.

Therapeutic angiogenesis, therefore, remains a technique which is not fully recognized but has a great potential for future use in medicine.

In summary we have demonstrated that VEGF expression increases during the first two days after ulcer development and remains high for the next two days. Angiogenesis increases gradually during the first three days after ulceration and
remains high throughout the fourth day. On the fifth day VEGF expression and angiogenesis decrease and both return to normal levels six days after ulcer development.

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