UC 577,1; 61 ISSN 0354-3447

Jugoslov. Med. Biohem. 21: 339–343, 2002 *Originalni naučni rad*
Original paper

ALTERATION OF CIRCULATING INSULIN-LIKE GROWTH FACTORS IN PATIENTS INFECTED WITH BACTERIA *HELICOBACTER PYLORI* OR *FRANCISELLA TULARENSIS*

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Summary: Alterations of insulin-like growth factors (IGF-I and -II) and their binding proteins (IGFBP) in patients infected with *Helicobacter pylori* or *Francisella tularensis* are reported in this paper. Infections were diagnosed immunochemically, by determination of specific antibodies to each bacterial species. It was shown that IGF-I, IGF-II and IGFBP-3 concentrations were lower in patients with bacterial infections, while IGFBP-2 concentration increased in comparison with healthy adults. Although the effect was more pronounced in the case of *H. pylori* infection, statistically significant reductions of IGF-I and IGF-II were found in both groups of patients. For IGF-I $p < 0.0001$ and for IGF-II $p = 0.037$ in patients with *H. pylori,* while $p = 0.017$ and $p = 0.032$ in patients with *F. tularensis*. Alterations of the IGF system can be regarded as a combined effect of bacterial infection on immuno, gastrointestinal, hepatobiliary and nutritional axes in the organism.

Key words: insulin-like growth factors, bacterial infections.

Introduction

Insulin-like growth factors (IGF-I and -II) are polypeptide hormones (7.5 kD) with an important role in the regulation of cell growth and metabolism (1). They show 70% of structural homology and their total serum concentration is 700 800 ng/mL (approximately 100 nmol/L). Growth hormone (GH) and IGF molecules are not only involved in endocrine control of the immune system, but also play a role as local growth and differentiation factors (cytokines) (2). In addition, IGF-I expression in the immune system has been shown to be regulated by cytokines, such as interleukin-1 (IL-1), interleukin-6 (IL-6), tumour necrosis factor α (TNF-α) and interferon γ (INF-γ) (3, 4). The biological activity of IGF-I and -II depends on specific binding proteins (IGFBP-1 to -6) present in physiological fluids and tissues (5). In the peripheral circulation the IGFBPs serve as carriers of IGFs and modulators of their actions. The liver is the main source of circulating IGFs and IGFBPs, but the components of the IGF/IGFBP system are also produced in a specific manner by different cells and tissues, including the immune system. The structurally homologous IGFBPs are secretory (glyco)proteins that bind both IGFs with high affinity and specificity, but the binding sites and affinity toward IGF-I and -II are not identical (6). In healthy persons IGFBP-3 is the most abundant in serum (80 100 nmol/L), while the levels of other IGFBPs are: IGFBP-2 (8 20 nmol/L); IGFBP-1 (0.3 2.0 nmol/L) and IGFBP-4 (5 30 nmol/L). IGFBP-3 is produced largely in Kupffer's cells of liver in two glycoforms of 45 and 40 kD and may be phosphorylated (7). Both glyco forms bind most of the circulating IGFs in a stable ternary complex (approximately 150 kD) with an acid-labile subunit (ALS) synthesised in hepatocytes. It probably serves to provide a reservoir of IGF molecules by prolonging the half-life and limiting bioavailability of the IGFs. IGFBP-2 is a 34 kD protein synthesised in hepatocytes and neither phosphorylated nor glycosylated. It forms binary complexes with IGF peptides (40 50 kD) which can cross capillary walls and take part in the regulation of body distribution of IGF molecules (8). Serum IGFBP-2 concentrations tend to increase when there is less IGFBP-3.

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In addition to the primary immune response, bacterial infections often cause secondary consequences on the other physiological systems of the organism. Most pathogenic bacteria secrete endo- or exotoxins. Lipopolysaccharide LPS (endotoxin) is an integral component of the outer membrane of gram-negative bacteria and stimulates numerous immunobiological and pharmacological processes. LPS purified from most pathogenic bacteria readily activates macrophages, B lymphocytes, neutrophils and T cells indirectly for proliferation and/or production of a variety of cytokines and chemokines (IL-1, IL-6, TNF-a) (9). Many exotoxins, actively secreted by bacteria, are primarily cytotoxic.

Helicobacter pylori, a gram-negative, microaerophilic bacillus, is the predominant cause of chronic gastritis, gastric and duodenal ulcers and gastric adenocarcinoma (10). Due to its spiral shape and the presence of flagellae, this organism can colonise the surface epithelium of the gastric crypts and proximal duodenum. Toxic products of *H. pylori* at first cause cell oedema, then shorten or lengthen microvillae, as well as expanding the intracellular compartments. There may be a host acute inflammatory response in which polymorphonuclear granulocytes react by releasing enzymes and free oxygen species, thus intensifying the toxic effects of *H. pylori*. However, the bacteria survive by producing many virulent factors including: 1) complex enzyme activity (presence of urease, catalase, oxidase, phospholipase and protease); 2) synthesis of specific adhesin proteins that enable them to adhere to mucous and epithelial cells; 3) presence of vacuolating cytotoxin (VacA gene product); 4) production of a high molecular weight (120 180 kD) major protein antigen-cytotoxin-associated protein (CagA), which is not toxic itself (11). The humoral immune response is characterized by a marked increase in plasma IgG and IgA, which bind to the surface antigens of *H. pylori in vitro,* and coat the bacterium in vivo. The cellular immune response is associated with B and T lymphocyte activation, especially in the expression and secretion of IL-8. *H. pylori* infection also increases pepsinogen secretion and may result in damage to epithelium integrity (12). It is now apparent that *H. pylori* can also infect the skin, liver and heart, and that these infections may produce a number of different disease states (13).

Francisella tularensis is a gram-negative, aerophilic coccobacillus causing tularaemia in humans and animals (14). Humans are not the usual host for this bacteria, but they become infected through contact with animals (rabbits and ixodidaes) and their environment. The main ways of transmission are: a) direct contact with rabbits, mice and other infected animals; b) contaminated water; c) inhalation way; d) ixodidae bite. Erythema appears at the place of entry to the organism, followed by local inflammation. The inflammatory process proceeds primarily through lymphatic organs, including an increase of regional lymph nodes and their necrosis (15). *F. tularensis* is transmitted to distant organs, engulfed in phagocytes of the reticuloendothelial system. The cellular immune response to *F. tularensis* is regarded more important than the humoral immune response (16). The humoral immune response appeared during the second week of infection and is characterized by synthesis of IgM, IgG and IgA antibodies. Cellular immunity is associated with activation of $CD4^+$ and $CD8^+$ lymhocytes and production of IL-2 and INF-γ.

The main purpose of this work was to examine alterations of circulating IGF/IGFBP system in persons infected with bacteria *Helicobacter pylori* or *Francisella tularensis*.

Materials and Methods

Serum samples were obtained from healthy adult people (N = 81; 45 women, 36 men) aged 20 75 years and persons infected by *H. pylori* (N = 10 in a preliminary study and N = 103 in a detailed analysis; 67 women, 36 men covering the same age range as in the control group) and by *F. tularensis* (N = 10). The sera were stored at 20 °C until examination.

Helicobacter pylori rapid immunochromatographic test (H. PYLORI-CHECK-1, VEDA-LAB) was used for the detection of human IgG antibodies to *H. pylori* in serum. In this test we used serum samples diluted to 1 : 20 000, and results can be explained as +/ . Serodiagnosis, i.e. the presence of antibodies to *F. tularensis* was established using a specific indirect immunofluorescence assay, IFA (VMA-Belgrade) with FITC-labelled secondary antibodies. This assay was also used for determination of the greatest serum dilution in which the antibodies could be detected.

IGF-I and -II concentrations in sera were measured by radioimmunoassay (RIA; INEP-Zemun) with ^{125}I labelled IGF-I and IGF-II, respectively. Serum proteins were separated by electrophoresis (SDS-PAGE) using a 10% (w/w) gel, under non-reducing conditions (50 mA, 150V, 6h) (17) and transferred to PVDF membrane (1.2 mA/cm^2, 1h). IGFBP patterns on the membrane were characterized by interaction with ligand ^{125}I-IGF-I (PVDF membranes were incubated in a solution containing 5×10^6 cpm ^{125}I-IGF-I at 4 °C overnight). Membranes were autoradiographed on raentgen film (Du Pont de Nemours GmbH, Germany) for 6 weeks at 80 °C.

Numerical data were expressed as the mean, median and standard deviation (SD). Differences between mean values were analyzed by Student's t-test. The relative intensity of autoradiographic bands was estimated using the Glyco Band Scan computer programme (Version 5, 1998).

Results

IGF-I and IGF-II concentrations in the analyzed group of healthy adult people (control group) ranged from 8.2 to 43.0 nmol/L and from 40.4 to 101.5 nmol/L, respectively (18). Mean value, median and standard deviation were 23.07, 21.60 and 7.995 nmol/L for IGF-I, and 72.05, 73.50 and 4.410 nmol/L for IGF-II. Statistically significant differences between women and men were not found. Autoradiographic analysis showed that sera from all control subjects contained IGFBP with molecular masses of 40 and 45 kD, corresponding to the well-known IGFBP-3 doublet, as well as a weak single band at 34 kD agreeing with mass of IGFBP-2. In order to examine if bacterial infection induced alterations of the IGF/IGFBP system, ten sera from patients with each infection (with immunological reaction to bacteria) were analysed.

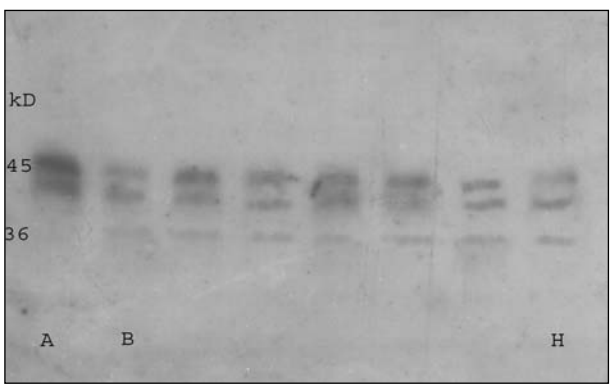

Figure 1. IGFBP profiles in human sera.
A: Serum of healthy person; B H: Sera of persons infected with *Helicobacter pylori*

Statistical analysis (Student's t-test) showed that the differences in mean IGF-I and IGF-II concentrations between the healthy group and persons infected either with *H. pylori* or with *F. tularensis,* were statistically significant. For the group infected with *H. pylori* $p < 0.0001$ was obtained for IGF-I and $p = 0.037$ for IGF-II, while in the group infected with *F. tularensis* $p = 0.017$ for IGF-I and $p = 0.032$ for IGF-II in comparison with the control group. IGFBP patterns analysed by autoradiography were characterized by decreased intensity of the IGFBP-3 bands and an increased level of IGFBP-2, especially in persons infected with *H. pylori*. No correlation between the antibody titer and serum IGF-I and IGF-II concentrations was found in patients with antibodies to *F. tularensis*.

Since *H. pylori* infection is accompanied by greater changes of the IGF system, the number of analysed samples with this infection was increased to 103. IGF-I concentrations were within the range of 1.0 to 28.8 nmol/L, mean value was 13.38 nmol/L, median 12.10, while standard deviation was 5.169 nmol/L. Variations of IGF-II were 27.3 120.5 nmol/L, mean value was 66.91, median 63.30, standard deviation 17.702 nmol/L. These results confirmed the results from the previous study. Decreasing median for IGF-I is slightly expressed in women than in men population, but it is not statistically significant ($p > 0.05$). IGFBP profiles in human serum infected with *H. pylori* are shown in *Figure 1.*

Discussion

The results of this study showed that the circulating IGF/IGFBP system may be altered during infection with gram-negative bacteria *(H. pylori* or *F. tularensis)*. In both cases mean IGF-I and IGF-II levels were significantly reduced ($p < 0.05$), but the decrease of IGF-I was relatively greater than the decrease of IGF-II. *H. pylori* appeared to have a greater influence on circulating IGF molecules and their binding proteins than *F. tularensis*. These results may be related to the localization of bacteria and their mode of entry (19, 20). *H. pylori* colonize the gastric epithelial layer and the gastrointestinal system is closely connected with the hepatobiliary system, responsible for the synthesis of most IGF and IGFBP molecules.

It is known that inflammation, liver disease, hypopituitarism and malnutrition lead to decreasing IGF-I concentration and alteration of the IGF binding protein ratio. Gram-negative bacterial endotoxin (lipopolysaccharide, LPS) is an integral component of the outer membrane. During bacterial infection, host cells recognize LPS, which activates macrophages, B lymphocytes, neutrophils and T cells to proliferate and/or synthesise different cytokines and chemokines (21). Kupffer's cells, tissue macrophages responsible for IGFBP-synthesis, may be continuously in contact with soluble bacterial products, mainly endotoxins, thus

Table I Alterations of IGF/IGFBP system in sera of patients with different bacterial infections

Infection (antibody titer)	IGF-I (nmol/L) $\bar{x}$	Me	Sd	IGF-II (nmol/L) $\bar{x}$	Me	Sd	IGFBP-2 (relative ratio)	IGFBP-3
Healthy	23.07	21.60	7.995	72.05	73.50	4.410	+	+++
H. pylori (20 000)	10.93	11.84	3.054	55.0	56.90	5.944	++/+++	++
F. tularensis (320 2 560)	16.45	14.40	9.265	61.50	59.25	15.856	+/++	++

leading to intensive secretion of inflammatory mediators, such as reactive oxygen species, nitrogen oxide, carbon monoxide, TNF-α, IL-6, IL-1. In that way macrophages control the early phase of liver inflammation and play an important role in the innate immune response (22). TNF-α decreased liver expression of mRNA for both IGFBP-3 and IGF-I, thus leading to decreased synthesis up to 40% (23). In acute endotoxaemia, with increased level of TNF-α in blood, resistance to GH receptor is appearing. It has been confirmed in isolated hepatocytes that TNF-α and IL-1 are capable to decrease the synthesis of IGF-I by negative regulation of GH signal transduction (24).

Increased IGFBP-2 and decreased IGFBP-3 were detected in both infections. IGFBP action can be regulated both systematically and locally by IGFBP proteases. Specific proteases for IGFBP-1 through -6 have been identified, including kallikreins, cathepsins, matrix metalloproteinases and other families of proteolytic enzymes (25). It has been shown that IGFBPs can be processed by cathepsin D. In pregnancy and several disease states such as severe critical illness, activities of IGFBP proteases are increased. Ligand blotting revealed that none of the IGFBP-1 to -3 fragments formed by cathepsin D retain their ability to bind IGFs (26). In addition, IGFBPs are produced by many cells of the immune system, including normal human lymphocytes (27). Unstimulated lymphocytes express only IGFBP-2 and -3, whereas after stimulation IGFBP-4 and -5 are detectable as well. This opens up the possibility that the actions of endocrine or locally produced IGFs can be modulated via production of IGFBPs by cells from the immune system.

Alterations of the IGF system also may be associated with malnutrition and starvation. Malnutrition suppresses IGF-I levels, but also interaction between growth hormone and its receptors. Reduced food intake, impaired digestion and/or absorption of nutrients, digestive disturbance (diarrhoea, vomiting), but also changes in secretion of harmful metabolic products, have an influence on the IGF system (28). The alterations of the IGF system observed in this work are probably induced by a combined reaction of the organism to nutritive, gastrointestinal, hepatobiliary and immune changes.

Acknowledgements: This work was supported by the Ministry of Science, Technologies and Development of Serbia, project 1504: Glycobiological aspects of physiological and pathophysiological processes.

PROMENA INSULINU SLIČNIH FAKTORA RASTA U CIRKULACIJI OSOBA INFICIRANIH BAKTERIJOM *HELICOBACTER PYLORI* ILI *FRANCISELLA TULARENSIS*

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Kratak sadržaj: U ovom radu opisane su promene insulinu sličnih faktora rasta (IGF-I i -II) i njihovih vezujućih proteina (IGFBP) kod pacijenata inficiranih bakterijama *Helicobacter pylori* ili *Francisella tularensis*. Infekcije su konstatovane imunohemijski, određivanjem specifičnih antitela. Pokazano je da su se koncentracije IGF-I, IGF-II i IGFBP-3 smanjile usled bakterijske infekcije, dok je koncentracija IGFBP-2 rasla. Efekat je bio izraženiji u slučaju infekcije *H. pylori,* mada je statistički značajan pad IGF-I i IGF-II konstatovan u obe grupe pacijenata. Za IGF-I i IGF-II $p < 0,0001$, odnosno $p = 0,037$ kod osoba sa *H. pylori,* odnosno $p = 0,017$ za IGF-I i $p = 0,032$ za IGF-II kod osoba sa *F. tularensis*. Promene IGF sistema se mogu smatrati posledicom kombinovanog efekta koji bakterijska infekcija izaziva na imunskom, gastrointestinalnom, hepatobilijarnom i nutritivnom nivou u organizmu.

Ključne reči: insulinu slični faktori rasta, bakterijske infekcije.

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Received: July 5, 2002

Accepted: September 15, 2002