LIGAND-BINDING ACTIVITY AND IMMUNOREACTIVITY OF INSULIN-LIKE GROWTH FACTOR BINDING PROTEINS IN PATIENTS WITH COLORECTAL CARCINOMA AND POSTOPERATIVE SEPSIS

LIGAND-VEZUJUĆA AKTIVNOST I IMUNOREAKTIVNOST VEZUJUĆIH PROTEINA ZA INSULINU SLIČNE FAKTORE RASTA KOD PACIJENATA SA KOLOREKTALNIM KARCINOMOM I POSTOPERATIVNOM SEPSOM

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Summary

Background: Postoperative sepsis alters the growth hormone (GH)/insulin-like growth factor (IGF) axis leading to reduction in IGF-I and IGF-binding protein (IGFBP) 3 and elevation of GH-independent IGFBPs. The aim of this work was to investigate the differences in ligand binding and immunoreactivity of the circulating IGFBPs between surviving and non-surviving septic patients that underwent colorectal surgery.

Methods: Ligand binding was detected only for IGFBP-3 and IGFBP-2 and, consecutively, immunoreactivity was assayed for these IGFBPs.

Results: In survivors both ligand- and immunoblotting revealed two IGFBP-3 isoforms (40 and 45 kDa). In non-survivors ligand-blotting hardly detected the IGFBP-3 doublet, intact immunoreactive IGFBP-3 was not detected and the major species was the 29 kDa fragment. Immunoblotting with an anti-IGFBP-2 antibody indicated an increase in the amount of intact and fragmented IGFBP-2 along the progression of illness in the non-survivors. The amount of intact IGFBP-2 in the survivors did not differ during the treatment. The amount of immunoreactive IGFBP-2 corresponded to its ligand-binding activity.

Conclusions: According to the results reported here, ligand-reactivity and immunoreactivity of IGFBP-3 should be taken into account as variables that affect the GH/IGF axis in critically ill patients. The degree of their variation is not directly

Kratak sadržaj

Uvod: Postoperativna sepsa izaziva promene u sprezi hormon rasta (GH)/insulinu slični faktori rasta (IGF). Smanjuju se koncentracije IGF-I i IGF vezujućeg proteina (IGFBP) 3, a povećavaju IGFBP koji ne zavise od GH. Cilj rada bio je da se ispita ligand-vezujuća aktivnost i imunoreaktivnost IGFBP molekula u cirkulaciji pacijenata operisanih od kolorektalnog karcinoma sa postoperativnom sepsom i različitim ishodom u smislu preživljavanja.

Metode: Ligand-vezujuća aktivnost detektovana je ligand blotom za IGFBP-2 i IGFBP-3, te je za ove IGFBP određivana i imunoreaktivnost.

Rezultati: Kod preživelih, ligand i imunoblotom utvrđeno je prisustvo dve IGFBP-3 izoforme (40 i 45 kDa). Kod umrlih, u ligand blotu IGFBP-3 dublet bio je jedva vidljiv, dok je u imunoblotu najjača traka pripadala fragmentu (29 kDa). Ukupna količina IGFBP-2 (intaktni molekuli i fragmenti) bila je znatno veća kod pacijenata koji nisu preživeli i progresivno je rasla sa približavanjem letalnog ishoda. Ligand-vezujuća aktivnost je takođe bila srazmerno veća.

Zaključak: Dobijeni rezultati upućuju na značaj određivanja ligand-vezujuće aktivnosti i imunoreaktivnosti IGFBP-3 kod pacijenata u kritičnom stanju. Promene IGFBP-3 aktivnosti nisu direktno odraz promene koncentracije IGFBP-3 određene imunotestom, te su stoga važne u proceni metaboličkog potencijala pacijenta da se suprotstavi bolesti.

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correlated to the total amount of IGFBP-3 determined by the immunoassay, but they may be related to patients' metabolic potential to combat a disease.

Keywords: colorectal carcinoma, IGFBPs, sepsis, survival

Ključne reči: IGFBP, kolorektalni tumor, sepsa, preživljavanje

Introduction

Colorectal cancer followed by surgery is a medical condition that carries a significant risk of complications. A strong systemic inflammatory response can occur, followed by infection and malnutrition. The human colon has a large bacterial content and septicemia may develop postoperatively. The metabolic response to sepsis includes breakdown of skeletal muscle protein, rapid mobilisation of fat and carbohydrate reserves, hepatic protein synthesis shifts from constitutive to acute-phase proteins, pituitary–adrenal axis favours synthesis of glucocorticoids, and all these reactions are initiated or mediated by cytokines (1). Sepsis alters the growth hormone (GH)/insulin-like growth factor (IGF) axis dramatically (2). The resistance to GH and a less pulsatile secretion pattern develops, leading to reduction in GH-dependent molecules, such as IGF-I, IGF-II and IGF-binding protein (IGFBP)-3 and elevation of GH-independent IGFBPs such as IGFBP-1, IGFBP-2, IGFBP-4 and IGFBP-6 (3). Different therapeutic approaches are administered to overcome complications of critical illnesses including administration of GH, GH-releasing peptide, hepatocyte growth factor, IGF-I, IGFBP-3, binary complex IGF-I/IGFBP-3 and insulin (4–6). Unfortunately, the improvement of anabolic processes is often not sufficient to overcome complications (7) and there are studies that report an increased mortality risk in GH-treated patients (5).

Mechanisms that underly health complications and therapeutic treatment are multifactorial and depend on both the quantity of specific molecules and their activity. The aim of our work was to analyse the circulating pattern of IGFBPs in septic patients that underwent colorectal surgery. The relationship between the ligand-binding activity and immunoreactivity of IGFBPs and the final survival/non-survival outcome was examined.

Materials and Methods

The patients chosen for this study (n=26, 15 males, 11 females, age range 54–73 years, BMI 15–29 kg/m^2) were diagnosed with colorectal carcinoma (graded as ASA III in 70% of cases or ASA IV in 30%). They all underwent open surgery (emergency n=16, elective n=10) under general anesthesia and were later diagnosed with sepsis. Ten patients received colorectal resection and primary anastomosis and 16 received diverting colostomy with proximal end colostomy and distal closure of the rectal stump (Hartmann's procedure). The diagnosis of sepsis was performed in the »Bežanijska Kosa« Clinical-Medical

Centre according to the following criteria: (i) clinical (body temperature: >39 °C or <36 °C; tachycardia: >90 beats/min; tachypnea: >20 breaths/min with respiratory alkalosis; petechial bleeding; organ dysfunction; abdominal tenderness; bowel movements), (ii) inflammatory (increased erythrocyte sedimentation: >20 mm/h; increased leukocyte count: >12×10^9 cells/L or decreased leukocyte count: <4×10^9 cells/L; increased fibrinogen concentration: >4 g/L; procalcitonin: >2 µg/L and C-reactive protein: >150 mg/L), (iii) endocrinological (increased insulin resistance in some cases), (iv) abdominal ultrasound, (v) cardiac echotomography and (vi) microbiological. The increase of C-reactive protein at post operative days 2 and 4 was a predictor of anastomotic leak which was recorded in 4 patients. Other septic complications were: wound infection, abdominal abscess and diffuse stercoral peritonitis. A urinary catheter was placed in all patients for 5 to 7 days. In 20 patients sepsis was diagnosed due to infection caused by *Enterococcus*, *Pseudomonas* and/or *Staphylococcus* species. Two patients had urosepsis caused by *Escherichia coli* and in one patient *Candida* species was detected. All patients were treated with broad-spectrum antibiotics, appropriate fluids and inotropics.

At the end of the study the patients were grouped according to the final outcome of their disease and treatment into survivors (n=12) and non-survivors (n=14). Emergency surgical intervention was a factor that increased the risk of septic complications and the mortality rate, and so was the coexistence of other diseases, such as cardiovascular (in 4 patients), pulmonary (in 2) or hepatic disease (3 patients had liver metastases), and/or previous abdominal or pelvic radiation therapy. Postoperative blood loss and weight loss were unfavourable factors for the survival outcome. The type of the surgery was not responsible for complications.

Serum samples were collected from patients preoperatively, postoperatively (24 h after surgery), several times after the onset of sepsis (the first sample was taken within 24 h after diagnosis) and during treatment. The last sample was obtained after termination of therapy for sepsis and, in the case of non-survivors, the last before death was used. Urine samples (24 h urine) were collected on the same occasions. All samples were collected according to the regular procedures of Clinical-Medical Centre and their use for determination of the IGF/IGFBP status in patients was approved by the local ethical committee. Concentrations of IGF-I, IGFBP-3, total protein, albumin, glucose and insulin were measured in sera and

the amount of excreted cortisol in 24 h urine. Serum samples were used in ligand and immunoblotting for the determination of the ligand-binding activity of IGFBPs and their relative presence.

The concentration of IGF-I was measured by radioimmunoassay (RIA-IGF-I, INEP, Belgrade, Serbia) standardised against WHO reference material 02/254 (8). The IGFBP-3 concentration was measured by an immunoradiometric assay (DSL-6600 IGFBP-3 IRMA kit, Diagnostic Systems Laboratories, Webster, TX, USA). The concentration of cortisol was determined by radioimmunoassay (RIA-CT-Cortisol, INEP, Belgrade, Serbia) that was standardised against the ICN RIA-CT Cortisol kit (ICN Biomedicals Inc., Costa Mesa, CA, USA). The concentration of insulin was measured by radioimmunoassay (RIA-Insulin, INEP, Belgrade, Serbia) standardised against WHO reference material 83/500. Total protein was determined using Biuret reagent, albumin was measured using bromocresol green reagent and glucose using GOD-PAP assay (Randox Laboratories, Crumlin, UK). The reference ranges for the analysed parameters are the following: serum IGF-I: 9–45 nmol/L, IGFBP-3: 58–130 nmol/L, insulin: 5–25 mU/L, total protein: 62–82 g/L, albumin: 35–55 g/L, glucose: 4.2–6.2 mmol/L and urinary cortisol: 27–275 nmol/24 h.

Numerical data are expressed as the median and the reference interval (determined as the central 95% range between the 2.5th and 97.5th percentiles)

as some of the values were not normally distributed. To evaluate differences in the results between groups of patients the Mann-Whitney U test was used. To evaluate differences between time intervals a non-parametric repeated measurement ANOVA test was performed. In order to assess statistically significant differences between groups of results the Friedman test was employed. Data were analysed by the Primer of Biostatistics software (version 5).

Serum proteins were subjected to SDS-PAGE prior to transfer to nitrocellulose membranes (9). Ligand-blotting was performed using ^{125}I-IGF-I (1 $\times 10^6$ cpm) and visualised by autoradiography. Immunoblotting employed affinity-purified goat polyclonal anti-IGFBP-3 and anti-IGFBP-2 antibodies (Diagnostic Systems Laboratories) and an HRP-conjugated swine anti-goat antibody (Biosource, Camarillo, CA, USA). Immunoreactive proteins were visualised using enhanced chemiluminescence (Pierce, Minneapolis, MN, USA). Molecular mass markers were from BioRad Laboratories (Hertfordshire, UK).

Results

The degree of sepsis in the patients was confirmed by measuring biochemical parameters in serum and urine (*Table I*). The concentrations of total protein and albumin in preoperative patients were below the reference ranges. The values further

Table I IGF-I, IGFBP-3, total protein, albumin, glucose and insulin concentrations in serum, and cortisol excretion in 24 h urine in patients diagnosed with postoperative sepsis grouped according to the survival (S)/non-survival (NS) outcome of their disease

	Preoperative sample Me (2.5th–97.5th)		24h postoperative sample Me (2.5th–97.5th)		Sample at onset of sepsis Me (2.5th–97.5th)		Sample after therapy Me (2.5th–97.5th)	
	S	NS	S	NS	S	NS	S	NS
IGF-I (nmol/L)	12.7 9.1–15.0	8.4a 5.2–11.0	10.6 6.3–13.0	7.2a 3.2–10.7	7.2b 5.0–9.9	5.3b 3.3–7.7	11.4b 7.3–16.7	5.4a 3.1–7.0
IGFBP-3 (nmol/L)	80.3 72.1–90.1	63.2 41.1–75.2	72.1 62.6–79.7	55.2a,b 37.8–73.0	58.7b 47.3–66.7	46.0a,b 32.1–57.8	80.3b 62.9–99.9	36.2a,b 25.4–43.8
Total protein (g/L)	52.9 47.2–59.7	53.7 48.1–57.0	52.4 43.9–61.8	53.3 48.1–59.2	47.6b 41.0–54.5	46.7b 43.1–53.0	49.1 41.7–53.0	45.6 39.4–50.8
Albumin (g/L)	32.6 30.0–36.9	30.1 27.3–33.7	32.9 28.3–38.4	30.6 27.1–34.5	28.6b 24.1–32.8	26.0b 23.3–27.7	33.1b 30.1–38.0	21.1a,b 19.0–23.1
Glucose (mmol/L)	4.8 4.1–5.8	4.7 3.7–5.9	5.1 3.9–6.5	5.6 4.1–7.4	5.2 3.5–6.2	5.8 4.0–8.4	5.2 3.7–7.0	5.5 3.6–8.2
Insulin (mU/L)	12.6 9.3–14.8	12.9 7.1–16.4	13.8 10.4–16.7	14.4 10.0–20.7	13.4 9.4–17.1	14.6 9.7–20.4	13.5 9.0–17.4	14.6 10.8–16.9
Cortisol (nmol/24h)	710 420–871	765 508–1041	713 407–927	692 373–892	860b 563–1043	911b 467–1278	726b 491–928	1173 403–2117

Results are presented as medians (Me) and the reference interval (determined as the central 95% range between the 2.5th and 97.5th percentiles). Statistically significant differences (at p<0.05) between patients that have survived and those that have not survived, for the same sampling period, are indicated as (a), while differences between successive sampling periods, for the same group of patients, are indicated as (b).
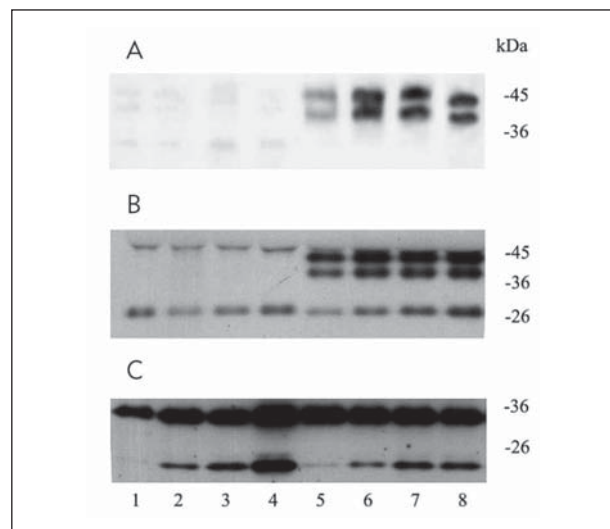
Figure 1 Ligand-blotting (A) and immunoblotting with anti-IGFBP-3 (B) and anti-IGFBP-2 (C) antibodies of the representative sera from critically ill patients, non-survivors (lanes 1–4) and survivors (lanes 5–8). Serum samples were taken 24 h postoperatively (lanes 1 and 5), at onset of sepsis (lanes 2 and 6), seven days after therapeutic treatment (lanes 3 and 7) and after termination of treatment (lanes 4 and 8). Positions of the molecular mass markers are shown on the right side.

decreased upon surgery and the onset of sepsis and then slightly increased after therapeutic treatment in the survivors or continued to decrease in the non-survivors. The concentration of urinary cortisol was higher than the reference range before surgery, it did not change upon operation itself, but increased due to sepsis and continued to increase in the non-survivors. The patients, therefore, exhibited an expected pattern of acute-phase reactants (10, 11). The concentrations of glucose and insulin were within the reference range during the observation period, although slight resistance to insulin was noted in some patients that did not recover from sepsis.

Ligand-blotting was performed to investigate ligand-binding activity of serum IGFBPs (*Figure 1*). Four samples from a non-surviving patient (lanes 1–4) and 4 from a surviving patient (lanes 5–8) are shown in the figure. Two protein bands of approximately 40–45 kDa corresponding to the IGFBP-3 doublet and a band slightly below 36 kDa corresponding to IGFBP-2 were seen (12). Other IGFBPs were not detected (*Figure 1A*). In the non-survivor the amount of IGF-I-reactive IGFBP-3 was very low during the course of critical state. IGF-I-reactive IGFBP-2 was clearly seen. In contrast, in the survivor the amount of IGF-I-reactive IGFBP-3 was the lowest at the postoperative time point, during the recovery period it increased and remained at a constant level even in the case of sepsis. IGF-I-reactive IGFBP-2 was almost undetectable.

Immunoblotting with anti-IGFBP-3 antibodies (*Figure 1B*) confirmed the results of ligand-blotting. The intact IGFBP-3 in the non-survivor was undetectable. The greatest proportion of the IGFBP-3 immunoreactivity was due to the 29 kDa proteolytic fragment. In the surviving patient the IGFBP-3 doublet was present. An immunoreactive proteolytic fragment of IGFBP-3 was also detected in all samples. Immunoblotting with an anti-IGFBP-2 antibody (*Figure 1C*) indicated an increase in the amount of intact and fragmented IGFBP-2 along the progression of illness in the non-survivor. The amount of intact IGFBP-2 in the survivor did not differ during the treatment, whereas increased IGFBP-2 fragmentation was seen along the course of sickness.

As both ligand and immunoblotting data indicated that IGFBP-3 differed more than IGFBP-2 between the survivors and non-survivors, the next step was to quantify this protein. The amount of IGFBP-3 was measured in all patients together with the concentration of IGF-I (*Table I*). The amount of IGFBP-3 (and IGF-I) was lower pre- and postoperatively in the non-survivors than in the survivors, it continued to fall as sepsis was diagnosed and became even lower at the time of death. In the survivors, the concentration of IGFBP-3 often remained within the reference range, albeit close to the lower reference range limit. In these patients the levels of both IGFBP-3 and IGF-I increased upon therapy.

Our data were analysed according to gender and nature of the infection. Seven of the survivors were females while 5 were males. The non-survivors were 10 males and 4 females. The measured biochemical parameters were not significantly different between females and males (data not shown). Furthermore, a correlation between the type of infectious bacteria and the final outcome of the critical illness could not be established.

Discussion

Circulating levels of GH (13), IGFs and IGFBPs (14–16) are often taken into consideration as prognostic biomarkers in critically ill patients. Lower levels of IGF-I and IGFBP-3 and higher levels of GH, IGFBP-1 and IGFBP-2 are found in non-survivors compared with survivors. The paradoxical relationship between GH and GH-dependent molecules is explained by trauma/endotoxin/cytokine-mediated hepatic resistance to GH coupled with increased catabolism. The results of our study underline the importance of the quantity/structure/function relationship of IGFBPs in these patients.

The quantity and the IGF-I-binding capacity of IGFBP-3 in the survivors matched and both IGFBP-3 isoforms were ligand- and immunoreactive. In the non-survivors IGFBP-3 (measured by IRMA) was at the most three times lower than in the survivors, but the

intensity of IGFBP-3 protein in the ligand-blot was much fainter than in the survivors. Furthermore, in the non-survivors the most immunoreactive species was at 29 kDa. We can postulate that besides an altered rate of synthesis, posttranslational modifications of IGFBP-3 may be responsible for reduced ligand-binding and an inappropriate metabolic response in some critically ill patients. Surgery and critical illness were found to be associated with increased IGFBP-3 proteolysis (17, 18) and, using lectin-affinity chromatography, structural alterations in the carbohydrate moiety of IGFBP-3 in these patients have been noted (19).

IGFBP-2 is not posttranslationally modified, whereas IGFBP-3 is both glycosylated and phosphorylated (12). IGFBP-2 does not form complexes with other circulating proteins, while IGFBP-3 may interact with transferrin (20). In our study the amount of IGFBP-2 reflected its ligand-binding activity. In contrast, IGFBP-3 detected by immunochemical methods (immunoblotting or IRMA) did not necessarily match the reactivity with its ligand. This altered reactivity may be partially responsible for the inadequate response of some critically ill patients to therapy. A marked inter-patient variability in the concentration of IGF-I and IGFBP-3 was found in response to exogenous recombinant human IGF-I (7). The most severely ill patients had the least response to recombinant human IGF-I.

In unfavourable metabolic conditions females appear to have higher levels of IGF-I and IGFBP-3 (21). There are indications that pathogenic differences between bacteria may result in different cytokine profiles and mortality rates associated with sepsis (22). No correlation, however, between gender or the nature of the infection and the mortality rate of the critically ill patients was established.

Ligand- and immunoreactivity of IGFBP-3 should be taken into account as variables that affect the GH/IGF axis in critically ill patients, as both activities do not correlate with the total amount of IGFBP-3 determined by the immunoassay. Such variations may be related to patients' metabolic potential to combat a disease and, possibly, to respond to a therapy.

Conflict of interest statement

The authors stated that there are no conflicts of interest regarding the publication of this article.

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