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Molecular forms of the insulin-like growth factor-binding protein-2 in patients with colorectal cancer

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ABSTRACT

The components of the insulin-like growth factor (IGF) system and molecules with which they interact are associated with the neoplastic transformation of cells in colorectal cancer. The IGF-binding protein-2 (IGFBP-2) plays a significant role in mitotic stimulation of the cancer cells and its concentration is significantly elevated in tumor states. Little is known about IGFBP-2 at the molecular level and the purpose of this study was to examine the interactions between IGFBP-2 and some other proteins, the fragmentation pattern and posttranslational modifications that might have occurred due to a disease. Results have shown that the amount of monomer IGFBP-2 was 20–30% greater in patients with cancer and the amount of fragmented IGFBP-2 was doubled compared to healthy people, whereas the portion of IGFBP-2 in complex with α2 macroglobulin (α2M) was 2.5 times lower in cancer patients. According to this distribution, IGFBP-2 was not only increasingly synthetized in patients with cancer, but also the amount involved in complexes with α2M was reduced favoring the existence of binary IGFBP-2/IGF complexes, free to leave the circulation. Both IGFBP-2 and α2M were significantly more oxidized in patients with colon cancer than in healthy individuals and α2M was additionally sialylated. It can be speculated that the formation of IGFBP-2/α2M complexes is part of the control mechanism involved in the regulation of IGFBP-2 and, consequently, IGF availability. It also seems that posttranslational modifications are more important factors in determining the amount of IGFBP-2/α2M complexes than the actual quantity of these two proteins.

Introduction

Insulin-like growth factor (IGF) system includes a group of proteins which play a key role in biological processes essential for the survival of an organism, such as cellular growth, proliferation, differentiation, migration, apoptosis and metabolism. It consists of two IGF peptides (IGF-I and IGF-II), six high affinity IGF-binding proteins (IGFBP-1 to -6), and four IGF receptors [IGF receptor type I (IGF-1R), IGF receptor type II (IGF-2R), insulin receptor (IR) and hybrid receptor (IR/IGF-1R)] (Federici et al., 1997; Le Roith, 2003).

IGFBP-3 has the highest concentration of all binding proteins in the circulation of healthy persons, followed by IGFBP-2 (Firth and Baxter, 2002). IGFBP-2 is a simple protein (36 kDa) and, although many cell types can synthesize it, in the circulation it originates mostly from the hepatocytes (Ross et al., 1996). The regulation of IGFBP-2 expression is very complex, as several hormones and growth factors have the influence (Hoeflich et al., 2001; Rajaram et al., 1997). In our previous work we have shown that IGFBP-2 forms complexes with α2 macroglobulin (α2M) (Šunderić et al., 2013), a large glycoprotein that protects bound ligands from exogenous proteases and even enhances their action (Schüt et al., 2004).

IGFBP-2 possesses a RGD (Arg–Gly–Asp) sequence that enables its attachment to the elements of the extracellular matrix and integrin receptors. IGFBP-2 can react with several members of the integrin family, such as α5β1 and αvβ3 (Pereira et al., 2004; Srichai and Zent, 2010). IGFBP-2 binding to the extracellular domain of an integrin induces signal transmission that influences intracellular processes (Schüt et al., 2004).

Numerous studies have shown that IGFBP-2 acts as a promoter of proliferation, migration and invasion of cancerous cells (El Atiq et al., 1994). Positive correlation between elevated serum concentration of IGFBP-2 and cellular proliferation was found in patients with colorectal cancer (El Atiq et al., 1994), ovarian (Flyvbjerg et al., 1997; Karasik et al., 1994) and breast tumor (Busund et al., 2005; So et al., 2008), glioma (Fuller et al., 1999; Song et al., 2003; Wang et al., 2003) and leukemia (Mohnike et al., 1996). Colorectal cancer is on the third place by diagnosis of all cancers in the world, and its prevalence is higher in developing countries (WHO, 2013). It was noted that in many colon cancer cell lines, and in human colorectal adenocarcinoma, the expression of IGF-II and IGFBP-2 mRNA was elevated (Hoeflich et al., 2001). Proteolysis of IGFBP-2 regulates the amount of free, biologically active IGFs. It is known that transformed colonocytes increasingly secrete matrix

Abbreviations: α2M, alpha 2 macroglobulin; DNP, dinitrophenyl hydrazine; IGF, insulin-like growth factor; IGFBP, IGF-binding protein; MAL, *Maackia amurensis* lectin; MMP, matrix metalloprotease; PCO, protein carbonyl; SNA, *Sambucus nigra* agglutinin.

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metalloprotease-7 (MMP-7), which is responsible for the degradation of IGF-II/IGFBP-2 complexes bound to the extracellular matrix. The release of IGF-II was shown to stimulate colonocytes to divide uncontrollably (Miyamoto et al., 2007).

Cancer development was reported to be associated with posttranslational modifications of proteins (Wang, 2005). The gastrointestinal tract is exposed to large quantities of free radicals (Yeh et al., 2010). Protein carbonyls (PCO) are products of the oxidative damage of almost all proteins, and PCO levels were found to be higher in patients with colorectal cancer than in healthy persons (Shacter, 2000). Also, the aberrant glycosylation of proteins is a common event in many types of cancer. Recent studies have demonstrated that altered glycopattern induces oncogenic transformation and stimulates invasion and metastasis of tumor cells (Qui et al., 2008). Pronounced sialylation of glycoproteins allows malignant cell to disguise its immunogenic sites and to evade the immune system (Abdul and Abbass, 2009).

Increased concentration of IGFBP-2 in serum from patients with colorectal cancer makes IGFBP-2 a biomarker for this pathology, but little is known about molecular events that are possibly connected to the metabolic outcome of this elevated presence. The purpose of this study was to examine IGFBP-2 at the molecular level in patients with colorectal cancer, namely the interaction between IGFBP-2 and α2M in serum, and IGFBP-2 and α5β1 integrin on colon cell membranes, the IGFBP-2 fragmentation pattern, the presence of MMP-7 and posttranslational modifications of IGFBP-2 and α2M that might have occurred due to a disease.

Materials and methods

Serum samples

Serum samples were obtained from two groups of persons: healthy volunteers (42, 22 males and 20 females, age 45–75 years, BMI 20–32 kg/m^2) and patients with colorectal cancer (29, 16 males and 13 females, age 54–76 years, BMI 16–30 kg/m^2), after 12 h fasting. The patients were diagnosed with colorectal cancer apart from this study in Clinical-Medical Centre "Bežanijska Kosa". Their sera were obtained preoperatively on the day of surgery. All serum samples were stored frozen at −80 °C until analysis.

Tissue samples

Patients with colorectal cancer were subjected to open surgery under general anesthesia in Clinical-Medical Centre "Bežanijska Kosa". The incision length due to colon resection was 10–50 cm. Colon samples for further experiments were collected from 15 patients and differentiated into cancerous and non-cancerous tissue immediately post surgery. After thorough washing in physiological solution supplemented with protease inhibitors, tissue samples were frozen at −80 °C until use. The experiments with the human samples were approved by the Institute Review Board.

Determination of IGFBP-2 and protein concentration

The concentration of IGFBP-2 in serum was measured by the IGFBP-2 ELISA kit (Abcam, Cambridge, UK). Total protein concentration in serum was determined by Biuret assay (Randox Laboratories, Crumlin, UK), whereas total protein concentration in solubilized membrane preparations was determined by bicinchoninic acid assay (Abcam, Cambridge, UK).

Isolation of membrane proteins

Frozen tissue sample was chopped and homogenized in 250 mM sucrose solution in 50 mM HEPES buffered saline pH 7.4 (HBS) supplemented with protease inhibitors. The homogenate was centrifuged at 600 ×*g* for 20 min at 4 °C to discard tissue debris, and the supernatant was further ultracentrifuged at 190 000 ×*g* for 1 h at 4 °C (Beckman Coulter Ultracentrifuge type Ti 50.2) to precipitate cell membranes. The pellet was suspended in HBS and washed twice, with ultracentrifugation in between. The final precipitate was suspended in HBS supplemented with 1% Triton X-100 and the suspension was mixed for 1 h at 4 °C to achieve membrane solubilization. The suspension was ultracentrifuged as described, and the supernatant with solubilized membrane proteins was used in experiments. Protein concentration was adjusted to 1 mg/mL (Takano et al., 1975).

Derivatization of PCO

For dinitrophenyl hydrazine (DNP) derivatization of serum proteins (Levin et al., 1990), 10 mg/mL protein solution in deionized water was used, whereas DNP derivatization of membrane proteins was performed with 1 mg/mL solution. The sample (0.5 mL) was mixed with 0.25 mL of 10% trichloroacetic acid (TCA) and centrifuged at 1500 ×*g* for 5 min to precipitate proteins. DNP reagent (0.25 mL of 0.01 M 2,4-DNP in 2 M HCl) was added to the pellet, the suspension was vigorously mixed to homogeneity and incubated at 25 °C for 30 min with periodic mixing. TCA solution (0.5 mL) was added and proteins precipitated by centrifugation as described. The pellet was washed with 1:1 ethanol/ethylacetate solution (2 × 1 mL), with vigorous mixing and centrifugation to remove the unreacted DNP, and dissolved in 1.5 mL of 2% SDS in 0.1 M phosphate buffer pH 8 after incubation at 37 °C for 10 min (Robinson et al., 1999).

Immunoprecipitation

Immunoprecipitation was carried out using Pierce® Co-Immunoprecipitation Kit (Pierce Biotechnology, Rockford IL, USA). Goat anti-IGFBP-2 antibody (Santa Cruz Biotechnology, Santa Cruz, USA) or rabbit anti-α2M antibody (AbD Serotec, Kidlington, UK) was immobilized onto preactivated matrix, Aminolink®Plus Coupling Resin, according to manufacturer's instructions. The prepared immunoaffinity matrix (0.05 mL) was incubated with 0.2 mL of diluted serum (1:20 in a supplied dilution buffer) native or DNP-derivatized, or membrane protein sample (1:1, only with the IGFBP-2 affinity matrix), again native or

Table 1
Concentration of serum IGFBP-2 (ELISA), relative abundance (densitometric evaluation) of monomer, fragmented and complexed forms of IGFBP-2, and relative abundance of α2M and MMP-7 in sera from healthy persons and patients with colorectal cancer, expressed as the mean value ± SD.

	Healthy persons n = 42	Patients with colorectal cancer n = 29
Concentration of IGFBP-2 (μmol/L, μmol/g protein)	12 ± 8, 0.17 ± 0.11	22 ± 14, 0.39 ± 0.25*
Monomer IGFBP-2 (ADU)	796 ± 132	1087 ± 79*
Fragment IGFBP-2 (ADU)	494 ± 84	982 ± 19*
IGFBP-2/α2M complex (ADU)	342 ± 54	136 ± 54*
α2M monomer (ADU)	249 ± 31	221 ± 65
MMP-7 (ADU)	136 ± 35	111 ± 12

* Statistically significant difference ($P < 0.05$).