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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 ($P < 0.05$).

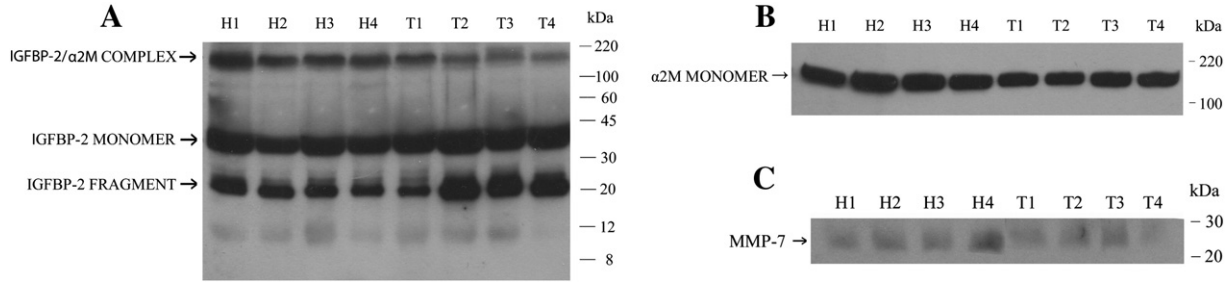

Fig. 1. The expression of A. IGFBP-2, B. α2M and C. MMP-7 in serum from healthy persons (H1–H4) and patients with colorectal cancer (T1–T4).

DNP-derivatized at 4 °C overnight. Unbound proteins were removed by centrifugation at 2000 ×*g* for 30 s, the matrix was washed three times with a dilution buffer and specifically bound proteins were eluted with the supplied elution buffer pH 2.5 (3 × 0.05 mL). Both the acidic eluate and the immunoaffinity matrix were neutralized with 2 M Tris–HCl buffer pH 8, and the matrix was washed with the dilution buffer before application of the next sample.

Electrophoresis, immuno- and lectin-blotting

Sera, solubilized membrane proteins and various protein fractions obtained by immunoaffinity chromatography or immunoprecipitation were subjected to SDS-PAGE under reducing conditions (A Guide to Polyacrylamide Gel Electrophoresis and Detection, BioRad). Separated proteins were transferred to nitrocellulose membrane and analyzed by immuno- and lectin-blotting. For immunoblotting, the following primary antibodies were used: goat anti-IGFBP-2, rabbit anti-α5 integrin subunit (Santa Cruz Biotechnology, Santa Cruz, USA), rabbit anti-α2M (AbD Serotec, Kidlington, UK), rabbit anti-MMP-7 (Abcam, Cambridge, UK) and rabbit anti-DNP (Sigma-Aldrich, St. Louis, USA). The corresponding HRP-conjugated secondary antibodies used were swine anti-goat IgG (Biosource, Camarillo, USA) and sheep anti-rabbit IgG (AbD Serotec, Kidlington, UK). Lectin-blotting was performed by using biotinylated lectins SNA (*Sambucus nigra*) and MAL (*Maackia amurensis*), coupled with HRP-avidin (Vector, Burlingame, USA). Enhanced chemiluminescence (ECL) reagent (Pierce Biotechnology, Rockford, USA) was employed for visualization of the immunoreactive proteins and their masses were determined using molecular markers from Sigma-Aldrich (St. Louis, USA).

Statistical analysis

Numerical data are expressed as the mean (X) and standard deviation (SD), or as the range, where appropriate. The differences between two groups of results were assessed by Student's t test, with statistical significance set at $P < 0.05$.

Table 2
Concentration of membrane-bound IGFBP-2, relative abundance of monomer and fragmented IGFBP-2, and relative abundance of α5 integrin subunit and MMP-7 in solubilized membrane proteins obtained from healthy and cancer colon tissue, expressed as the range.

	Healthy tissue n = 15	Tumor tissue n = 15
Concentration of IGFBP-2 (nmol/g protein)	0.03–0.08	0.14–0.28*
Monomer IGFBP-2 (ADU)	9–15	15–354
IGFBP-2 fragment (ADU)	9–503	10–722
α5 integrin subunit (ADU)	16–109	28–880
MMP-7 (ADU)	32–136	73–292

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

Results

Concentrations of serum IGFBP-2 and relative abundance of monomer, fragmented and complexed forms of IGFBP-2 in sera from healthy persons and patients with colorectal cancer are given in Table 1. Representative IGFBP-2 profiles obtained by immunoblotting are shown in Fig. 1A (four samples from each group). As it can be seen, the concentration of IGFBP-2 (determined by ELISA) and the relative abundance of IGFBP-2 monomer and its fragment (determined by densitometry) is greater in sera from patients with colorectal cancer than in controls. On the contrary, the amount of IGFBP-2 in complex with α2M is reduced. In order to investigate whether diminished amount of IGFBP-2/α2M is due to decreased concentration of α2M in patients with cancer, α2M immunoblotting was performed (Fig. 1B). According to densitometric analysis, there were no statistically significant differences between two groups of persons (Table 1). As the amount of IGFBP-2 fragments was much greater in patients' sera and MMP-7 is known to be IGFBP-2 protease, MMP-7 immunoblotting was used to investigate the presence of this enzyme (Fig. 1C). Densitometric evaluation did not reveal increased presence of MMP-7 in patients' sera.

The concentration of IGFBP-2 in the final membrane preparations was estimated by densitometry, as it was below the sensitivity of ELISA. Serially diluted ELISA IGFBP-2 standard and a serum with known concentration of IGFBP-2 were subjected to electrophoresis and IGFBP-2 immunoblotting in parallel with solubilized membrane proteins. The concentration of IGFBP-2 was expressed per gram of membrane proteins (Table 2). In general, all results for membrane preparations exhibited huge differences between samples. When paired specimens (non-cancerous and cancer) from one individual were analyzed, there were cases with the similar amount of IGFBP-2 in both samples, with greater amount in tumor tissue or even greater amount in a tissue classified as non-cancerous. Representative IGFBP-2 immunoblot illustrating these cases is shown in Fig. 2A (four paired samples). Due to large differences, data obtained for membrane proteins are given as the range (Table 2). Since IGFBP-2 is known to interact with α5β1 integrin, the idea was to investigate whether there is correlation between the amount of IGFBP-2 and this integrin on colon membranes (this interaction was previously confirmed by dotblot, data not shown). Immunoblotting with anti-α5 subunit antibody demonstrated significant interindividual differences. There were persons with greater or smaller amounts of α5β1 integrin, both in non-cancerous and cancer portions of the colon (Fig. 2B, Table 2), and there was no regularity that could link the quantities of IGFBP-2 and α5β1 integrin. Immunoblotting with anti-MMP-7 antibody was performed to assess the amount of membrane-associated protease and it revealed primarily interindividual differences, not between non-cancerous and tumor samples (Fig. 2C, Table 2).

Finally, the existence of posttranslational modifications of the investigated proteins was examined. IGFBP-2 oxidative change expressed as carbonylation, α2M carbonylation and α2M sialylation were analyzed after immunoprecipitation of IGFBP-2 from serum and membrane samples, α2M from serum samples, and IGFBP-2 and α2M from sera

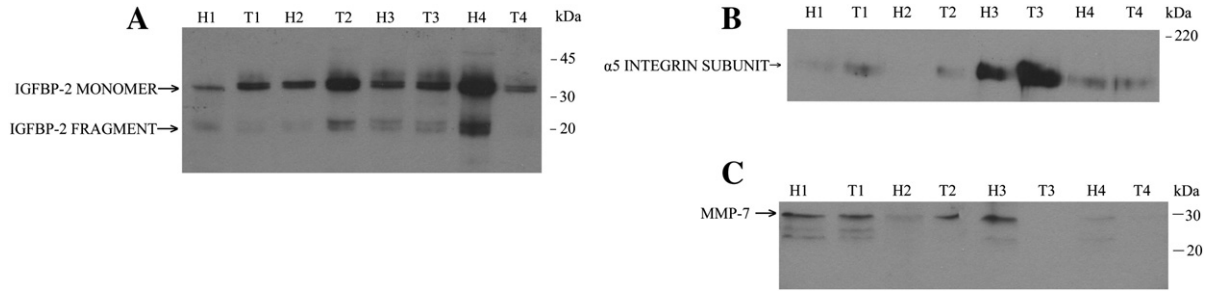

Fig. 2. The expression of A. IGFBP-2, B. α5 integrin subunit, and C. MMP-7 in membrane solubilizates obtained from non-cancerous (H1–H4) and cancerous colon tissue (T1–T4) from patients with colorectal cancer.

previously derivatized with DNP. The experimental conditions for immunoprecipitation were sub-optimal and they were chosen to isolate the same amounts of IGFBP-2 or α2M from both groups of samples. Posttranslational changes, therefore could be directly compared. Immunoblotting with anti-DNP antibody demonstrated that both IGFBP-2 and α2M were increasingly oxidized in sera from patients with colorectal cancer (Fig. 3A and B, Table 3), whereas no immunoreactive proteins were seen with IGFBP-2 immunoprecipitated samples obtained after DNP-derivatization of membranes. As shown by lectin-blotting, serum α2M was increasingly sialylated in patients (Fig. 3C and D, Table 3). Densitometric analysis of signal intensities enabled calculation of the relative ratios between signals, and it was found that α2M in sera from patients with colorectal cancer had almost twice as much α2,3 sialic acid than α2M in sera from healthy persons.

Discussion

The cancer arises as a consequence of uncontrollable cell division, which occurs when the body control mechanisms fail (National Cancer Institute, Defining Cancer). There are many events involved in cancer development and overproduction of growth factors is among the most important (Michell et al., 1997). Several studies have shown that IGFs have stimulatory effect on the cell division (Collett-Solberg and Pinchas, 1996) and that their availability is regulated by IGFBPs. Majority of IGFs in the circulation of healthy persons forms ternary complexes which include IGFBP-3 and, due to their size of approximately 150 kDa, these complexes are retained in blood vessels representing IGF reservoir (Grimberg and Cohen, 2000). In patients with colorectal (and some other) carcinoma, the concentration of IGFBP-3 is significantly reduced (Ma et al., 1999) and the concentration of IGFBP-2 increased (Liou et al., 2010). When the molar concentrations of these two binding proteins are compared, it can be seen that the amount of IGFBP-3 is several times greater than IGFBP-2 in healthy persons, whereas in patients with colon cancer their concentrations are similar (El Atiq et al., 1994). IGFBP-2, in contrast to IGFBP-3, forms binary complexes with IGFs whose size is approximately 43 kDa. These complexes can freely cross the endothelial barrier and transport IGFs to local tissues. Therefore, the role of IGFBP-2 seems to be much more important in patients with cancer than in healthy individuals.

Our results have confirmed that the concentration of serum IGFBP-2 (determined by ELISA) is higher in patients with tumor than in the control group, but the distribution of different molecular forms is not the same. The amount of monomer IGFBP-2 (determined by densitometry) is, on average, 20–30% greater in patients, the amount of fragmented IGFBP-2 is doubled compared to healthy people, whereas the portion of IGFBP-2 in complex with α2M is 2.5 times lower in cancer patients. According to this distribution, IGFBP-2 is not only increasingly synthetized in patients with cancer, but also the amount involved in complexes with α2M is significantly reduced favoring the existence of binary IGFBP-2/IGF complexes, from which IGFs are liberated due to intensive IGFBP-2 proteolysis. When the relative amounts of different IGFBP-2 forms are compared, in healthy persons IGFBP-2/α2M complexes make approximately 20% of the total amount of IGFBP-2 molecules. 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. At the moment, it is unknown whether IGFBP-2 in complexes with α2M binds IGFs.

In tissues, IGFBP-2 can interact with the elements of the extracellular matrix via its RGD sequence, enabling the existence of the local IGF reservoir from which IGF molecules may be slowly released upon local cell demand. In this work we have investigated the amount of colon cell membrane-associated IGFBP-2 and the possible correlation with the cell-membrane bound α5 subunit of the integrin α5β1, which is known to be expressed on colon cells (Srichai and Zent, 2010). Results have shown great variability between patients, which disallowed firm conclusions to be made. A possible source of variability may be the stage of a disease and the relative proportion of different cells in tumor. It is known that the transformed cells of the colon can increasingly express and secrete MMP-7, a protease which was shown to degrade IGFBP-2/IGF-II complexes liberating IGF-II (Miyamoto et al., 2007). It was also found that MMP-7 can associate with the cell membrane where it modifies surface proteins inducing loose aggregation of tumor cells (Kioi et al., 2003). Our results did not reveal difference in the level of membrane-associated

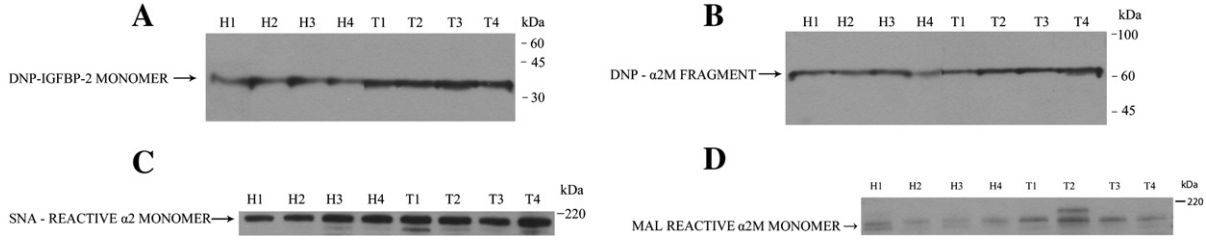

Fig. 3. The presence of the carbonylated, DNP-derivatized A. IGFBP-2 and B. α2M, and sialylated C. SNA-reactive and D. MAL-reactive α2M in serum from healthy persons (H1–H4) and patients with colorectal cancer (T1–T4).

Table 3
Relative abundance of carbonylated IGFBP-2 and α2M, and sialylated α2M 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
Carbonylated IGFBP-2 (ADU)	117 ± 17	202 ± 20[a]
Carbonylated α2M (ADU)	109 ± 16	189 ± 53[a]
SNA-reactive α2M (ADU)	287 ± 116	444 ± 51[a]
MAL-reactive α2M (ADU)	29 ± 13	70 ± 30[a]

[a] Statistically significant difference ($P < 0.05$).

MMP-7 between non-cancerous and cancer tissue, nor in the content of MMP-7 in serum from healthy persons and patients with colorectal cancer. Although MMP-7 was identified as IGFBP-2 protease, it may be suggested that it is not the only protease responsible for so pronounced IGFBP-2 degradation.

Posttranslational modifications of proteins may cause changes in their affinity for ligands, the rate of their enzymatic cleavage, and the reactivity towards other biomolecules (Westwood et al., 2001). Protein oxidation and altered glycosylation are the most common modifications related to cancer development (Shacter, 2000; Qui et al., 2008). An increased oxidative stress is one of the hallmarks of cancer (Yeh et al., 2010). Protein oxidation occurs due to increased exposure of molecules to free radicals (Shacter, 2000). Several factors determine protein susceptibility to oxidation: the nature and concentration of a specific protein, the (patho)physiological surrounding, the nature and concentration of free radicals, and the defense capacity to inactivate them (Stadtman and Levin, 2000). Protein carbonylation is a modification that generates the most reliable markers of protein oxidation (Levin et al., 1990). A degree of IGFBP-2 and α2M carbonylation was studied in this work and it was found that both proteins were significantly more oxidized in patients with colon cancer than in healthy individuals. Increased oxidation of IGFBP-2 and α2M may be important for the IGFBP-2/α2M complex dissociation, which was found to be more intensive in patients. There is evidence that the amount of thioredoxin, a redox protein that promotes dissociation of α2M from some of its ligands (Borth et al., 1990), is increased in some tumors, including colorectal cancer (Reuter et al., 2010), so it may be postulated that thioredoxin is also involved in the regulation of the amount of IGFBP-2 bound to α2M.

As it was stated in the introductory part, increased sialylation of proteins is commonly found in cancer disease, as this modification enables longer lifespan of certain molecules and cells (Abdul and Abbass, 2009). Increased reactivity of α2M isolated from patients' sera towards SNA (specific for α2,6 terminal sialic acid) and MAL lectin (specific for α2,3 terminal sialic acid) was detected. As in the case of carbonylation, it may be postulated that increased sialylation of α2M, besides affecting the half-life of α2M, may also influence the stability of IGFBP-2/α2M complexes.

In this study we have raised some questions on the molecular events that are connected with the tumor-related involvement of IGFBP-2, which may be part of the etiology and/or adaptive response to the development of colorectal cancer. Our investigation revealed that, besides being increasingly synthesized, IGFBP-2 in patients with cancer tends to dissociate from complexes with α2M. This dissociation controls IGFBP-2 availability and its freedom to leave blood vessels and/or its degradation, which enables liberation of bioactive IGFs. Also, oxidation of IGFBP-2 and α2M, and possibly increased sialylation of α2M, seem to be more important factors in determining the amount of IGFBP-2/α2M complexes than the actual quantity of these two proteins.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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