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Analysis of Four Circulating Complexes of Insulin-Like Growth Factor Binding Proteins in Human Blood during Aging

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Abstract—The primary role of insulin-like growth factor binding proteins (IGFBPs) is to regulate availability of IGFs for interacting with receptors, but IGFBPs perform IGF-independent actions as well. The availability and activity of IGFBPs in the circulation is influenced primarily by their concentration and structural modifications, but possibly also by interaction with major plasma proteins such as transferrin, alpha-2-macroglobulin (α2M), and fibrinogen. Four types of circulating IGFBP complexes were examined in this study by immuno- and ligand-binding assays in adults of different age. The amounts of IGFBP-3/transferrin and IGFBP-1/fibrinogen complexes were similar in middle- and old-aged persons, whereas the amounts of IGFBP-1 (or -2)/α2M monomer complexes were lower in the old-aged group and negatively correlated with total IGFBP-1 (or -2) amounts in blood. In contrast to IGFBP-1, IGFBP-2 was present in significantly greater quantities in complexes with α2M dimer than α2M monomer in older individuals. IGFBP complexes did not bind ^{125}I-labeled IGF-I in amounts detectable by ligand blotting. According to the results of this study, the quantities of IGFBP-1 and IGFBP-2, which interact with α2M, are age-dependent and, in the case of complexes with α2M monomer, they are negatively correlated with the total circulating levels of these two IGFBPs.

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Reduced activity of the growth hormone/insulin-like growth factor (IGF) axis has been recognized as a biomarker of delayed aging, longer life, and reduced risk of age-related diseases [1-3]. Activity of IGF peptides depends on their availability to interact with receptors, which is regulated by IGF-binding proteins (IGFBPs) [4]. IGFBPs, however, perform IGF-independent actions as well, after binding to cell membranes or components of the extracellular matrix, initiating signaling pathways that do not activate IGF receptors [5-7]. There are six high- and one low-affinity binding IGFBPs [7-9]. IGFBP-3 (43-45 kDa of glycosylated forms) is the dominant IGFBP in the circulation, IGFBP-1 (28 kDa), IGFBP-2 (35 kDa), and IGFBP-4 (26 kDa) are present in several times lower concentrations, whereas levels of IGFBP-5 and IGFBP-6 are very low in blood [5].

The activity of IGFBPs depends on posttranslational modifications [5, 6, 10]. For analysis of IGFBPs, their concentrations and structures are routinely considered. There is, however, another factor that may influence IGFBP availability and function – interaction with other plasma proteins. Several complexes were detected under physiological conditions, some with major plasma proteins (concentrations ranging from 2 to 4 g/liter): IGFBP-3/transferrin [11, 12], IGFBP-1 or IGFBP-2/alpha-2-macroglobulin (α2M) [13, 14], and IGFBP-1/fibrinogen [15]. These complexes are relatively stable and can be detected by immunochemical methods.

Aging is generally characterized by a decrease in the concentration of IGFBP-3 and an increase in IGFBP-1, IGFBP-2, and IGFBP-4. Reduced total protein concentration is often seen in elderly, the concentration of transferrin (79 kDa of the glycosylated form) may remain unchanged or slightly decrease [16], whereas concentrations of α2M (glycosylated monomer is 179 kDa; tetramer form is dominant under physiological conditions) and fibrinogen (340 kDa of the native glycosylated molecule) can increase in response to age-related inflammation [17, 18].

Considering the importance of the IGF system and its involvement in aging, it seems relevant to investigate whether formation of IGFBP complexes with plasma proteins changes with aging.

Abbreviations: α2M, alpha-2-macroglobulin; BMI, body mass index; DU, densitometric unit; IGF, insulin-like growth factor; IGFBP, IGF-binding protein.

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MATERIALS AND METHODS

Study population and samples. The population studied in this work included healthy adult volunteers divided into two groups: age 30-60 years (n = 40, 22 women and 18 men, BMI 20-31 kg/m^2) and 61-90 years (n = 40, 21 women and 19 men, BMI 19-32 kg/m^2). Their blood samples were collected after overnight fasting. Routine biochemical and hematological examination of the samples was carried out to confirm the absence of significant deviation of the analyte concentrations from the reference values and the surveyed denied awareness of illness. The persons underwent brief medical examination in the Clinical-Medical Centre "Beñanijska Kosa" to exclude "unknown" or "hidden" diseases (such as diabetes, cardiovascular or pulmonary disease, or cancer). The participants were not under any specific nutritional regime or professionally involved in sports. They gave informed consent to use their samples for this investigation. The study was approved by the ethics committees of Clinical-Medical Centre "Beñanijska Kosa" and Institute INEP. Plasma and serum were separated from whole blood samples (collected with and without anticoagulant) and used for further experiments.

Measurement of protein and protein-carbonyl concentrations. Protein concentrations were measured using commercial reagents: biuret for total proteins (Human GmbH, Germany), immunoturbidimetric assay for transferrin (Elitech, France), ELISA for IGFBP-1, IGFBP-2, IGFBP-3, and α2M (Abcam, UK), and Fowell reagent for fibrinogen (Alfapanon, Serbia). According to the manual that accompanies each ELISA IGFBP test, the producer declares that the assay is "an *in vitro* enzyme-linked immunosorbent assay for the quantitative measurement of human IGFBP in serum, plasma, and cell culture supernatants", without specification whether it is detecting only monomeric form or IGFBP in complexes can be recognized to some extent as well. Protein carbonyls were determined by the DNPH method [19]. Concentrations of all analytes were measured in serum except for fibrinogen, which was measured in plasma.

Electrophoresis and immunoblotting. SDS-PAGE (on 8% gels) under reducing condition and immunoblotting were performed to analyze IGFBP complexes with transferrin or α2M in serum, whereas complexes with fibrinogen were investigated by native electrophoresis after isolation of fibrinogen from plasma, as native fibrinogen is needed for binding of IGFBP-1 [15]. For the analysis of IGFBP/α2M complexes, serum samples were diluted 1 : 20, while dilution of 1 : 40 was optimal for investigation of IGFBP-3/transferrin complexes. The presence of IGFBP-1/fibrinogen complexes was studied in 1 g/liter solutions of the isolated fibrinogen. The following primary antibodies were used: goat anti-IGFBP-2 (Santa Cruz Biotechnology, USA) and anti-IGFBP-3 (DSL, USA), rabbit anti-IGFBP-1, anti-α2M (AbD Serotec, UK) and anti-fibrinogen (Abcam), and sheep anti-transferrin (INEP, Serbia). HRP-conjugated secondary antibodies were anti-goat IgG, anti-sheep IgG (Biosource, USA) and anti-rabbit IgG (AbD Serotec), and ECL substrate (Pierce, Thermo Scientific, USA) enabled protein detection by chemiluminescence.

To investigate one type of complexes, two parallel electrophoretic plates with the same set of samples were run and; after electrotransfer, one membrane was immunoblotted with anti-IGFBP antibody and the other with antibody to one of the binding partners. Membranes were exposed to X-ray film for the same time; longer periods were needed to visualize complexes than monomer forms (20-30 min). Protein bands whose positions overlapped with two antibodies corresponded to complexes. Positions of complexes were determined in previous studies (mostly by double immunoprecipitation) and published elsewhere [11-13, 15].

Densitometric evaluation of protein bands was performed (Phoretix 1D software; TotalLab, UK) and signals (in densitometric units, DU) normalized taking into consideration protein concentrations (assessed by Ponceau S staining of membranes prior to immunodetection). One set of samples consisted of seven individual samples from the middle-aged group and seven from the old-aged group of persons. All individual samples were analyzed, divided into sets, and direct comparison between two groups was made for each set. Results were expressed in three ways: (i) as densitometric intensity of signals originating from IGFBP in complexes (densitometric units, DU), (ii) as portion of particular IGFBP in complexes compared to total amount of that IGFBP measured in serum by ELISA (DU/nM), and (iii) as relative ratio of signals IGFBP/binding partner in protein bands containing complexes (DU/DU).

Electrophoresis and ligand blotting. SDS-PAGE (on 12% gel) under nonreducing condition and ligand blotting with ^{125}I-labeled IGF-I (10^6 cpm) were performed to analyze IGF-I binding to IGFBPs in plasma [20]. Chosen conditions were optimal, as monomer IGFBPs were not clearly separated from each other in electrophoresis on 8% gel, and nonreducing conditions preserved ligand-binding sites in IGFBPs. For this experiment, two plasma pools were made, one for each age group, containing equal portions of individual plasma samples. Ligand-binding of ^{125}I-labeled IGF-I to IGFBPs was detected by autoradiography.

Statistical analysis. All data were checked by the Shapiro–Wilk normality test. Some results were normally distributed, whereas others were not. Thus, all results are presented as medians (Me) and 2.5-97.5 percentile ranges, and the differences between two groups were assessed by the Mann–Whitney U-test (with statistically significant difference set at $p < 0.05$).

RESULTS

Measurement of protein and protein-carbonyl concentrations. Determination of protein concentrations indicated that individuals in the old-aged group had significantly lower concentrations of total proteins and transferrin and higher concentrations of α2M, IGFBP-1, and IGFBP-2 than the middle-aged persons (Table 1).

Concentration of fibrinogen was higher and concentration of IGFBP-3 lower in elderly people, but not statistically significantly. Protein carbonyls were determined to assess robustly the extent of protein oxidation, which can possibly interfere with interaction between binding partners. As seen in Table 1, the amount of protein carbonyls in the old-aged group was greater than in the middle-aged persons, but not significantly, confirming that redox status in our participants was not seriously affected by aging and probably did not impair complex formation.

Electrophoresis and immunoblotting. Immunoblotting of serum and fibrinogen samples pointed to certain differences in complexes in the old-aged compared to the middle-aged group. Representative immunoblots for complexes are shown in Fig. 1 (7 + 7 individual samples from each age group). IGFBP-3/transferrin complexes were separated as distinct molecules from their constituent monomers in electrophoresis on 8% gels (between molecular markers of 100 and 180 kDa; Fig. 1a), whereas IGFBP complexes with α2M (close to marker of 180 kDa with α2M monomer and above marker of 300 kDa with α2M dimer; Figs. 1c and 1d) or fibrinogen (Fig. 1b) could not be clearly resolved from IGFBP-free α2M or fibrinogen species due to their similar masses. The predominant type of IGFBP-1/α2M complexes seen was with α2M monomer (Fig. 1c), whereas IGFBP-2 was detected in complexes with both α2M monomer and dimer (Fig. 1d). No complexes were detected with α2M tetramer (most likely due to tetramer decomposition during SDS electrophoresis). This last result was deduced from Fig. 1d, taking into consideration the existence of two α2M immunoreactive protein bands above marker of 300 kDa and just one IGFBP-2 immunoreactive band corresponding to the lower mass of these two α2M bands.

The amounts of IGFBP-3/transferrin (Fig. 1a) and IGFBP-1/fibrinogen complexes (Fig. 1b) were similar in the two population groups, whereas the amount of IGFBP-1/α2M monomer complexes (Fig. 1c) was lower in the old-aged group. In contrast to IGFBP-1, IGFBP-2 was present in significantly greater quantities in complexes with α2M dimer than α2M monomer (Fig. 1d). A comparison of densitometric signals originating from binding partners was performed and the results expressed in three ways: (i) as densitometric intensity of signals originating from IGFBP in complexes (DU), (ii) as portion of particular IGFBP in complexes compared to total amount of that IGFBP measured in serum by ELISA (DU/nM), and (iii) as relative ratio of signals IGFBP/binding partner in protein bands containing complexes (DU/DU).

As seen from Table 2, the relative amounts of IGFBP-1 and IGFBP-2 in complexes with α2M monomer were lower in the older people than in the middle-aged. The portion of these two IGFBPs in complexes compared to total amounts of IGFBPs was twice lower in both cases (expressed as DU/nM), i.e. they were negatively correlated ($r > 0.7$). The amount of IGFBP-2

Table 1. Protein and protein-carbonyl concentrations in serum/plasma from healthy middle- and old-aged persons ($n = 40$ in each group, Me and 2.5-97.5th range)

Concentration	Age group 30-60 years	Age group 61-90 years
Total protein, g/liter	73.1 (68.75-79.57)	67.9 (55.51-80.35)*
Transferrin, g/liter or μM	2.7 (2.40-2.97) or 33.7 (30.01-37.12)	2.1 (1.19-3.21) or 26.2 (14.87-40.12)*
α2M, g/liter or μM	2.1 (1.56-4.25) or 2.9 (2.12-5.78)	5.2 (2.68-9.96) or 7.0 (3.64-13.54)*
Fibrinogen, g/liter or μM	3.1 (2.42-4.55) or 9.1 (7.09-13.33)	3.7 (2.90-4.55) or 10.8 (8.50-13.33)
IGFBP-1, μg/liter or nM	7.75 (3.100-22.692) or 0.25 (0.100-0.732)	15.50 (1.240-59.055) or 0.50 (0.040-1.905)*
IGFBP-2, μg/liter or nM	428 (163.8-628.28) or 11.9 (4.55-17.73)	594 (263.16-1317.24) or 16.5 (7.31-36.59)*
IGFBP-3, μg/liter or nM	4044 (3039.1-4331.9) or 96.3 (72.36-103.14)	3755 (2267.2-4197.5) or 89.1 (53.98-99.94)
Protein carbonyls, μmol/g protein	0.44 (0.344-0.554)	0.55 (0.287-1.024)

* Statistically significant difference between the two groups (at $p < 0.05$).

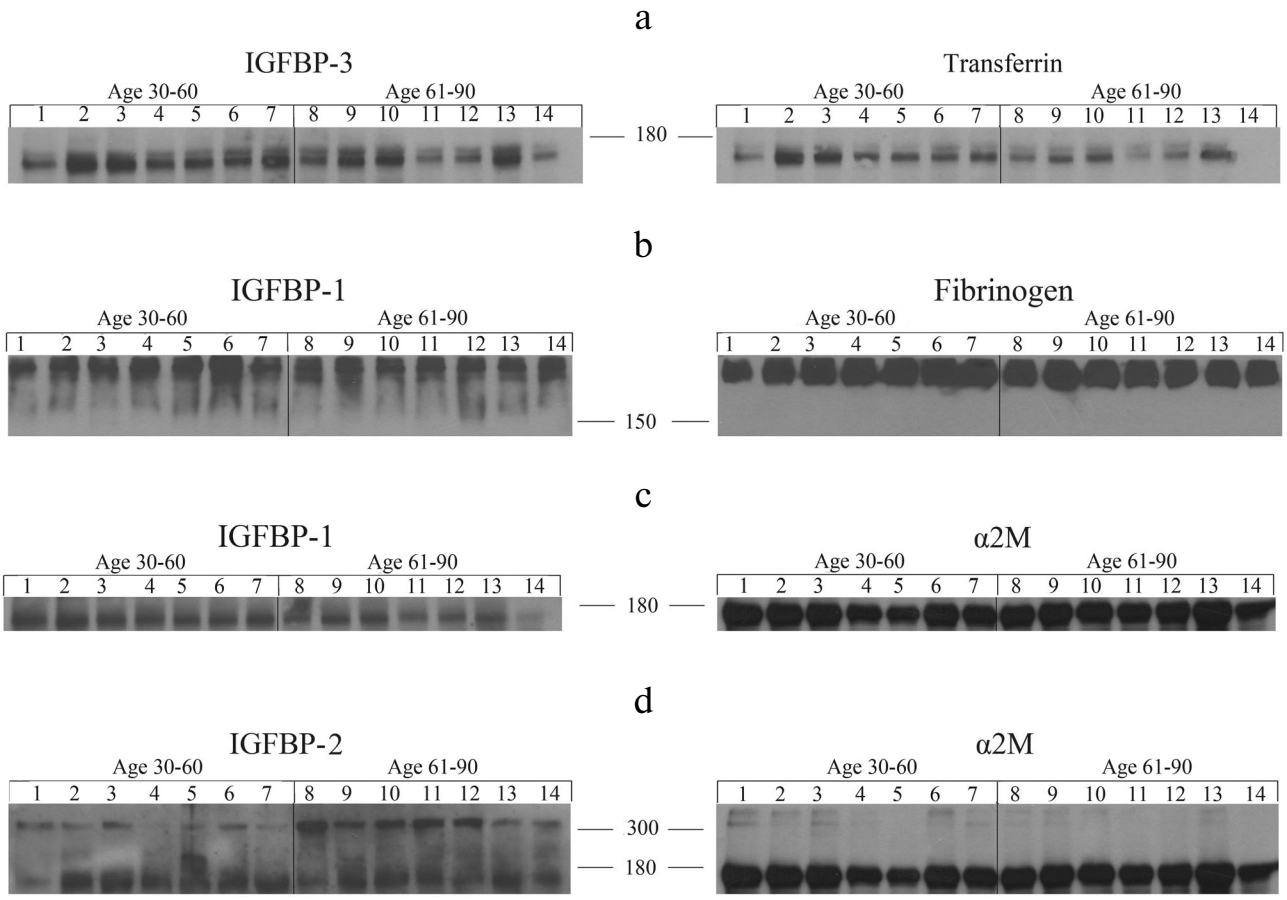

Fig. 1. Representative immunoblotting results for complexes: IGFBP-3/transferrin (a), IGFBP-1/fibrinogen (b), IGFBP-1/α2M (c), and IGFBP-2/α2M (d) in the circulation of healthy middle-aged (1-7) and old-aged (8-14) persons. Panels on the left side monitor the presence of specific IGFBP (-1, -2 or -3) in complexes and on the right side of its binding partner (transferrin, fibrinogen, or α2M). SDS-PAGE of serum samples (on 8% gels) under reducing condition was performed to analyze IGFBP complexes with transferrin or α2M, whereas complexes with fibrinogen were investigated by native electrophoresis after isolation of fibrinogen from plasma. Molecular mass markers are shown between panels; different set of markers were used for SDS and native PAGE.

bound to α2M dimer was greater in the persons above 60 years than in the younger individuals, but when that quantity was normalized to total IGFBP-2 concentration, the relative portion of IGFBP-2 in complexes with α2M dimer was found to be unchanged due to aging. In other words, smaller involvement of IGFBP-1 and IGFBP-2 in complexes with α2M monomer was detected in the older persons than in the middle-aged, whereas increased concentration of IGFBP-2, which accompanied aging, correlated with its increased association with α2M dimer.

No differences were seen between males and females. In preliminary data analysis, individuals and their results were grouped into age groups with shorter time frames of 10 or 20 years. Sharp limits between groups were not discernible and some degree of heterogeneity was noticed in each of them, thus not pointing to any better division of participants than the one reported in this study.

Electrophoresis and ligand-blotting. Ligand-blotting with ^{125}I-labeled IGF-I (Fig. 2) clearly indicated IGF-I binding to monomer IGFBPs in plasma, but not to higher molecular species (the entire gel is shown), suggesting that IGFBP complexes with transferrin, α2M, or fibrinogen either did not bind ^{125}I-labeled IGF-I or bound very little quantities, not detectable by the ligand-blotting technique. The difference between the two study groups in IGFBP-3 signal intensity was greater than the difference found when IGFBP-3 concentration in sera was measured by ELISA, but one must bear in mind that ligand blotting was performed with pooled samples and that signal intensity depended on both IGFBP-3 quantity and reactivity (which could also be affected by aging).

DISCUSSION

Aging is associated with significant changes in metabolism affecting almost all physiological systems and resulting in gradual alteration (reduction) of functions. Regulatory mechanisms involved in (healthy) aging and

Table 2. Relative amounts of IGFBPs in complexes determined from chemiluminescent signals originating from IGFBPs and their binding partners in particular complex types in serum/plasma from healthy middle- and old-aged persons (n = 40 in each group, Me and 2.5-97.5th range)

Relative amounts	Age group 30-60 years	Age group 61-90 years
IGFBP-3 in complexes with transferrin, DU	3.8 (3.29-4.52)	3.6 (2.92-4.21)
IGFBP-3 in complexes/concentration, DU/nM	0.05 (0.040-0.077)	0.04 (0.031-0.057)
IGFBP-3/transferrin in complexes, DU/DU	1.1 (1.00-1.25)	1.3 (1.13-1.81)
IGFBP-1 in complexes with fibrinogen, DU	8.3 (6.72-10.45)	8.7 (7.74-9.71)
IGFBP-1 in complexes/concentration, DU/nM	37 (23.4-75.0)	24 (17.8-33.3)
IGFBP-1/fibrinogen in complexes, DU/DU	0.8 (0.42-1.07)	0.8 (0.58-0.85)
IGFBP-1 in complexes with α2M monomer, DU	2.0 (1.85-2.15)	1.6 (0.85-1.90)*
IGFBP-1 in complexes with α2M monomer/concentration, DU/nM	6.3 (2.91-19.0)	3.2 (0.87-5.99)*
IGFBP-1/α2M in complexes with α2M monomer, DU/DU	0.24 (0.223-0.271)	0.18 (0.115-0.205)*
IGFBP-2 in complexes with α2M monomer, DU	1.9 (0.97-1.82)	1.7 (0.91-1.95)
IGFBP-2 in complexes with α2M monomer/concentration, DU/nM	0.12 (0.060-0.379)	0.05 (0.030-0.082)*
IGFBP-2/α2M in complexes with α2M monomer, DU/DU	0.23 (0.118-0.241)	0.20 (0.107-0.230)
IGFBP-2 in complexes with α2M dimer, DU	0.8 (0.38-1.01)	1.9 (0.86-1.99)*
IGFBP-2 in complexes with α2M dimer/concentration, DU/nM	0.06 (0.042-0.080)	0.06 (0.030-0.098)
IGFBP-2/α2M in complexes with α2M dimer, DU/DU	2.2 (1.25-4.12)	4.1 (2.02-7.55)*

* Statistically significant difference between the two groups (at $p < 0.05$).

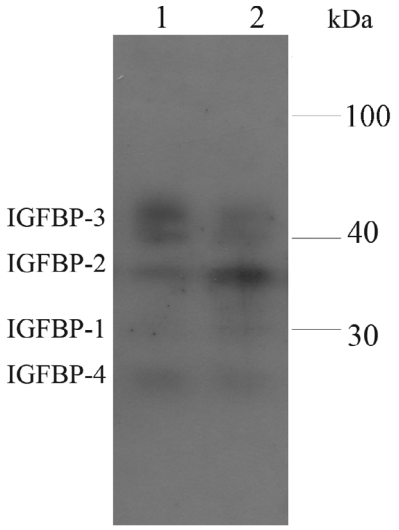

Fig. 2. Ligand-blotting results for IGFBPs in plasma. Two plasma pools were made: for age group 30-60 (1) and age group 61-90 (2), containing equal portions of individual plasma samples. SDS-PAGE (on 12% gels) under nonreducing condition and ligand blotting with ^{125}I-labeled IGF-I (10^6 cpm) were performed. Ligand binding was detected by autoradiography. Identities of monomer IGFBPs are shown on the left side and molecular mass markers on the right side.

lifespan are complex and remain unclear. Many age-related changes have been characterized, but their influence on health is insufficiently understood. In the study described here, four types of circulating IGFBP complexes were examined in relation to aging. Two exhibited age-related changes in their quantity, i.e. those containing α2M. By examining the ranges of concentrations of the investigated proteins in blood, it becomes evident that concentrations of IGFBPs are 10^3 times lower than concentrations of their binding partners, implicating that they are variables that determine the extent of complex formation. Higher concentrations of total IGFBP-1 and IGFBP-2 were measured in older individuals than in middle-aged persons (Table 1), and if the formation of complexes was only concentration-dependent, they would increase with age. As this was not the case with IGFBP/α2M monomer complexes, and only IGFBP-2 was present in significant quantities in complexes with α2M dimer (Fig. 1 and Table 2), an active mechanism may be supposed to regulate interaction of IGFBP-1 and IGFBP-2 with α2M.

IGFBP complexes with plasma proteins, therefore, appear as additional players to be taken into consideration when studying the IGF system. Besides well-known roles of IGFBPs as regulators of IGF activity and direct

participants in specific interactions and processes, the roles of their complexes are still unresolved. According to our ligand-blotting experiment, complexes were not found to bind ^{125}I-labeled IGF-I. Westwood et al. [14], however, were able to identify minute amounts of IGF-I in IGFBP-1/α2M complexes by using a very sensitive technique – surface plasmon resonance. Thus, α2M does not modify IGF/IGFBP interaction and the activity of IGF significantly.

Transferrin and, especially, α2M are large molecules that can be only actively transported from the circulation to extravascular space where they interact with cell-surface receptors [21, 22]. Fibrinogen, on the other hand, is activated *in situ* in the case of bleeding or injury [23]. IGFBPs in complexes, therefore, can possibly reach target tissues possessing transferrin or α2M receptors and/or become located at specific positions, such as a fibrin cloth. According to our data, formation of IGFBP complexes with α2M is influenced by aging.

Each subunit in tetramer α2M possesses several binding sites specific for capturing proteases, cytokines, growth factors, amyloid β, and zinc ions, and for interaction with receptors [24]. Growth factors associated with native α2M are inactive, but, since this interaction is reversible, α2M complexes may be seen as a reservoir involved in the regulation of free growth factors [25]. Ligands bound to α2M may be also directed toward a signaling or degradation pathway [26, 27]. Even though we do not know the role of IGFBPs in complexes, reduced amounts of IGFBP/α2M complexes in old-aged people compared to middle-aged, which co-exist with increased total concentrations of IGFBP-1 and IGFBP-2, suggest weakening of the reservoir potential with age.

Receptor for α2M itself plays a dual role, as a scavenger receptor that mediates endocytosis of ligands, and as signaling receptor that transduces intracellular signals [27]. It is involved in integrin signaling, which regulates survival of healthy and cancer cells and their proliferation, adhesion, and migration [28]. Both IGFBP-1 and IGFBP-2 perform their IGF-independent roles by interacting with integrins [5]. Only these two IGFBPs possess a tripeptide Arg-Gly-Asp known as RGD sequence. This coincidence is worth mentioning.

The degree of inflammation was recently proposed to be a measure of biological aging, the so-called inflame-aging predictor of mortality in old people [29]. Investigation on naked mole rat, a long-lived animal resistant to cancer, pointed to possible role of α2M as anti-aging and anti-cancer agent [30]. On the other hand, increased concentrations of IGFBP-1 and IGFBP-2 with aging are linked to metabolic diseases and tumor development [5, 31]. According to the results of this study, the quantities of IGFBP-1 and IGFBP-2, which interact with α2M, are age-dependent and, in the case of complexes with α2M monomer, they are negatively correlated with total quantities of these two IGFBPs in blood.

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