Development and Functional Characterization of Human Antibodies against Galectin-3

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Abstract- Galectin-3 (Gal-3) is a 26 kDa galactoside binding protein found both inside and outside of cells. An increase of Gal-3 level in serum correlates with progression of heart failure, fibrosis, and some types of malignant tumors. The aim of this work was to obtain recombinant human monoclonal antibodies to galectin-3. Using the phage display method, we obtain six unique anti-Gal-3 antibodies with affinities ranging from 33 to 0.12 nM. All the antibodies specifically increase the ability of galectin-3 to agglutinate red blood cells. Two of the obtained antibodies reduce collagen expression in human fibroblasts in a dose-dependent manner. Thus, the obtained antibodies represent a useful tool for the future research of Gal-3 functions, and, potentially, for diagnostics and therapeutic application.

Keywords- galectin-3, antibodies, phage display, agglutination, fibrosis.

I. INTRODUCTION

Galectin family of proteins includes 14 galactoside-binding members [1]. Human Gal-3 has molecular mass 26 kDa and its structure differs from the structures of other galectins [2]. It consists of a single polypeptide chain, which is folded into two domains: atypical N-terminal domain and carbohydrate-recognizing C-terminal domain [2]. This protein can act as a modulator of cellular adhesion, because it binds glycoproteins and glycosylated components of the extracellular matrix. It also has been shown that Gal-3 binds laminin, fibronectin and collagen IV [3]. Gal-3 can stimulate capillary tube formation of endothelial cells in vitro, and this effect is inhibited by modified citrus pectin, an inhibitor specific to Gal-3 [4]. In healthy individuals, Gal-3 is present in epithelium of many organs, eosinophils and macrophages. Its concentration in normal serum varies 3.2±1.6 ng/ml [6].

It is well known that Gal-3 is involved in the progression of cancer, in liver, renal and idiopathic pulmonary fibrosis and heart failure [7, 8, 9]. Tumors overexpressing this protein are characterized by good blood supply and fast metastasis [10].

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There is good amount of evidence supporting that Gal-3 may be druggable target for the therapy of a number of diseases [7, 8, 9, 10, 11, 12]. The purpose of this work was a development, production and functional characterization of human monoclonal antibodies for human Gal-3 that are cross-reactive to Gal-3 monkey (rhesus macaque) ortholog.

II. METHODS

A. Design of expression vectors for recombinant production of human and macaque galectins-3.

To obtain cross-reactive antibodies to Gal-3 in humans and macaques (in order to expand the possibilities of preclinical testing in animals), two variants of Gal-3 were cloned.

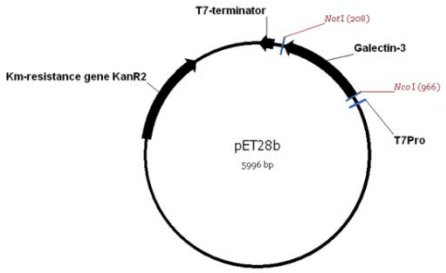

Fig. 1. A schematic map of the Gal-3-pET28b plasmid (Antherix, Puchshino, Russia) used for the E.Coli expression of human and macaque orthologs of Gal-3.

The codon-optimized genes coding for human and macaque Gal-3s were synthetized by GenScript (Piscataway, NJ, USA).

The complete Gal-3 genes were cloned in pET28b plasmid (Fig.1). Insert DNA verification sequencing was carried out by Evrogen (Moscow, Russia).

B. Expression and Purification of Recombinant galectins-3

E. coli DE3 RIL cells were transformed by electroporation and grown on a selective medium containing kanamycin. After 12 hour of incubation one colony of *E. coli* was placed in 50 ml liquid 2TY medium (16 g/L tryptone, 10 g/L yeast extract, and 5 g/L NaCl) with 0.2% glucose, and the culture was incubated overnight at 37°C under active mixing. On the next day, 8 ml of night culture were inoculated in 800 ml of 2TY without glucose. When optical density of bacterial cells suspension reached 0.5-0.6 the IPTG was added up to the final concentration of 1nM. The expression was carried out at room temperature overnight.

The bacterial cells were sedimented at 3 000 g, resuspended in 20 ml of lysis buffer (300 mM NaCl, 25 mM NaP, 8 mM DTT, 1 mM PMSF), and disintegrated by French-press. The lysate was clarified by centrifugation for 30 minutes at 9 000 g at 4^{o} C. Recombinant Gal-3 proteins

were purified by affinity chromatography on lactosyl-sepharose, as described previously [13].

C. Affinity Selection of Fab-fragments from Phage Library

The selection of Fab-fragments was carried out according to the standard protocol [14]. Gal-3 was sorbed on an immunotube (Greiner bio-one) in PBS substituted with 8 mM mercaptoethanol to prevent a dimerization of Gal-3 molecules. To obtain cross-reactive antibodies for human and macaque Gal-3, the human antigen was used in the rounds I and III, while the macaque antigen was used in the round II.

D. Screening of Individual Fab Clones Obtained from Phage Library

Phagemid DNA was isolated from cell suspension after the third round of selection by mini-prep kit (BioSilica, Novosibirsk, Russia) under the vendor's protocol. DNA encoding Fab-fragments of antibodies was amplified by PCR. The fragments were restricted, purified by gel-electrophoresis on LMP agarose (Amresco, France) and treated with β-agarase (Fermentas, Vilnius, Lithuania) at 42°C for 4 hours. The inserts were cloned in pLL vector containing ampicillin and kanamycin resistance genes. Further, *E. coli* F' were transformed by electroporation and seeded on an agarized medium containing 100 μg/ml of selective antibiotics, and 0.2% glucose. Bacterial cells were incubated at 37°C overnight and then DNA was isolated from them for subsequent screening. For screening *E. coli BL Gold* were transformed by electroporation with pLL vector obtained in the recloning process. The screening was carried out by ELISA.

E. Expression of full-size IgG1 in CHO-1 cells

The unique sequences of Fab-fragments were converted to full-size human IgG_1. LPEI, the stable polymer of polyethilenimin (polyethilenimin "Max", Polysciences, England), was used as a transfection agent. The transfectant has a positive charge therefore it can form complexes with negatively charged nucleic acid molecules and cell surface.

The mixture of DNA and LPEI was added to the cells in the exponential phase of growth. Antibodies expression was carried out for 7-10 days. The antibodies were purified by affinity chromatography using Mab-select (Sigma-Aldrich, USA) according to the standard protocol [15].

F. Surface Plasmon Resonance (SPR)

SPR measurements were performed at 25°C using Bio-Rad ProteOn™ XPR36 protein interaction array system and ProteOn GLH sensor chip (Bio-Rad, Hercules, CA, USA). 40 μg/ml human or macaque Gal-3 in 10 mM sodium acetate, pH 4.5 buffer, was immobilized on the chip surface (up to 10,000 resonance units, RUs) by amine coupling, according to the manufacturer's instructions. The remaining activated amine groups on the chip surface were blocked by 1 M ethanolamine solution. Analyte (antibodies for Gal-3, 2 to 32 nM) in a running buffer (10 mM HEPES, 150 mM NaCl, 2 mM $MgCl_2$, 0.01% TWEEN 20, pH 7.4) flowed over the chip at a rate of 30 μl/min for 350 s, followed by flushing the chip with the running buffer for 2,400 s. The double-referenced SPR sensograms were globally fitted according to a heterogeneous ligand model, which assumes existence of two populations of the ligand (L1 and L2) that bind single analyte molecule (A):

$$L1 + A \underset{kd1}{\overset{Kd1}{\longleftrightarrow}} L1A; L2 + A \underset{kd2}{\overset{Kd2}{\longleftrightarrow}} L2A \quad (1)$$

where K_d and k_d refer to equilibrium and kinetic dissociation constants, respectively. K_d, k_d and R_{max} (maximum response) values were evaluated using Bio-Rad ProteOn Manager™ v.3.1 software. The sensor chip surface was regenerated by passage of 0.5% SDS water solution for 50s.

G. Circular Dichroism Measurements

Far-UV circular dichroism (CD) studies were carried out at 20°C with a J-810 spectropolarimeter (JASCO, Inc.). The optical path of the quartz cuvette was 1 mm. The concentrations of antibodies were 0.59-0.92 μM. Each sample was measured three times. The contribution of the buffer was subtracted from the experimental spectra. Measured ellipticity of a sample was converted into specific units using the formula:

$$[\theta] = \theta/(C \times l \times z) \quad (2)$$

Where C is molar concentration of protein, l is length of optical path (mm), z is amount of amino acid residues in the protein. Quantitative estimates of the secondary structure fractions were performed using CDPro software package [16].

H. Measurements of the Temperature Dependences of Intrinsic Fluorescence of the Proteins

Fluorescence spectra of Trp residues of galectin antibodies (excitation wavelength was 280 nm; excitation monochromator bandwidth was 2.5 nm) were measured using a Cary Eclipse spectrofluorimeter (Varian, Inc.). All fluorescence spectra were corrected for spectral sensitivity of the instrument and fitted by a log-normal function (Burstein 1996). Fluorescence spectrum maximum positions (λ_{max}) were obtained from these fits. Temperature dependences of intrinsic fluorescence in the region from 20°C to 98°C were treated by the method described in [18].

I. Agglutination of Rabbit Erythrocytes

Erythrocytes agglutination was used as a qualitative test reaction for lectins. The rabbit blood (10 ml) with 30 action units of heparin was washed three times by addition of cold PBS and centrifugation at 200 g for 10 min. Agglutination analysis was carried out in 96-well V-shape plates. Various dilutions of Gal-3 and antibodies were prepared in the PBS with 8 mM mercaptoethanol to prevent a dimerization of Gal-3 molecules. After incubation of the samples at room temperature and low speed stirring for ten minutes erythrocytes were added to the mixture up to 4%. Agglutination was carried out at room temperature for 1 hour. The plate was scanned on an office scanner and the image was processed using the ImageJ program. Three typical wells with different degree of agglutination are shown in Fig. 2.

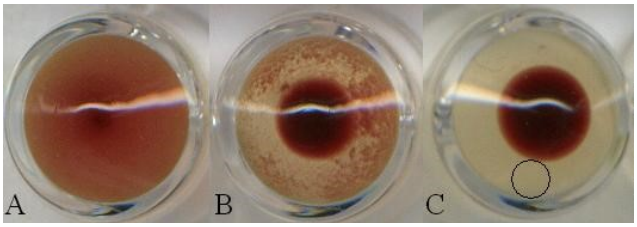

Fig. 2. A. The complete agglutination: all erythrocytes are precipitated. B. Partial agglutination: part of the erythrocytes are aggregated with each other and the rest are concentrated at the bottom of the well forming a compact circle. C. No agglutination: all red blood cells are concentrated at the bottom of the well. The agglutination intensity was evaluated using an area at the edge of the well (1380 pixels shown in Figure 3C).

Agglutination degree was assessed by analysis of image obtained with the scanner. Namely, with the help of the ImageJ program the brightness in relative units was determined in the selected area. Such an approach allows us accurate evaluating of the degree of agglutination without application of more sophisticated methods.

J. Study of the effect of antibodies on collagen expression in human fibroblasts

Fibroblasts isolated from healthy donor were kindly provided by Dr. Fadeev from Laboratory of Structure and Functions of Muscle Proteins, Institute of Theoretical and Experimental Biophysics. Cells were cultured in DMEM F12 medium. Fibroblasts were sown on 96-well plates, 5000 cells per well. It was cultivated for three weeks in DMEM F12 medium with 10% serum and various concentrations of antibodies or with phosphate buffered saline as a negative control. During this time, the medium was changed three times (once a week) while the investigated dose of antibodies was saved. Fibroblasts monolayer were washed three times by 200 µl of PBS and blocked by 50 µl of Boulin solution (75 ml of saturated picric acid, 25 ml of 38-40% formaldehyde, 5 ml of glacial acetic acid per 100 ml of solution). Fibroblasts were incubated for 1 hour at room temperature. The cells were washed three times by 200 µl of deionized water, and stained by 1 mg/ml of Sirius Red solution (Sigma Aldrich, USA) in 0.1% acetic acid and incubated for 1 hour [19]. Then fibroblasts were washed 5 times by 0.1% acetic acid and dissolved in 50 µl of 0.2 n NaOH for optical density measuring. It was carried out using a plate spectrophotometer (ThermoScientific, Waltham, MA, USA) at a wavelength of 550 nm. The results were processed using the Origin program.

III. RESULTS

A. Production of Recombinant Human galectin-3 Antibodies

We successfully produced human and macaque Gal-3 with molecular weights 26 and 27 kDa, respectively. The presence of minor proteolysis did not affect the results of further experiments. The identity of the antigen was confirmed by Western blot using commercial mouse antibodies against human Gal-3 (BioLegend, USA, Fig. 3).

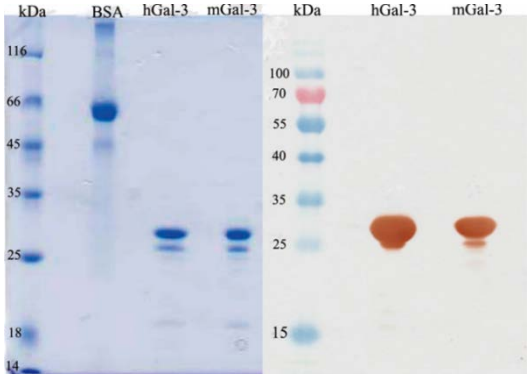

Fig. 3. Results of affinity chromatography of human and macaque galectins. Left part: electrophoresis of the proteins; Right part: Western-blots.

Production of recombinant antibodies was performed by means of affinity selection using solid-phase carriers. Conditions of the selection are shown in Table 1.

Table 1. Conditions of enrichment rounds of synthetic Fab libraries.

Round	Phage concentrati	Antigen concentrat	Blocking solution	Washings by PBST	Colonies, pcs.
I	10^{12}	5	SuperBlock	10	$2x10^5$
II	10^{12}	2	Milk2.5%	20	10^5
III	10^{12}	1	BSA 2.5%	30	$4x10^5$

Six antibodies for Gal-3 were found. Expression of antibodies D2 and H9 was very low therefore, these molecules were not used in the experiment with fibroblasts.

B. Structural Characterization of the Antibodies. Affinity Measurements

All antibodies bound both human and macaque Gal-3. The results are presented in Table 2.

Table 2. Affinities of the antibodies to Gal-3 (dissociation constant of the complexes) obtained by the SPR method.

	Equilibrium dissociation,constant, nM	
Antibody	Human Galectin-	Macaque Galectin-
D2	33.6±2.50	19.2±9.81
B3	9.11±1.35	1.29±0.34
C4	0.237±0.04	0.276±0.04
C7	1.65±0.37	2.25±0.59
H8	0.122±0.021	0.121±0.02
H9	4.45±0.63	6.44±1.17

C. Secondary Structure of Antibodies

The results of analysis of the far-UV circular dichroism (CD) spectra at 20°C for Gal-3 antibodies with the use of CDPro software [18] are shown in Table 3.

Table 3. The content of secondary structure elements in Gal-3 antibodies

Antibody	α-helices, %	β-structure, %	Turns, %	Unordered structure, %
D2	0.50±0.10	41.43±0.23	22.23±0.12	36.20±0.17
B3	0.37±0.02	40.93±0.12	22.43±0.06	35.30±0.26
C4	1.23±0.25	43.00±0.44	22.73±0.12	32.39±0.28
C7	1.00±0.26	46.23±0.40	22.17±0.12	29.87±0.60
H8	-0.13±0,10	41.03±0.32	22.73±0.12	36.33±0.06
H9	3.37±0.31	38.03±0.32	20.50±0.44	36.87±0.21

The negative values in the α-helices graph for H8 antibody indicate that this structure is not present or negligible. It is well known [20] that all IgGs are characterized by the predominance of β-fold structure (47-48%) and a small contribution of α-helices just like the antibodies obtained in our work.

D. Thermal Stability

Table 4 contains half-transition temperatures of thermal denaturation of antibodies obtained. This data can be useful for development of storage conditions of the antibodies.

Table 4. Half-transition temperatures of thermal denaturation of antibodies. Protein concentration 0.2-0.5 μM.

Antibody	$T_{1/2}$ °C
D2	73.3±0.80
B3	73.8±0.20
C4	71.8±0.60
C7	73.2±0.30
H8	67.3±0.50
H9	71.4±0.70

E. Agglutination Assay

Human Gal-3 causes agglutination of human erythrocytes at concentrations of 3-10 μg/ml, or 120-380 nM [21]. We have found that Gal-3 in concentrations starting from 800 nM causes an agglutination of rabbit red blood cells both in the presence and in the absence of antibodies (Figure 4).

Each antibody was used in three concentrations: 1000, 500 and 70 nM and it all cause a similar effect. Figure 4 shows effects of 70 nM antibodies on rabbit red blood cells agglutination.

Surprisingly, the antibodies do not block, but, stimulate the agglutination of erythrocytes. All the antibodies tested increase the ability of Gal-3 to agglutinate red blood cells by approximately 25 times (from 770 nM to 30 nM). This effect is specific for the Gal-3 antibodies. Nonspecific IgG, added in the same concentrations does not exert any effect on agglutination. Figure 5 demonstrates dependence of cell agglutination on antibody concentration at a fixed concentration of Gal-3.

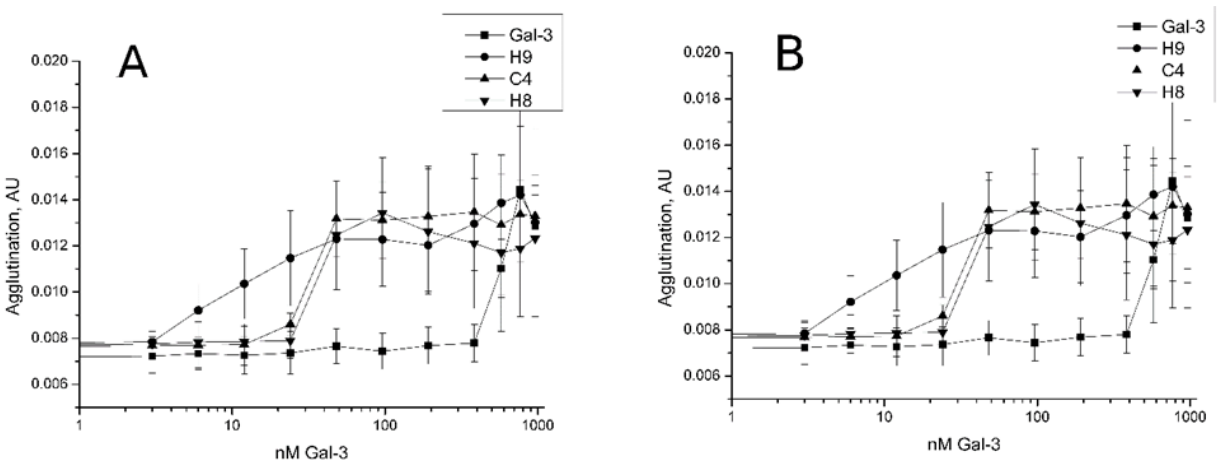

Fig. 4. The effect of antibodies on agglutination of rabbit erythrocytes by Gal-3. Antibody concentration was 70 nM in each experiment. The Y axis shows inverse value of plate brightness at selected area in arbitrary units obtained with the use of the ImageJ program. A – B3 antibody; B - D2 antibody; C - C7 antibody; D – H9 antibody, E – C4 antibody; F – H8 antibody.

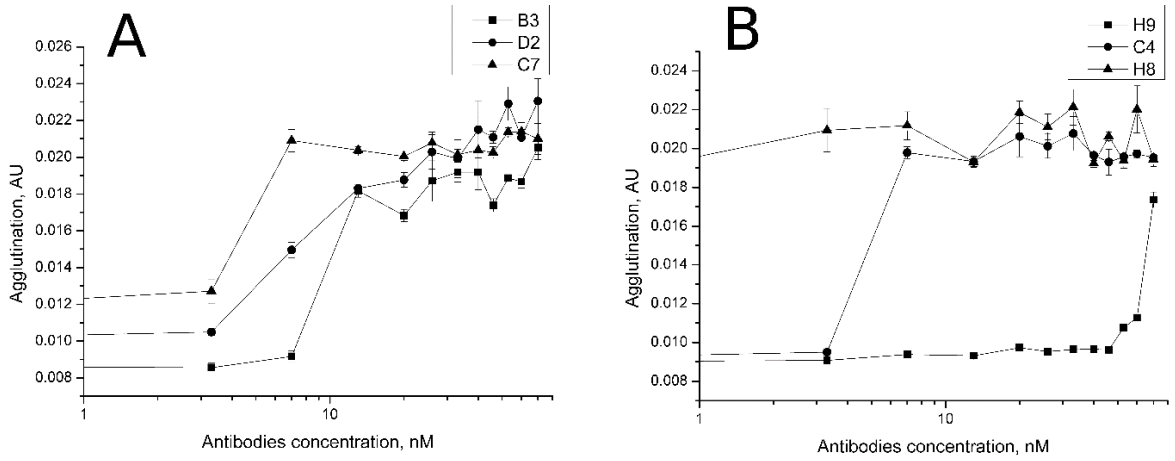

Fig. 5. The dependence of cell agglutination on antibody concentration at a fixed concentration of Gal-3. Concentration of Gal-3 was 200 nM in each experiment. The Y axis shows inverse value of plate brightness at selected area in arbitrary units obtained with the use of the ImageJ program.

F. Effects of Antibodies on Collagen Expression in Human Fibroblasts

Fig. 6. shows of the antibodies effect on collagen expression in human fibroblasts.

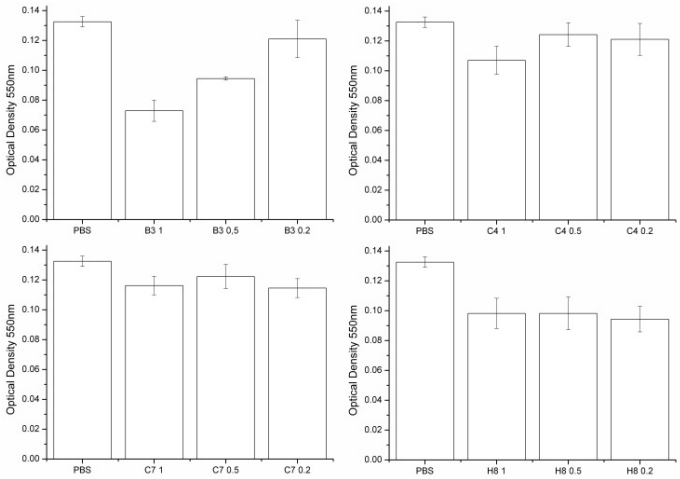

Fig. 6. Collagen expression by human fibroblasts in the presence of antibodies specific to Gal-3. Antibodies were used in the concentration of 1, 0.5 and 0.2 μM.

The antibodies C4 and C7 did not demonstrate any significant effects on the expression of collagen by fibroblasts, while B3 and H8 reduced the amount of collagen. The reduction of collagen expression observed with the B3 antibody was dose-dependent.

IV. DISCUSSION

Here we described the generation of fully human monoclonal antibodies equally well cross-reactive with both human and macaque galectin-3 ortologs. These antibodies were selected from phage display Fab antibody libraries, then produced and characterized as purified Fab antibody fragments. The full length antibodies appeared with the affinities ranging from ~120 pM to ~30 nM. We suggest that antibodies we describe here can be useful in research and diagnostics, in such assays as immunohistochemical staining, immunoprecipitation, western-blot, flow cytometry, ELISA and others.

Similar agglutination results were described in [24]. Agglutination of erythrocytes with Gal-3 was observed in the presence of antibodies specific for carbohydrates. In our case the presence of antibodies with Gal-3 provoked agglutination of erythrocytes, while in the absence of Gal-3 the addition of antibodies did not lead to agglutination. Figure 5 shows the dependence of cell agglutination on antibody concentration at a fixed concentration of Gal-3. 200 nM of Gal-3 was chosen for these experiments since this concentration of Gal-3 causes a strong agglutination of red blood cells in the presence of antibodies. The effective stimulating concentration for all antibodies except H9 was 3-10 nM (65 nM for H9).

Is known, CRD of Gal-3 has one canonical β-galactosides binding site (S-face) and one non-canonical (F-face) which bind relatively large galactomannans [25]. Zhang at al. showed that with the addition of certain ligands, the avidity of galectin-3 to them increases [26] It is assumed that this is due to conformational changes in the gal-3 complex with the ligand, additional binding sites on the S-face are opened. Perhaps the same process we observed during agglutination of red blood cells with galectin-3 in the presence of the obtained antibodies.

Besides the importance of blood serum Gal-3 measurements in diagnostics, this protein is also considered to be an important therapeutic target for fibrosis, heart failure and cancer. At this moment, several inhibitors of Gal-3 are known, including a modified citrus pectin GCS-100 [5], high molecular weight poly-carbohydrates GM-CT-01 and GR-MD-02 with molecular weights of 54 kDa and 120 кDa, respectively [6], and peptides G3-A9, G3-C12 (26). In xenograft model, when added to the drinking water of mice with MDA-MB-435-induced tumor, GCS-100 reduces the growth rate of the tumor, and inhibits metastases. Besides, the modified citrus pectin inhibits morphogenesis of umbilical cord endothelial cells in a dose-dependent manner in vitro [4].

All the described inhibitors have a number of disadvantages as therapeutic agents. The derivatives of polysaccharides can have a high probability of occurrence of immunogenicity, since carbohydrates are the main component of the bacterial cell wall, and this is the most frequent antigen to immune cells [23]. For this reason, application of drugs on their basis likely will be limited to oral administration. The peptides lack this disadvantage; however, small molecules usually have a short half-life, which leads to an increase in their dose or to frequent administration of the drug.

Application of therapeutic antibodies is the most quickly growing field of medicine. A human recombinant antibody less likely causes allergic reactions and has a sufficiently long half-life in blood to fulfill its function in human body [24]. Our Gal-3 specific antibodies do not block its ligand binding since the agglutination process even increases in its presence. The interaction between the N-terminal domain of Gal-3 is perhaps weaker than that between Gal-3 and antibodies, so we see the absence of erythrocyte agglutination at low concentrations of Gal-3.

Our data on suppression of collagen synthesis by anti-Gal-3 antibodies show that antibodies C4 and C7 do not affect the collagen synthesis on fibroblasts neither positively no negatively. Furthermore, B3 antibody suppresses collagen synthesis in a dose-dependent manner like other inhibitors of Gal-3 described in the literature [7].

Possible therapeutic properties of the obtained antibodies such as effects on cancer cells proliferation, or regression fibrosis in vivo are yet to be studied. However, there is no doubt that the antibodies open a new ways for investigation of the role of Gal-3 in spread of cancer metastases, development of fibrosis and methods of treatment of these diseases.

V. CONCLUSIONS

1. Using phage display technology, recombinant monoclonal cross-reactive human antibodies specific for human and macaque galectins-3 were first obtained;
2. The increased agglutination of red blood cells in the presence of antibodies was shown;
3. A decrease in collagen production by normal human fibroblasts was shown when they were cultured in the presence of two antibodies: B3 and H8.

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REFERENCES

[1] Kazuya E., Kohnoe S., Tsujita E., Watanabe A., Nakashima H., Baba, H., Maehara, Y. (2005) Galectin-3 expression is a potent prognostic marker in colorectal cancer. Anticancer Research 25:3117-3122.

[2] Houzelstein D., Goncalves I.R., Fadden A.J., Sidhu S.S., Cooper D.N., Drickamer K., Leffler H., Poirier F. (2004) Phylogenetic analysis of the vertebrate galectin family. Mol. Biol. Evol., 21:1177–1187.

[3] Sato S., Hughes R.C., (1992) Binding specificity of a baby hamster kidney lectin for H type I and II chains, polylactosamine glycans, and appropriately glycosylated forms of laminin and fibronectin. J. Biol. Chem., 267:6983–6990.

[4] Nangia-Makker P., Hogan V., Honjo Y., Baccarini S., Tait L., Bresalier R., Raz A. (2002) Inhibition of Human Cancer Cell Growth and Metastasis in nude Mise by Oral Intake of Modified Citrus Pectin. J Natl Cancer Inst., 24:54-62.

[5] Xue J., Gao X., Fu C., Cong Z., Jiang H., Wang W., Chen T., Wei Q., Qin C. (2013) Regulation of galectin-3-induced apoptosis of Jurkat cells by both O-glycans and N-glycans on CD45. FEBS Lett., 24:86-94.

[6] Shimura T., Shibata M., Gonda K., Nakajima T., Chida S., Noda M., Suzuki S., Nakamura I., Ohki S., Takenoshita S. (2016) Association between circulating galectin-3 levels and the immunological, inflammatory and nutritional parameters in patients with colorectal cancer. Biomed Rep., 5:203-207.

[7] Traber P., Chou H., Zomer E., Hong F., Klyosov K., Frel MI., Friedman SL. (2013) Regression of fibrosis and reversal of cirrhosis in rats by galectin inhibitors in thioacetamide-induced liver disease. Plos One, 10:778–789.

[8] Lotz MM., Andrews CW., Korzelius CA., Lee EC., Steele Jr. GD., Clarke A., Mercurio AM. (1993) Decreased expression of Mac-2 (carbohydrate binding protein 35) and loss of its nuclear localization are associated with the neoplastic progression of colon carcinoma. Proc. Natl. Acad. Sci. U. S. A. 90:3466–3470.

[9] Kramer F. (2013) Galectin-3: clinical utility and prognostic value in patients with heart failure, Research reports in Clinical Cardiology, 4:13-22.

[10] Danguy A., Camby I., Kiss R. (2002) Galectins and cancer. Biochim. Biophys. Acta, 1572:285–293.

[11] Delaine T., Collins P., MacKinnon A., Sharma G., Stegmayr J.. Rajput VK., Mandal S., Cumpstey I., Larumbe A., Salameh BA., Kahl-Knutsson B., van Hattum H., van Scherpenzeel M., Pieters RJ., Sethi T., Schambye H., Oredsson S., Leffler H., Blanchard H., Nilsson UJ. (2016) Galectin-3-Binding Glycomimetics that Strongly Reduce Bleomycin-Induced Lung Fibrosis and Modulate Intracellular Glycan Recognition, Chembiochem., 18:59-70.

[12] Paul M., Bao-Ling Christine A., Gourin G., Moretz W. H., Bollag R. J., Wang B. Y., Liu Z., Lee J. R., Terris Weinberger D. J. (2007) Association of Nuclear, Cytoplasmic Expression of Galectin-3 With Catenin/Wnt-Pathway Activation in Thyroid Carcinoma, Arch Otolaryngol. Head Neck Surg., 133:503-510.

[13] Connie M.A., Rodrigues L.C., Baruffi M.D., Sullivan C.H., Heimburg-Molinaro J., Smith F.D., Cummings D.R., Stowell R.S. (2015) Examining Galectin Binding Specificity Using Glycan Microarrays. Methods Mol Biol., 1207:115–131.

[14] Barbas C.F., Burton D.R., Silverman G.J. (2004) Phage Display: A Laboratory Manual. New York, NY: Cold Spring Harbor.

[15] Affinnity chromatography. Vol. 1: Antibodies. Handbook from CE Healthcare Life Science.

[16] Sreerama N., Venyaminov S.Y., Woody R.W. (2000) Estimation of protein secondary structure from circular dichroism spectra: inclusion of denatured proteins with native proteins in the analysis. Anal. Biochem., 287:243–251.

[17] Burstein E.A., Emelyanenko V.I. (1996) Log-normal description of fluorescence spectra of organic fluorophores. Photochem, Photobiol. 64:316-320.

[18] Permyakov E. A., Burstein E. A. (1984) Some aspects of studies of thermal transitions in proteins by means of their intrinsic fluorescence. Biophysical chemistry 19:265-271.

[19] Chen C., Yang S., Zhang M., Zhang Z., Zhang B., Han D., Ma J., Wang X., Hong J., Guo Y., Okunieff P., Zhang L. (2013) In vitro Sirius Red collagen assay measures the pattern shift from soluble to deposited collagen. Adv Exp Med Biol., 765:47-53. DOI: 10.1007/978-1-4614-4989-8_7

[20] Immunobiology: The Immune System in Health and Disease. 5th edition.

[21] R&D systems: official site// URL: https://www.rndsystems.com/productsrecombinant-human-galectin-3-protein_1154-ga// (date of the application 3.05.18).

[22] Vemuri P. K., Veeravalli S. (2014) Expression, Purification and Characterization of Human Recombinant Galectin3 in Pichia pastoris. Iranian Journal of biotechnology, 2:3-8.

[23] Amon R., Reuven EM., Leviatan Ben-Arye S., Padler-Karavani V. (2014) Glycans in immune recognition and response, Carbohydr Res. 7:15-22.

[24] Chames P., Van Regenmortel M., Weiss E., Baty D. (2009) Therapeutic antibodies: successes, limitations and hopes for the future. Br. J. Pharmacol. 157:220–233.

[25] Miller, M. C., Ippel, H., Suylen, D., Klyosov, A. A., Traber, P. G., Hackeng, T. et al. (2016) Binding of polysaccharides to human galectin-3 at a noncanonical site in its carbohydrate recognition domain. Glycobiology 26:88-99.

[26] Zhang T., Miller M.C., Zheng Y., Zhang Z., Xue H., Zhao D., Su J., Mayo K.H., Zhou Y., Tai G. (2017) Macromolecular assemblies of complex polysaccharides with galectin-3 and their synergistic effects on function. Biochem J. Nov 9;474(22):3849-3868.