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Study of the antitumor effect of the monoclonal antibody 8D1 against the membrane-associated heat shock protein Hsp70 in vivo and in vitro

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Abstract. It is known that the 70 kDa heat shock protein (Hsp70) is localized on the membranes of cancer cells and can serve as a target for tumor theranostics. When developing new generation drugs, priority is given to drugs for "targeted" therapy. The most interesting and promising objects in this area of pharmacology are therapeutic antibodies that directly interact with the pathogen molecule, neutralizing its effects. The present study investigated the antitumor activity of the 8D1 monoclonal antibody, specific to the membrane-associated form of Hsp70, in an in vivo model of mouse myeloma Sp2/0. It was shown that the introduction of the antibody increased the expected lifespan of animals by approximately 20% compared to the control group. The possibility of enhancing the action of the 8D1 antibody has been demonstrated on an in vitro model of suppressing the viability of human lung carcinoma cell line A549 using a conjugate of this antibody with doxorubicin.

1 Introduction

The family of homologous heat shock proteins 70 (Hsp70, HSPA1A) is found in various cellular and subcellular compartments, including the cytosol, nucleus, endoplasmic reticulum, and plasma membrane, where it is involved in the folding of synthesized proteins, preventing their aggregation, and facilitating transport across membranes [1, 2].

Particular interest in membrane-bound Hsp70 arose after studies conducted by Prof. Gabriele Multhoff showed that Hsp70 is present exclusively on the plasma membrane-embedded surface of tumor cells [3]. The same group of researchers mapped the Hsp70 epitope exposed on the outer side of the membrane, which was identified as a "TKD" peptide (TKDNNLLGRFELSG) [4]. A new antibody cmHsp70.1 was obtained for this epitope, which made it possible to identify many tumors that carry Hsp70 on their surface. In addition, there are now many reports demonstrating the presence of representatives of other HSP families on the surface of various cells [5].

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It has been experimentally established that in cancer, part of the synthesized Hsp70 is exported to the environment along with tumor peptides, and the proportion of protein not bound to the substrate is transported to the cytoplasmic membrane [6,7]. It is obvious that the Hsp70 fixed in the membrane is conformationally different from the free analog, and this difference depends on the lipid microenvironment. There is evidence that Hsp70 has high selectivity for negatively charged phospholipids. This fact was confirmed in model experiments analyzing the interaction of recombinant human Hsp70 with model phospholipid membranes. It has been established that Hsp70 has a strong specificity for membranes consisting of negatively charged phosphatidylserine (PS) compared to membranes made of neutral phosphatidylcholine [8,9]. The ion-hydrophobic environment should significantly change the conformation of the protein, which led us to the idea of immunizing mice with the Hsp70 protein immobilized on a hydrophobic carrier. As a result, hybridomas producing monoclonal antibodies of different specificity were obtained. As a result of targeted screening, antibodies with low affinity for free Hsp70 in solution (Kd of the order of 10^{-5}M) were selected [10]. At the same time, it was shown by methods of flow cytofluorometry that antibodies bind to linear tumor cells of humans and animals. The monoclonal antibody 8D1, which recognizes Hsp70, was most effective on the membranes of human carcinoma A549 and mouse myeloma Sp2/0 cells [10].

The use of monoclonal antibody conjugates associated with a toxic agent should certainly be more effective than the use of free antibodies, the action of which, as a rule, leads to the death of cancer cells due to natural effector functions due to the Fc fragment of the antibody, such as complement-dependent cytotoxicity (CDC) and antibody-dependent cell cytotoxicity (ADCC) [11].

The purpose of this research was to study the antitumor activity of the mouse monoclonal antibody 8D1 against membrane-associated Hsp70 in in vivo experiments on a model of the ~~continuous~~ tumor cell line Sp2/0 and to evaluate the cytotoxic effect of the antibody conjugate with the cytotoxic agent doxorubicin in vitro on a model for assessing the viability of human lung adenocarcinoma cells A549.

2 Research methodology

2.1 Preparation and analysis of a monoclonal antibody

To obtain a monoclonal antibody, Balb/c mice were injected with clone 8D1 cells in the amount of 2 million per mouse. The resulting ascitic fluid was collected to isolate immunoglobulins. The antibody purification process included the following steps: separation of fluid taken from the abdominal cavity of animals by centrifugation (10,000 rpm); precipitation of the supernatant with ammonium sulfate (45% of saturation); chromatography of the dissolved sediment on a sorbent with immobilized protein A (MabSelect, GE). The obtained monoclonal mouse antibody 8D1 was characterized by SDS PAAG electrophoresis and gel permeation chromatography.

2.2 Cells

Human lung carcinoma A549 and mouse myeloma Sp2/0 cells were obtained from the Russian Collection of Cell cultures (Institute of Cytology of the Russian Academy of Sciences, St. Petersburg, Russia).

2.3 Study of the antitumor activity of monoclonal antibody 8D1

For in vivo experiments, Sp2/0 mouse myeloma cells were cultured in culture flasks with a surface area 175 cm2 (Corning) in a final volume of 230 ml in RPMI-1640 culture medium supplemented with up to 10% fetal bovine serum. Cell were cultured to a concentration of 0.6×106 per ml was carried out in an incubator at a temperature of 37°C in an atmosphere of 5% CO2 under conditions of absolute humidity up to the concentration. Next, the cells were centrifuged at 1000 rpm. The cell pellet was resuspended in RPMI-1640 culture medium to a concentration of 1x106 per ml and 3x106 per ml, after which the cells were ready for further studies.

To set up an experiment on antitumor effect, 30 mice were used, which were divided into 3 subgroups of 10 animals. Mice of subgroups 1 were injected with only myeloma cells of 1×106 cells/mouse. Mice of subgroup 2 were injected with normal mouse immunoglobulins of the same isotype as 8D1 in an amount of 1.0 mg/mouse in a volume of 0.5 ml immediately after injection of tumor cells and then with a frequency of once every two days. Mice of subgroup 3 were injected with monoclonal antibody 8D1 in an amount of 1.0 mg in a volume of 0.5 ml after injection of tumor cells and then continued injections with the same frequency.

2.4 Conjugation of an antibody with doxorubicin

To obtain the 8D1 antibody conjugate with doxorubicin (DOX), the biodegradable (linker 4-[N-maleimidomethyl]cyclohexane-1-carboxylhydrazide (M2C2H, Pierce Chemical Co., Rockford) was used. The conjugation process was carried out in several stages.

At the first stage, a chemical reaction was carried out between the carbonyl group of DOX and the hydrazide group of linker to form the hydrazone M2C2H – DOX with an active maleimide group. The reaction was carried out in an equimolar ratio in DMSO at a temperature of 55 ± 5° for 30 minutes. The resulting conjugate was separated from the unreacted RP-HPLC ingredients in a gradient of 25 mM ammonium acetate (10-60%) acetonitrile, pH 4.4.

At the second stage, the synthesis of an activated DOX conjugate with an 8D1 antibody was carried out. In this regard, the antibody was thiolated before conjugation by treatment with the Trout reagent. For this purpose, the Trout reagent (Pierce Prod 26101) was dissolved in water at a concentration of 2 mg/ml (14.5 mM) and added to 1 ml of 8D1 antibody with a concentration of 10 mg/ml, in a buffer solution containing 50 mM sodium phosphate buffer solution, pH 8.0, 45.8 µl of Trout reagent solution was added, which corresponds to a 10-fold excess molar quantity. The reaction was carried out for 1 hour at room temperature. After the reaction, the thiolated protein was released from the unreacted Trout reagent by gel permeation chromatography on a 1.5x15cm column filled with Sefadex G25 sorbent in desalination mode. The number of thiol groups in the modified antibody was determined using the Ellman's reagent by the optical density level of the compound formed after the reaction at 412 nm. The results of the analysis indicated that there are 8 thiol groups per antibody molecule.

The conjugation reaction of a thiolated antibody with maleimide –DOX was carried out in a 15% DMSO solution on a phosphate-salt buffer solution by mixing them in a ratio of 10 mg of antibody 8D1:0.5 mg, which corresponded to a 20-fold excess molar quantity of maleimide-M2C2H-DOX relative to the antibody and were incubated for 2 hours at 37°C. At this ratio, almost all antibody molecules bound to activated DOX, which was confirmed by electrophoresis data in SDS PAAG.

The antibody-DOX conjugate formed as a result of the reaction was separated from the unbound maleimide-DOX by gel permeation chromatography in desalination mode on a column with sephadex G25 balanced with a solution of 0.2 M ammonium acetate, pH 4.4.

3 Research results

The purified monoclonal antibody 8D1 was characterized by a 98% purity level according to electrophoresis and GPC data (data are not provided). The results of cytofluorimetry [11] indicated the interaction of the antibody with the membranes of A549 and Sp2/0 cells, which provided grounds for conducting a study of the direct antitumor effect of the antibody on a model of continuous Sp2/0 tumor in mice, as well as for assessing the effectiveness of the antibody-DOX conjugate in the in vitro experiment using human lung carcinoma cells with the prospect of further application.

During the experiments on the antitumor effect, the animals were monitored daily. The effectiveness of the 8D1 antibody was assessed by animal survival. The results of the animal survival assessment are presented in Table 2, as well as in the Kaplan-Meier curve (Figure 1).

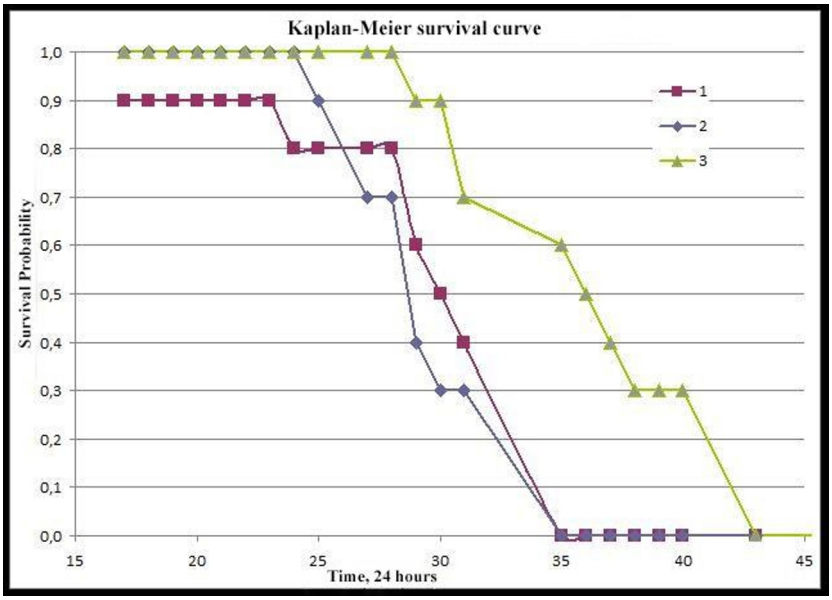

Fig.1. Kaplan-Meier curve of mouse survival after injection:1 - myeloma Sp2/0 (1×10^6 cells/mouse, once); 2 - myeloma Sp2/0 (1×10^6 cells/mouse, once) + normal IgG mice (1.0 mg/mouse, 1 day after myeloma injection); 3 - myeloma Sp2/0 (1×10^6 cells/mouse, once) + 8D1 antibodies (1.0 mg/mouse, 1 day after myeloma injection).

The survival function S(t)=P(T>t) was used in processing the results, where t is the observation time, the random variable T is the moment of death, S(t) is the probability of "death" later than t; cumulative survival function F(t)=1-S(t); median expected life time is the time t when F(t)=1-S(t)=S(t)=0.5

Based on the survival curves for each experimental group, the median expected lifespan was calculated - this is the time to which 50% of individuals live. The numerical values of the medians turned out to be different: 28.7 days - for mice injected with only Sp2/0 cells; 30.0 days - for mice injected with normal IgG of mice after cell injection; 37.0 days - for mice injected with 8D1 antibody after cell injection.

Thus, according to the results of the conducted studies, it is possible to observe an increase in the life span of a group of experimental animals with vaccinated myeloma Sp2/0 tumor cells, which were injected with monoclonal 8D1 antibodies, which may indicate their antitumor activity.

3.1 Preparation of 8D1-DOX antibody conjugate

As shown by the above studies, the 8D1 antibody has antitumor activity in the syngeneic Sp2/0 tumor model in Balb/c mice, characterized by a median survival of 37 days (compared to 28 days for control animals). The mechanism of action of the antibody is unknown, however, it can be assumed that it is associated with the activation of non-specific immunity.

It is assumed that the delivery of an antibody conjugated with a cytotoxic agent to the target cell should ensure the antitumor effect of the antibody in vitro. In this regard, the task of obtaining a conjugate of the monoclonal antibody 8D1 with the antitumor antibiotic of the anthracycline series, doxorubicin (DOX), which has an anti-mitotic and anti-proliferative effect on cells, was being solved. The mechanism of action of the antibiotic involves the formation of free radicals and direct action on cell membranes with suppression of nucleic acid synthesis in cells, which are predominantly in the S- and G2- phases due to intercalating action between DNA strands.

For the successful implementation of the conjugate action, it is necessary that as a result of conjugation, the antibody epitopes responsible for interacting with the membrane HSP70 are not damaged, and that the antibiotic detaches from the antibody after internalization of the complex that has passed through the cell membrane and interacts with DNA in a free state.

In the present study, this task was solved by attaching DOX to the antibody through the biodegradable linker 4-[N-maleimidomethyl]cyclohexane-1-carboxyl hydrazide (M2C2H - Pierce Chemical Co., Rockford, IL), forming a hydrazone bond in the conjugate, which easily hydrolyzes at acidic pH values in lysosomes, releasing DOX from the conjugate (Figure 2).

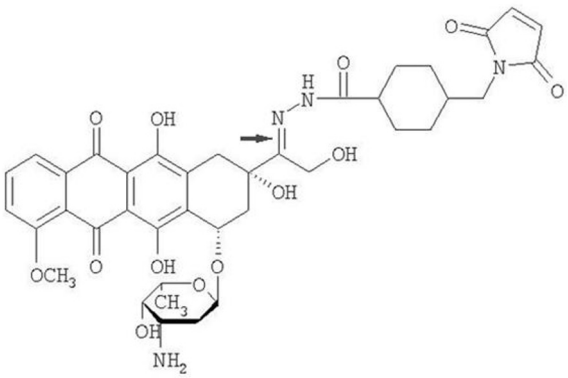

Fig. 2. The DOX complex after reaction with the M2C2H linker (DOX-maleimide). The arrow indicates the predicted rupture of the connection with the release of DOX in the body.

The purified conjugate was characterized by gel permeation chromatography on a Superose 12 column (10x300 mm) in a 100 mM phosphate buffer solution, which was pre-graduated by chromatography of the initial 8D1 antibody (void time 15 minutes). At the same time, it was assumed that the retention times of the conjugate in the form of a monomer and a cold antibody on chromatograms practically coincide. As can be seen from the chromatogram (Figure 3, Table 1), the conjugate was heterogeneous in molecular weight, with the proportion of monomer in it being 66.89%. Figure 3 shows a chromatogram at a wavelength of 500nm. Considering the fact that the optical density profile at a wavelength of 500 nm, corresponding to the absorption of DOX, coincides with the absorption profiles at wavelengths of 220 and 280 nm, which correspond to the distribution of protein components in the chromatogram, we can conclude that all fractions of the conjugate contain the antibody in the complex with DOX.

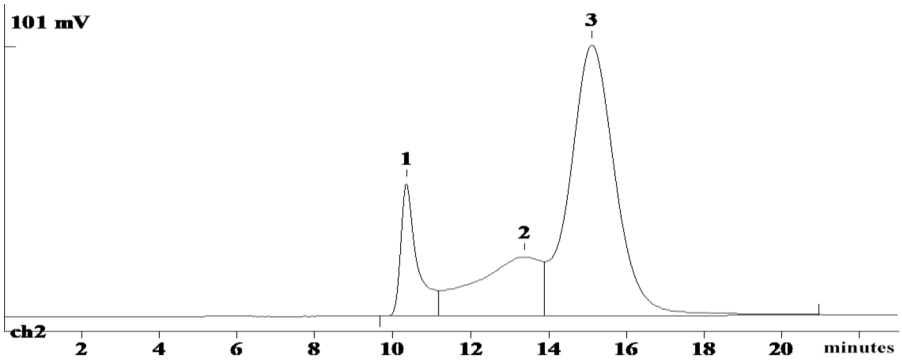

Fig. 3. Antibody-DOX conjugate chromatogram, Superose column 12 10*300 mm, 100 mM phosphate buffer solution pH=7.0, flow rate 0.8 ml/min, detection 500 nm, elution time 23 min.

Table 1. Characteristics of the elution bands of the chromatogram of the antibody-DOX conjugate

Elution band No.	Time, min	Area, mV*sec	Area, %
1	10.33	1 381.44	11.88
2	13.37	2 470.33	21.24
3	15.1	7 780.06	66.89
Total	23	11 631.83	100.00

The scheme of the resulting 8D1-DOX conjugate is shown in Figure 4. After isolation of the target substance from the reaction mixture, the antitumor activity of the conjugate was checked in vitro.

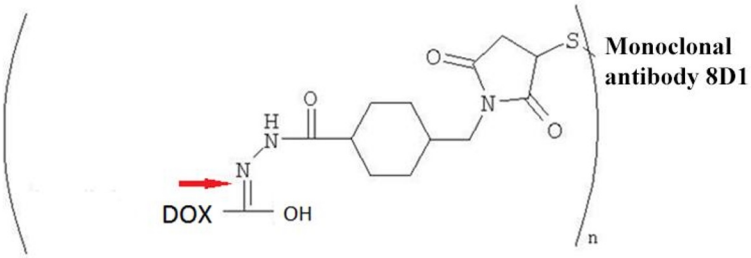

Fig. 4. DOX conjugate with antibody 8D1. The arrow indicates a possible disruption of the connection with the release of DOX in the body.

3.2 Evaluation of the effectiveness of the antitumor action of the 8D1-DOX antibody conjugate in vitro

The effectiveness of the antitumor action of the 8D1-DOX antibody conjugate in vitro was evaluated on lung carcinoma cells (A-549 line), comparing the viability of cells after treatment with free 8D1 antibody, free cytotoxic agent DOX and 8D1-DOX complex.

To perform the test, cells of the A-549 line were grown in culture flasks with a surface area of 75 cm2 (Corning) in 30 ml of a mixture of modified Eagle's medium and F-12 substrate (DMEM/F12) with 10% fetal calf serum. The cells were grown in an incubator at a temperature of 37°C in an atmosphere of 5% CO_2 under conditions of absolute humidity until a dense monolayer was formed. Next, the cells were removed from the plastic with a Versene solution and centrifuged at 1000 rpm. The cell pellet was resuspended with DMEM/F12 medium to a concentration of 3×10^5 μ/ml, after which the cells were ready for further studies. Next, cells of the A-549 line were introduced in an amount of 3×10^4 per well into a 96-well

flat-bottomed culture plate (Corning) in 100 µl of a mixture of modified Eagle's medium and F-12 substrate (DMEM/F12) with 10% fetal bovine serum. The plate was incubated for 24 hours in an incubator at a temperature of 37°C in an atmosphere of 5% CO2 under absolute humidity conditions.

Further, the studied drugs were added to the cells in various concentrations in a volume of 100 µl in a culture medium with 1% fetal calf serum. The concentrations of free antibody and conjugate were normalized to the protein, and the concentration of free DOX was calculated to correspond to the concentration of DOX in the antibody-DOX conjugate. Samples of each concentration were taken in four parallels. After 4 days, the culture medium was removed from the plate and the cells were washed 4 times with 200 µl of Hank's solution, and then 40 µl of a 0.2% Crystal Violet solution in 20% methanol was added and incubated for 15 min to stain living cells. After staining, the plates were washed under running water and 100 µl of lysis solution containing 2% sodium dodecyl sulfate and 5% glycerol in water was added. After dissolution of the stained cells, the optical density of the solution in the wells was measured at a wavelength of 490 nm on a Model 3550 Microplate Reader (BioRad). The obtained results are shown on the graph as the average of the four parallels. Based on the measurement results, cell survival curves were constructed.

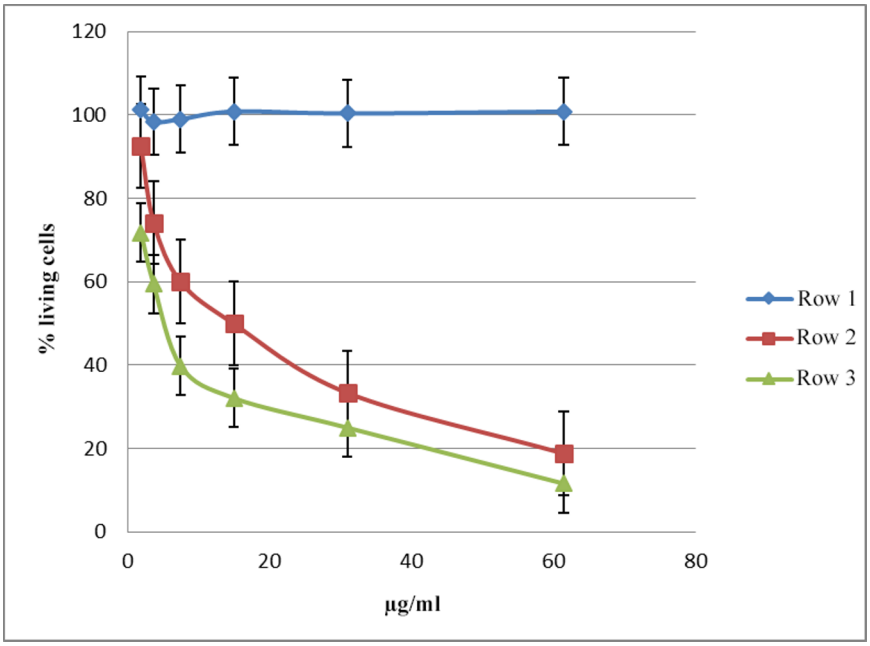

Fig. 5. Viability of lung carcinoma cells, A-549 line, after incubation with the studied drugs: Row 1 – Free 8D1 antibody, Row 2 – 8D1-DOX antibody conjugate, Row 3 – Free DOX.

As follows from Figure 5, on the fourth day, the viability of cells to which free DOX and 8D1-DOX antibody conjugate were added decreased dose-dependently, unlike cells to which free 8D1 antibody was added. This fact indicated that the free antibody that served as a control did not have a toxic effect, while the antibody-DOX conjugate interacted with the target cells of human lung carcinoma A-549 and caused their death with the same effect as the free antibiotic.

4 Discussion of results

It is known that cancer cells contain high levels of Hsp70, which, together with co-chaperones, protects these cells from apoptosis, contributing to increased proliferation and increased metastasis. The fact that some of the Hsp70 molecules remain associated with the membranes of cancer cells attracts researchers as a possible target for the teranostics of tumor cells [12,13]. It is well known that antibodies are one of the ways to deliver markers and cytostatic agents to cancer cells, which is currently widely used in the creation of anti-cancer drugs [14]. When considering the possibility of using antibodies to Hsp70 for anticancer therapy, it seems important to use antibodies that are able to react only with membrane-associated, but not with free Hsp70, since this fact predetermines the possible prospects for their pharmacological use. Known antibodies, including the monoclonal antibody cmHsp70.1 (MultiImmune, Germany) are not strictly selective only for the membrane form of Hsp70 and also recognize a protein not embedded in the membrane.

The studied antibody met the stated criteria, since the previously obtained results of surface plasmon resonance (SPR) indicated a low affinity of 8D1 for free Hsp70, and indicated its interaction with the Hsp70 protein exposed on the membranes of tumor cell lines of mouse myeloma Sp2/0 and human lung carcinoma A549. The results of the study of its antitumor potential showed that the administration of the 8D1 antibody after injection of Sp2/0 tumor cells to mice increased the expected lifespan of animals by approximately 20% compared with the control group. In addition, it was shown that the effectiveness of the antibody can be enhanced by the introduction of a cytotoxic agent, as evidenced by the results of protection against death of A549 cells using 8D1 conjugated with doxorubicin. These facts indicated the potential prospect of using the 8D1 antibody as an antitumor therapeutic antibody.

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