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Immunoliposomes and Their Targets

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Abstract—Immunoliposomes have been actively studied over the past two decades. However, researcher's attention has still been limited to intensive preclinical trials and no one immunoliposomal formulation has passed clinical trials. This fact can be explained by a fear of complications, when mouse monoclonal antibodies are used as delivery vehicles. The situation has radically changed over the past few years. Nonimmunogenic single-chain antibodies have become an accessible research tool. Moreover, antibodies can be easily modified and conjugated with liposomes. Therefore, a vigorous breakthrough in the field of development of new-generation immuno-liposomal formulations for oncological practice can be expected.

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Nanocarriers as new systems used to deliver chemotherapeutics to body cells and organs are presently a focus of translational research in oncology [1, 2]. The idea to encapsulate drugs into nanoparticles to extend therapeutic possibilities in oncology and allow drug release in the extracellular matrix or penetration through the cytoplasmatic membranes of tumor cells has been realized [3–5]. Liposomes are just such particles that are able to encapsulate various therapeutic substances, thereby increasing their bioavailability [6].

The main disadvantage of liposomes as drug carriers is that are actively entrapped by the reticulo-endothelial system and rapidly eliminated from the blood stream. To overcome this drawback, liposomes are coated with polyethylene glycol (pegilation) which creates increased osmotic pressure around liposomes, which prevents other cells from closely approaching the latter. Pegylated liposomes longer circulate in blood and are accumulated in tumor tissues to a greater extent than in normal tissues and organs, and enhance the permeability and retention effect [7]. Pegylated liposomes allow the dose of a drug to be increased several times. For example, the minimal cumulative dose of a pegylated antitumor drug doxorubicin, which does not cause cardiovascular complications, is 500–1500 mg/m^2 against 350–400 mg/m^2 for free doxorubicin [8, 9]. At the same time, pegylation hinders endosomal release of protein preparations and siRNA (double-stranded RNA) [10, 11].

Apart from drugs, such substances as magnetic nanoparticles, colloid gold, silver nanoparticles, and fluorescent labels can, too, be encapsulated in liposomes for diagnostics and microscopy analysis [12]. Magnetoliposomes ensure drug targeting to tumor and increased drug concentrations in the magnetic field region.

Liposomes are widely used in the development of various photosensitizers which are presently under preclinical and clinical trials [13–19]. They are also used as antigen carriers [20, 21].

The accumulation of liposomes in tumor tissues occurs due to enhanced permeability of tumor vessels. The reason for this phenomenon consists in that the endothelial cells of tumor vessels tend to proliferate 30–40 times faster than the endothelial cells of normal vessels, and therefore, solid tumor capillaries have large pores between endothelial cells (from 380 nm to 1.2 μm depending on the tumor type). Tumor capillaries are permeable for particles up to 300 nm in size, whereas the functional permeability barrier of normal capillaries can only be overcome by 7-nm nanoparticles [25]. In view of this difference in

permeability, liposomes are accumulated in tumor tissues but fail to break through the endothelial barrier in healthy tissues [22–24].

Vessel pores allow passive delivery of liposome-encapsulated drugs to tumor. This process can be intensified by means of ligand-assisted targeting. The role of such ligands can be played by oligosaccharides, peptides, proteins, vitamins, and antibodies [26]. The most promising of them are monoclonal antibodies or antibody fragments, which bind to antigens selectively expressed or overexpressed on tumor cells [27]. The cytotoxic effect of immunoliposomes is enhanced with increasing number of monoclonal antibodies on the surface of a pegylated liposome [28]. However, the low density and heterogenicity of antigen expression can pose limits to the efficiency of immunoliposomal formulations. Certain monoclonal antibodies used for liposome targeting can induce apoptosis of tumor cells, thereby enhancing the killing effect [29].

Immunoliposomes can carry more drug than antibody drug conjugates. Thus, for example, one antibody molecule can be conjugated with 10 drug molecules, whereas immunoliposomes can encapsulate several hundreds of such molecules. This increases the therapeutic efficiency of the drug, reduces antibody-induced side effects, and decreases the cost of treatment [2].

Normally, tumors accumulate 7–8% of the injected dose per 1 g of tumor tissue [30]. Experiments with gold-labeled liposomes showed that immunoliposomes are accumulated around tumor tissues, whereas ordinary liposomes are localized in stroma and macrophages. In the case of antigen-negative tumors, immunoliposomes, too, are localized in stroma. At the same time, in the case of antigen-positive tumor cells, the accumulation of immunoliposomes is 6 times greater compared to ordinary liposomes.

Immunoliposomes are delivered to tumor cells via monoclonal antibody-mediated endocytosis. If immunoliposomes include monoclonal antibodies to antigens which undergo no pinocytosis after antigen–antibody complex formation, drug release from liposomes occurs in the intercellular space rather than inside the tumor cell. Therefore, the therapeutic efficiency of immunoliposomes is due to selective accumulation of drug in tumor [31].

The procedure of binding monoclonal antigens to lipids is based on three fairly efficient and selective reactions: (1) reaction between activated carboxy and amido groups to form an amide bond, (2) reaction between pyridinedithiols and thiols to form disulfide bonds, and (3) reaction between maleimide derivatives and thiols to form thioester bonds [32]. Other approaches have also been reported, for instance, carbamate bond formation by the reaction of *p*-nitrophenylcarbonyl and amino groups [33]. Furthermore, ligands are noncovalently bound to liposome surface via biotin–avidin complex formation [34]. A recombinant antibody-binding protein was obtained from lipid-modified streptococcal protein G site-targeted by genetic engineering [35]. This lipoproteid was attached to liposome surface by simple mixing.

The immunoliposome targets can be various surface membrane antigens which are absent on normal cells but abundant on tumor cells or formed de novo on tumor blood vessels. Immunoliposomes which will target CD19, CD20, CD22, CD34, CD72, CD133, EGFR, Her2/neu, HB-EGF, VEGF-A, GD2, CA19-9, Muc-1, HLA-DR CD25, CD105, VGFR, VCAM, ICAM-1, and other antigens are presently under development [2, 36–41].

The B-cell antigen CD19 is expressed at all staged of differentiation of B cells and in all B-cell leukemias and lymphomas. Antigen–antibody complexes are subject to pinocytosis. All these features make the CD19 antigen an attractive target for immunoliposomes.

Several research groups used monoclonal antibodies targeting CD19 in immunoliposomal formulations targeting B-cell lymphoma [42–44]. The model of xenografts of a Namalwa (human B lymphoma) cell line in athymic mice was used to demonstrate a higher therapeutic efficiency of anti-CD19-immunoliposomes loaded with doxorubicin compared to conventional liposomal doxorubicin. Immunoliposomes were constructed using whole anti-CD19 monoclonal antibodies and their Fab' fragments or single-chain antibodies. Immunoliposomes on the basis of single-chain antibodies were found to better bind to cells and exhibit higher cytotoxicity with respect to the B cell line compared to conventional liposomes. The researchers expect that such immunoliposomes will find clinical application in therapy of B-cell leukemias and lymphomas.

Another B-cell antigen CD20 was expressed at the mature stages of differentiation of B cells and is not expressed on other cell types, and, therefore, it is an

attractive target for immunoliposomes. However, this antigen undergoes no pinocytosis on contact with monoclonal antibodies. Khugaeva et al. [45] prepared immunoliposomes using an anti-CD20 monoclonal antibody ICO-180 and the antitumor drug mitoxantrone. The obtained immunoliposomes specifically bound with antigen-positive cells Raji and did not bind with antigen-negative cells Jurkat. Triple washing of cells had no effect on immunoliposome binding but removed liposomes and free mitoxantrone. Comparative *in vitro* cytotoxicity tests of anti-CD20-immunoliposomes and free and liposomal mitoxantrone gave the following results. Liposomal mitoxantrone showed a lower cytotoxic activity with respect to the antigen-positive cells Raji compared to free mitoxantrone on incubation for 24 h; however, after 72-h incubation these two formulations showed close cytotoxicities. Immunoliposomes compared in cytotoxicity with free mitoxantrone already after 24 h [45].

The CD22 antigen is a B-cell-specific glycoproteid expressed at all stages of differentiation of B lymphocytes, except for their terminal differentiation to plasma cells. It functions as an adhesive molecule and also modulates signal transduction through the B-cell receptor. The CD22 antigen is internalized after binding with antibody [46]. Since B-cell lymphomas almost always express the CD22 antigen, the latter can be considered as an attractive target for immunoliposomes. O'Donnell and Martin [47] conjugated doxorubicin-loaded pegylated liposomes with monoclonal antibodies targeting the CD22 antigen. Specific binding of the obtained immunoliposomes with antigen-positive cells and lack of reaction with antigen-negative cells were noted. Immunoliposomes are more cytotoxic with respect to antigen-positive cells than pegylated liposomes. The half-inhibition concentration IC_{50} of immunoliposomes against antigen-negative cells is 3.1–5.4 times lower than against antigen-positive cells. However, the IC_{50} of immunoliposomes does not differ from the IC_{50} of liposomal doxorubicin in antigen-negative cells. Moreover, immunoliposomes remain to be bound with CD22+ cells after washing, unlike liposomal and free doxorubicin.

Monoclonal antibodies My10 targeting the CD34 antigen (marker of hemopoetic stem cells) were used to develop immunoliposomes for the therapy of acute myeloblastic leukemia. *In vitro* experiments gave evidence showing that such immunoliposomes are able to selectively deliver doxorubicin [48].

The transferrin receptor CD72 is a target of immunoliposomal preparations in the case of pancreatic cancer. Immunoliposomes prepared with single-chain antibody fragments were further used to develop systems for delivery gens to tumor cells [49].

The epidermal growth factor receptor (EGFR) exhibits over expression on various tumor cells, and, therewith, its expression correlates with an unfavorable disease course. This receptor is considered as a good target for immunoliposomes [50]. Gao et al. [11] made use of anti-EGFR immunoliposomes loaded with small interfering RNA to block *in vitro* and *in vivo* the gen which cause EGFR overexpression in breast cancer cells.

Much effort has been put on the development of immunoliposomes targeting the epidermal growth factor receptor Her2/neu. This receptor is over-expressed on breast cancer cells and associated with a bad disease prognosis. Monoclonal antibodies block the HER2/neu receptor and induce apoptosis of tumor cells. The humanized monoclonal antibodies trastuzumab (Herceptin) are used to success in the therapy of a HER2/neu-positive breast cancer, and, therefore, this receptor can be considered an attractive target for immunoliposomes. Immunoliposomes with whole monoclonal antibodies and their F(ab)2 and Fab' fragments, as well as single-chain antibodies have been designed [51]. The conjugation of antibodies and their fragments with liposomes has no effect on the biodistribution and circulation time of intravenously injected liposomes. Such immunoliposomes exhibit good therapeutic efficacy. The entrapment of immunoliposomes by tumor cells depends on the concentration of lipids and temperature and time of incubation, and, therewith, they are preferentially localized in lysosomes. It was found that the cytotoxic effect of doxorubicin-loaded immunoliposomes targeting the HER2/neu receptor is stronger compared to conventional liposomes loaded with the same drug. The internalization of immunoliposomes into tumor cells is blocked by endocytosis inhibitors, such as clathrin or caveol. Furthermore, immunoliposomes bind to umbilical vein cells. These immunoliposomes are suggested to act directly on tumor cells or by destroying newly formed vessels [52].

Lytic immunoliposomes with anti-HER2/neu monoclonal antibodies have been developed [53]. The term "lytic" in the name of such immunoliposomes means that they include a lytic peptide melittin. These

liposomes were found to target selectively the EpCAM adhesion antigen. Both types of immunoliposomes have a strong cytotoxic effect on breast cancer cell lines.

Bandekar et al. prepared doxorubicin-loaded pH-sensitive immunoliposomes targeting the HER2/neu receptor [54] and observed their good antitumor activity in subcutaneously growing BT474 tumor xenografts. According to the results of examination of the tumor node, the antitumor effect of immunoliposomes is associated with release of doxorubicin in an acidic intercellular space rather than with internalization of immunoliposomes into tumor cells.

The main drawback of anthracycline antibiotics used in the chemotherapy of breast cancer is their cardiotoxicity. Clinical trials showed that liposomal doxorubicin exhibits no cardiotoxicity. However, no information is available concerning cardiotoxicity of immunoliposomal doxorubicin: It has not yet passed clinical trials, and there is no validated *in vitro* test for cardiotoxicity. Reynolds et al. [55] made use of an experimental model on the basis of human stem cells to show that the immunoliposomal doxorubicin targeting the HER2/neu antigen does not bind to cardiomyocytes and causes no dysfunction.

The heparin-binding epidermal growth factor (HB-EGF) is frequently overexpressed in breast and ovarian tumors. This protein is considered a good target for antitumor drugs [56]. Doxorubicin-loaded immunoliposomes targeting the HB-EGF receptor react with antigen-positive Vero-H and MDA-MB-231 human breast cancer cells and do not react with antigen-negative cells. Intravenous injection of these immunoliposomes in MDA-MB-231 tumor-bearing mice caused regression of the tumor [56].

Endothelial cells of blood vessels formed de novo in tumors are a good target for immunoliposomes. Resent research showed that de novo blood vessels have antigens which are either lacking or present at undetectable levels in normal blood vessels. One of the representatives of such targets is type 1 matrix metalloproteinase. Monoclonal antibodies to type 1 matrix metalloproteinase react with tumor cells and endothelial cells of *de novo* blood vessels. Immunoliposomes with these antibodies showed good results in *in vitro* experiments [57].

The vascular endothelial growth factor A (VEGF-A) is a target for immunoliposomes based on the recombinant humanized monoclonal antibody bevacizumab (Avastin). Kuesters and Campbell [58] conjugated this antibody with pegylated cationic liposomes and tested them as a delivery vehicle to target human pancreatic cancer cells. Immunoliposomes targeting P-selectin were used to deliver VEGF in a damaged myocardium region in rats with induced experimental myocardial infarction [59].

The epidermal growth factor receptor (EGFR) is a validated target in cancer therapy [60]. This receptor is overexpressed on head and neck epidermoid carcinoma, non-small-cell lung cancer, large and small bowel cancer, as well as pancreatic cancer cells. The set of drugs used in the targeted therapy of these diseases includes tyrosine kinase inhibitors and monoclonal antibodies. However, the disadvantage of targeted therapy is rapidly developing resistance. This disadvantage can be overcome by means of dual-active anti-EGFR immunoliposomes with encapsulated kinase inhibitor AG538. Monoclonal antibodies block the EGFR receptor, whereas the immunoliposome-encapsulated kinase inhibitor blocks the insulin-like growth factor 1 receptor (IGF-1R). The immunoliposomes strongly inhibited tumor cell proliferation even upon short-term exposure [60].

The disialoganglioside receptor GD2 is a marker of neuroblastoma. Immunoliposomes targeting the GD2 receptor were injected in nude mice with human neuroblastoma xenograft. A good therapeutic effect was observed, specifically, tumor regression, destruction of tumor vessels, and prolonged lifespan of mice with orthotopic human neuroblastoma grafts [61].

In one of the first works on the preparation of immunoliposomes, Akaishi et al. [62] conjugated monoclonal antibodies to the CA19-9 antigene with adriamycin-loaded liposomes [62]. These immunoliposomes proved to be more active than pegylated liposomes.

A nonglycosylated antigen Muc-1 is a specific marker of breast and ovarian cancer cells, it is strongly expressed on tumor cells and slightly cells on normal cells. Sokolova et al. [63] prepared doxorubicin-loaded immunoliposomes using the monoclonal antibodies ICO-25 to the Muc-1 antigen. These immunoliposomes react with antigen-positive cells and do not react with antigen-negative cells. It was found that washing free from antigen-positive cells have no effect on their binding and cytotoxicity but adversely affects the binding and cytotoxic effect of liposomal or free

doxorubicin. The cytotoxicity of immunoliposomal doxorubicin to antigen-negative cells compares with that of liposomal doxorubicin.

The antigen Mucin-16 (Muc-16) is a stable marker of ovarian cancer. Antibodies to Muc-16 were used to create immunoliposomes for ovarian cancer diagnostics [64].

Sokolova et al. [65] prepared doxorubicin-loaded immunoliposomes targeted at the HLA-DR antigen, using the monoclonal antibodies ICO-1. On the average, one immunoliposome comprised 23 molecules of the ICO-1 antibodies. Such immunoliposomes react with 98% of antigen-positive cells. *In vitro* experiments showed that the highest cytotoxic effect is characteristic of doxorubicin in a traditional dosage form (IC_{50} 2.5 μg/ml). In this respect, immunoliposomal doxorubicin is close to liposomal doxorubicin: IC_{50} 5.5 and 6.1 μg/ml, respectively. This finding can be explained by the fact that the HLA-DR antigen is incapable for intracellular internalization, and, therefore, the drug releases to the extracellular space, like with a sterically stabilized formulation of doxorubicin. Similar results were obtained by Kirpotin et al. [66].

Stem tumor cells are a good target for immunoliposomes, because they express their CD133 antigen. Sokolova et al. [63] prepared immunoliposomes using monoclonal antibodies to CD133. These immunoliposomes react with antigen-positive cells and do not bind to antigen-negative cells. These immunoliposomes are suggested to be useful for siRNA delivery to tumor stem cells [67].

The efficiency of immunoliposomes can be enhanced by directing them to two targets [68]. To this end, either two monoclonal antibodies or one antibody targeted at both tumor and endothelial cells can be used. Another approach to the enhancement of the therapeutic effect of immunoliposomes consists in mixing immunoliposomes of two types, one targeted at tumor-associated antigens and the other, at blood vessel antigens.

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