Expert Opinion

Drug delivery to the lymphatic system: importance in future cancer diagnosis and therapies

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Cancer is the second leading cause of death in the US. Currently, protocols for cancer treatment include surgery to remove diseased and suspect tissues, focused radiation, systemic chemotherapy, immunotherapy and their combinations. With conventional chemotherapy, it is almost impossible to deliver anticancer drugs specifically to the tumor cells without damaging healthy organs or tissues. Over the past several decades, efforts have been made to improve drug delivery technologies that target anticancer drugs specifically to tumor cells. It has been known for over four decades that the lymphatics are the first site of metastasis for most solid cancers; however, few efforts have been made to localize chemotherapies to lymphatic tissues. Trials of several systemic targeted drug delivery systems based on nanoparticles containing chemotherapeutic agents (e.g., liposomal doxorubicin) have shown similar antitumor activity but better patient tolerance compared with conventional formulations. Animal studies have demonstrated that nanoparticles made of natural or synthetic polymers and liposomal carriers have higher accumulation in the lymph nodes and surrounding lymphatics compared to conventional intravenous therapies. This combination has the potential to both reduce nonspecific organ toxicities and increase the chemotherapeutic dose to the most likely sites of locoregional cancer metastasis.

Keywords: cancer diagnosis, chemotherapies, liposomes, lymphatics, nanoparticles

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1. Introduction

Cancer is the second leading cause of death in the US with treatments costing an estimated $219.2 billion nationally in 2007 as reported by the National Institutes of Health. Depending on the type of cancer and stage, the most common treatments involve surgical removal of the tumor, radiation therapy, chemotherapy, immunotherapy and combinations thereof. Surgical resection is the primary procedure to remove cancers large enough to detect and manipulate. However, surgical resection alone in most cases cannot remove every cancer cell present leaving behind microscopic tumor deposits that over time result in relapse and recurrent disease [1-3]. For many patients, severe side effects of anticancer drugs lead to reduction in dosing or shortened treatment cycles that are suboptimal and reduce efficacy as well as increase rates of recurrence and drug resistance. Such side effects usually result from the inability of current agents to selectively accumulate in cancerous tissues or cells, thereby damaging healthy organs and tissues that are exposed to these cytotoxic agents.

The lymphatic system is a chief component of the immune system and acts as a secondary circulation system to drain excess fluids, proteins and waste products from the extracellular space into the vascular system. The lymphatics have been exploited as a potential means of drug delivery as these channels can transport

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certain lipophilic compounds such as long-chain fatty acids, triglycerides, cholesterol esters, lipid soluble vitamins and some xenobiotics, including DDT (2,2-bis(p-chlorophenyl) 1,1,1-trichloroethane) [4]. The lymphatic system is active in the metastatic spread of cancer cells and dissemination of infection. The regional lymph nodes, once invaded by tumor cells, act as reservoirs where cancer cells take root and seed into other parts of the body [5-9]. The lymphatic system is not easily accessible by conventional intravenous infusion of chemotherapeutics, thus limiting the amount of drug that reaches lymphatic tissues including lymph node metastases.

Lymphatic capillaries play a vital role in particulate absorption and uptake into the lymphatic system and lymph nodes. The walls of capillary lymphatics are made up of a single layer of non-fenestrated endothelial cells that are extensively gapped and overlapped, forming numerous clefts and pores that allow passage of macromolecules into the capillary lumen when interstitial pressure exceeds the intraluminal lymphatic pressure [4]. Among various factors controlling particulate uptake into the lymphatics such as size, composition, dose, surface charge and molecular weight, particle size is the main factor determining *in vivo* behavior of particulates. For instance, there is an optimum range for lymphatic uptake of subcutaneously injected particles: particles > 100 nm in size will remain largely confined to the injection site, particles 10 – 80 nm in size are taken up well by the lymphatics and small particles and molecules (< 20 kDa) are absorbed primarily by rich capillary networks that drain into the systemic circulation [10]. Oussoren *et al*. report that ~ 80% of small liposomes (ca. 40 nm) are cleared from the injection site after subcutaneous administered, but < 20% of 400-nm liposomes left the injection site. Thus, nanocarriers administered subcutaneously have great potential to deliver anticancer drugs to lymphatic metastases.

The carrier system for targeted lymphatic delivery to the tumor should have the following characteristics: efficient uptake into the lymphatic system; high uptake in the lymph nodes; ability to release and accumulate chemotherapeutic agents to the tumor; and low toxicity to normal, healthy tissues. Many attempts have been made to enhance the therapeutic index of anticancer drugs while reducing the adverse side effects. *Trans*-lymphatic drug delivery systems using liposomes, micro-/nanoparticles and immunotherapy are examined in this review, with a focus on their potential impact in the clinical treatment of cancer.

2. Liposome-based drug delivery system

Liposomes are nanoscopic vesicles composed of amphiphilic monomers (e.g., phospholipids) that form a bilayered membrane, with the monomers arranging within the membrane so that the hydrophilic ends point towards hydrated surfaces. Liposomes can encapsulate hydrophilic drugs within their aqueous core or incorporate hydrophobic drugs within the lipid bilayers. Early liposomal delivery systems via the intravenous route were limited by the rapid uptake of drug by the reticuloendothelial system (RES) resulting in a shortened plasma half-life. Additionally, these drugs demonstrated plasma and gastrointestinal tract instability resulting in lipid degradation and drug leakage [11]. As the lymphatic system is difficult to target via the intravenous route, local parenteral administration, such as subcutaneous, intraperitoneal and intramuscular injections, is more extensively investigated for lymphatic delivery via liposomal carriers. This section focuses on liposome lymphatic targeting for the diagnosis and therapy of cancer.

Traditional visualization techniques, including MRI, CT and positron emission tomography (PET) imaging, have limited success in detecting lymph node metastases because these techniques are primarily based on the lymph node anatomy, mostly the size, to differentiate the benign and malignant nodes. Efforts have been made to improve the MR contrast agent by using Gd-labeled liposomes. Liposome-encapsulated gadolinium-diethylenetriamine pentaacetic acid (Gd-DTPA) was subcutaneously injected into the footpad of rabbits [12]. The MRI revealed higher accumulation of the Gd liposomes and enhanced MR contrast in the popliteal lymph node ipsilateral to the side of injection; the enhanced MR contrast was also achieved at the retroperitoneal lymph nodes 20 min after injection and were still detectable after 24 h. PEG-modified Gd liposomes were found to enhance MR contrast signal compared to unmodified liposomes after subcutaneous administration [13]. A higher proton relaxivity and stronger MR signal were achieved by using a polymeric chelator that had the ability to introduce several Gd ions per lipid molecule. In a subsequent study, only PEG Gd liposomes were able to visualize the subscapular lymph node 4 min post injection into the right forepaw of rabbits, compared to plain Gd liposomes [14]. Although only 50% of the PEG Gd liposomes were accumulated in the axillary lymph nodes 2 h after subcutaneous administration, the enhancement of the MR signal in the lymph nodes by PEG Gd liposomes, owing to higher relaxivity, was 3- to 3.5-fold higher than that of the plain Gd liposomes.

Liposomes encapsulating chemotherapeutics have shown promising results in animal models. Radiolabeled methotrexate (MTX) was encapsulated into liposomes or immunoliposomes (anti-rat IgG-conjugated liposomes) [15]. In this study, free tridiated [^{3}H] MTX (treatment I) or [^{3}H] MTX-liposomes (treatment II) or [^{3}H] MTX-immunoliposomes (treatment III) were injected at a dose of MTX 200 μg/kg body weight i.v. into rats. The phamacokinetic study demonstrated that [^{3}H] MTX-liposomes and [^{3}H] MTX-immunoliposomes were cleared from the plasma ($t_{1/2}$ 1,460 and 810 min, respectively) significantly slower than free [^{3}H] MTX ($t_{1/2}$ 336 min); and $AUC_{0-6\,h}$ values for treatment groups I, II and III were 32, 1,730, 212% of dose·min/ml, respectively. Free [^{3}H] MTX (treatment IV) or [^{3}H] MTX-liposomes (treatment V) or [^{3}H] MTX-immunoliposomes (treatment VI) were also

injected into the right thigh muscle at a dose of MTX 200 μg/kg body weight i.m. The free MTX disappeared rapidly from the injection site within 30 min and only 0.19% remained 2 h post injection; however, ~ 16.4 and 23.1% of the injected dose was found at the injection site 24 h after intramuscular administration for treatments V and VI, respectively. As a result of this slow release and sustained drainage of MTX from the liposomes, the $AUC_{0\ -24\ h}$ values in the regional lymph nodes (right lymph nodes) and nonregional lymph nodes (left lymph nodes) increased by 6.6-fold and 5.35-fold for the [^{3}H] MTX-liposomes intramuscular group compared with the free MTX group. Similarly, the $AUC_{0\ -\ 24\ h}$ values for the [^{3}H] MTX-immunoliposomes intramuscular group were 6.66-fold and 4.78-fold higher in the regional and nonregional lymph nodes, respectively. This study proved that MTX-encapsulated liposomes had better lymphatic targeting ability than free MTX. In a subsequent study, it was found that the surface charge of liposomes and the route of administration greatly influenced the biodistribution, lymph node targeting and pharmacokinetics of MTX-containing liposomes [16]. The positively charged MTX-liposomes had the highest localization in the lymph nodes 24 h post intramuscular injection into rats, followed by negatively and neutrally charged MTX-liposomes. After intramuscular injection of MTX-liposomes, the ratio of MTX-equivalents in regional lymph nodes to that in plasma was increased 10- to 20-fold compared to the ratio following intramuscular injection of free MTX. Although liposomes were mainly localized in the liver, spleen and lung following intravenous route administration [17,18], intramuscular injection of MTX-liposomes primarily targeted the lymph nodes with lower accumulation in the kidney, liver and other non-targeted tissues compared with free MTX.

Liposomes containing doxorubicin have been reported by several groups in animal models. Free [^{14}C] doxorubicin, empty [^{3}H] liposomes, free [^{14}C] doxorubicin plus empty [^{3}H] liposomes and [^{14}C] doxorubicin encapsulated into [^{3}H] liposomes were given intraperitoneally to rats. The lymphatic absorption and tissue distribution of doxorubicin and the lipid components were investigated over 24 h [19]. The measured radioactivity from the thoracic duct lymph 24 h post injection revealed a 6-fold increase of doxorubicin from the doxorubicin-encapsulated liposome compared to free doxorubicin treatment. Even though there was an increase in the amount of doxorubicin in the lymph owing to the encapsulation into the liposome, there was a decrease in the amount of radioactive lipid components recovered from the thoracic duct lymph (from 30% for empty liposomes to 10% for the doxorubicin-encapsulated liposomes). From the tissue distribution studies, the concentration of doxorubicin was found to be highest in the draining lymph nodes with most of the uptake being in the diaphragm, liver and spleen after intraperitoneal administration. Encapsulation of doxorubicin into liposomes increased the tissue uptake of drug by the diaphragm, liver and spleen compared to the free doxorubicin injection. Here the doxorubicin concentrations in the thoracic lymph nodes after treatment with doxorubicin-encapsulated liposomes were 6-fold at 4 h post injection and 3-fold higher at 24 h post intraperitoneal injection compared to treatment with free doxorubicin. These findings suggest that liposomes are absorbed from the peritoneal cavity by lymphatics and are retained by draining lymph nodes. Anticancer drugs encapsulated into liposomes that are administrated via the intraperitoneal route may, therefore, provide a means of locally delivering drugs to the lymphatics and tumor metastases. The route of administration and liposome entrapment both play roles in metabolism and disposition of doxorubicin in rats [20]. The concentration of doxorubicin in the draining lymph nodes (mediastinal and renal nodes) after intraperitoneal administration from the liposome-entrapped doxorubicin was 8- to 14-fold (4 h post dose), and 3- to 6-fold (24 h post dose) higher than the concentrations achieved by free doxorubicin. By contrast, there was no significant difference in the concentration of doxorubicin in the lymph nodes for both groups after intravenous administration. A > 38-fold increase in the doxorubicin concentration was seen in the draining lymph nodes for the intraperitoneal injected liposomal-doxorubicin when compared to the intravenous route at both 4 and 24 h post injection.

Liposomes containing doxorubicin have also demonstrated efficacy benefits in clinical trials. In patients with gastric carcinoma, doxorubicin was delivered specifically to the regional lymph nodes at high concentrations by a gastric submucosal injection of doxorubicin-liposomes [21]. A total of 34 patients received liposomal-doxorubicin by a gastric submucosal injection adjacent to the primary tumor and 18 received a similar dose of free doxorubicin by intravenous administration. After liposomal-doxorubicin treatment, the doxorubicin concentration in the primary and secondary draining lymph nodes, which are those most susceptible to metastases, was higher than in the other regional lymph nodes. In contrast, the concentration of doxorubicin in all the lymph nodes after intravenous treatment was ~ 0.22 – 0.72 μg/g, which was much lower than the levels of doxorubicin achieved by the liposomal formulation. When equivalent doxorubicin doses were given either by a submucosal injection of the liposome-doxorubicin or an by an intravenous injection of free doxorubicin, the doxorubicin concentrations in the primary draining lymph nodes were 15.1 ± 8.30 μg/g and 0.29 ± 0.10 μg/g on day 1 for liposome-doxorubicin and free doxorubicin groups, respectively; and the corresponding doxorubicin levels on day 4 were 11.9 ± 4.80 μg/g and 0.36 ± 0.0 μg/g, respectively. This study proved that local delivery of doxorubicin to the regional lymph nodes, which are susceptible to gastric tumor metastases, was possible by a submucosal injection of the liposome-entrapped doxorubicin.

Melphalan is a nitrogen mustard alkylating agent with extremely poor stability at physiological conditions (half-life < 1 h) owing to its rapid hydrolysis resulting in the inactive form of the drug, and has high systemic toxicity. It was

encapsulated into liposomes made of egg phosphatidylcholine (PC) and cholesterol with average entrapment efficiency of melphalan of 8.1% [22]. The release study in PBS at 37°C indicated a fast release of melphalan during the first 4 h (> 70%) and only 20% remained after 24 h. After subcutaneous injection of free melphalan into left leg of rats, different melphalan concentrations were seen between ipsilateral and contralateral nodes at 0.5 h; but no difference was observed at subsequent time points. In the liposomal-melphalan group, the liposome concentration in the ipsilateral lymph nodes was 200- and 100-fold higher than that in the plasma and contralateral nodes, respectively. The encapsulated melphalan demonstrated a sustained release from the liposome *in vivo*. At 24 h post dose, the melphalan level in the ipsilateral nodes was 20- and 10-fold greater than in the plasma and the contralateral nodes, respectively. In the tumor-bearing rats (mammary adenocarcinoma 13762), melphalan-encapsulated liposomes were more efficient in reduction of lymph node metastasis weight than for the same dose of free melphalan (0.125 mg/kg). An equivalent effect on the reduction of lymph node metastases was achieved comparing 0.125 mg/kg liposome-melphalan and 1 mg/kg free melphalan. The role of the liposomal size in lymphatic uptake was investigated in a separate study [23]. Melphalan-encapsulated liposomes produced by short (2 – 4 min) periods of sonication resulted in larger liposomes with sizes ≤ 250 nm. By contrast, liposomes prepared by a prolonged sonication time (40 – 50 min) were relatively uniform with a mean size of 34 nm. When melphalan-encapsulated liposomes were given by subcutaneous injection into the left thigh of rats, the melphalan level in the ipsilateral lymph nodes was increased such that 24 h post dosing, the melphalan levels reached a 10-fold higher concentration for small liposomes compared to large liposomes. The melphalan concentration in the plasma was relatively low for both small and large liposome groups.

In conclusion, factors including liposome size and route of administration were significant in determining lymph node uptake of liposomes and drug encapsulated. From the results on lymphatic targeting via liposomes, the same conclusions can be reached: that chemotherapeutics and imaging agents can by delivered specifically to the lymphatics with high concentrations by means of locally injected liposomes; and that this liposomal delivery system may improve treatment of lymphatic metastases by accumulating chemotherapeutics in the regional lymph nodes.

3. Polymer-based drug delivery systems

Chemotherapeutics and diagnostic agents have been encapsulated into polymeric nano- and/or microparticles to improve lymphatic targeting and to suppress tumor metastasis. Natural polymers such as dextran [24] and hyaluronic acid (HA) [25,26], as well as synthetic polymers such as poly(lactide-co-glycolide) (PLGA) [27], poly(L-lactic acid) (PLA) [28,29], polyhexylcyanoacrylate (PHCA) and polymethylmethacrylate (PMMA) [30] have been extensively investigated as drug carriers for targeted lymphatic delivery.

Lymphatic imaging using nano-/microparticles demonstrated enhanced lymphatic uptake and metastasis detection. Kobayashi *et al.* reported successful detection of lymphatic drainage of breast cancer in normal mice and spontaneous (BALB-neuT mice) and xenografted (PT-18) breast tumor models after a direct mammary gland or peritumoral injection of the generation-6 polyamidoamine dendrimer Gd contrast agent [31]. In a following study, Gd-labeled dendrimer-based contrast agents (1 – 12 nm in diameter) were compared in terms of the efficiency of delivery of the agents to the sentinel lymph nodes [32]. The generation 6 polyamidoamine (PAMAM-G6) Gd-dendrimer agent (ca. 9 nm) illustrated the axillary lymph nodes and lymphatic vessels more clearly in non-tumor-bearing mice than the other agents including PAMAM-G2, G4, G8 and generation 5 polypropylenimine dendrimer Gd agents. The peak concentration of the PAMAM-G6 agent (> 400 ppm) was achieved at 24 – 36 min post injection with high signal-to-background ratio (> 100). Although no significant difference was found between micrometastatic or non-metastatic lymph nodes for the accumulation of Gd in the axillary lymph nodes, the PAMAM-G6 Gd dendrimer showed the potential for targeted delivery to the lymph nodes.

The lymphatic system and lymph nodes can be accessed by polymeric nano-/microparticles. In 1992, Maincent *et al.* studied the lymphatic targeting of radiolabeled PHCA and PMMA nanoparticles after intraperitoneal administration in rats with a thoracic duct cannula [30]. The dramatic results from this study were that the measured radioactivity in the mediastinal nodes in rats receiving intraperitoneal injections of PHCA and PMMA nanoparticles was 70- to > 2,000-fold higher than the corresponding lymph nodes of rats receiving intravenous injections of the nanoparticles. In another study, three different kinds of micro- and nanoparticles made of activated charcoal, modified polystyrene (PS) or PLGA-rhodamine were injected into the pleural space of healthy rats, post-pneumonectomy rats and rats bearing orthotopic lung cancer [27]. In the pneumonectomy rat model, the accumulation of charcoal particles (700 – 1,500 nm) in the regional thoracic lymphatic system, the mediastinal lymph nodes and peri-aortic lymph nodes in the abdomen was observed after 3 h. It was found that the smaller the charcoal particle, the lesser the deposition that occurred in the thoracic lymphatics. In the healthy rat group, PS and PLGA-rhodamine particles were taken up into the regional lymphatics and evenly distributed in the lymph nodes. In contrast, particles were deposited mainly in the peripheral regions of the lymph nodes 24 h following injection into the metastatic lung tumor. These results indicated that regional thoracic lymphatics and lymph nodes can be accessed by colloidal particles, and thus, targeted delivery to the lymphatic system via micro- and nanoparticles might be a means to treat cancer metastases.

Surface properties of nano-/microparticles are one of the chief determinants of lymphatic uptake and biodistribution *in vivo*. Hawley *et al.* investigated the relationship between surface properties of nanoparticles and *in vivo* biodistribution [33]. Poly(lactide)-poly(ethylene glycol) (PLA:PEG) copolymers with PEG lengths of 750, 2,000 and 5,000 Da were used as a surface coating of PS and poly(DL-lactide-co-glycolide 75:25) (PLGA) nanoparticles. The PLGA–PLA:PEG nanoparticles were prepared by an oil-in-water emulsion of PLGA and PLA:PEG. All of the nanoparticles were sub-100 nm in size and negatively charged on their surfaces, with the PEG portion forming steric barriers. ^{125}I radiolabeled, uncoated PS or PLGA nanospheres were retained in the injection site ≤ 24 h after subcutaneous administration in rats. The lymphatic uptake of the nanoparticles was strongly dependent on the surface characteristics. Polystyrene or PLGA nanospheres coated with PLA:PEG (molar ratio 1.5:0.75, PEG length 750 Da) had the most lymph node uptake, ~ 15 – 20%, owing to a suitable hydrophilicity on the surface, which provided an adequate steric barrier to promote drainage from the injection site, while remaining hydrophobic enough to be recognized by the lymph node macrophages. Less than 3% of naked PLGA nanoparticles injected were retained in the lymph nodes, whereas the lymphatics retained 17 and 16% of the nanoparticles prepared by co-precipitation of PLGA and PLA:PEG (1.5:0.75) with 75 and 65% PLGA content, respectively. The uptake into the lymph nodes decreased with further increase (> 65%) of PLA:PEG content, possibly owing to the surface being too hydrophilic. The incorporation of hydrophilic PEG onto PS and PLGA nanoparticles improved their lymphatic drainage, but the rate of lymph node uptake was closely related to the length of the PEG chain and eventually the hydrophilicity of the nanoparticles.

Natural polymers, for example, dextran and HA, have been studied for their potential use as carriers for lymphatic drug delivery for cancer. Mitomycin C (MMC) was conjugated to dextran (average Mw of 10, 70 and 500 K), and distribution of the mitomycin–dextran (MMC–D) conjugate was compared to free MMC after injection into the left thigh muscle of rats [24]. The MMC–D conjugate slowly cleared from the injection site with a significant amount remaining in the muscle after 48 h, whereas only 0.56% of the free MMC remained at the injection site after 30 min. The free MMC reached a peak regional lymph node concentration of 11.8 μg/g after 5 min, but no drug was detectable after 30 min. MMC-D (T-70K and T-500K) showed a higher and sustained accumulation in the regional lymph nodes even ≤ 48 h post dose. In rats inoculated with L1210 leukemia cells in the left thigh, no significant tumor suppression was observed in the lymph node metastases with free MMC or MMC-D(T-10K) groups owing to low MMC uptake by the nodes. In contrast, the T-70K and T-500K MMC-D (2.5 mg/kg dose) reduced the weight of the left iliac node, indicating the suppression of tumor growth and metastasis via lymphatic system. In conclusion, a dextran conjugate of MMC may be useful for preventing lymphatic metastasis of cancer.

Hyaluronic acid is a natural polysaccharide of D-glucuronic acid and D-*N*-acetylglucosamine found in the interstitial space of tissues and in the synovial fluid of joints. Cisplatin was incorporated with HA by anionic polymer–metal complexation between the HA and cisplatin [25]. The nanoparticles were ~ 100 – 200 nm in size, with cisplatin loading from 3.9 – 11.8% (w/w). The cisplatin release kinetics demonstrated that the cisplatin–HA conjugate had an initial burst release in the first 12 h followed by a sustained release for 4 days. The cisplatin release rate was enhanced by the addition of hyaluronidases (HAses), the enzyme that degrades HA. Cai *et al.* reported an *in vivo* study of cisplatin–HA nanoparticles (25% w/w cisplatin), where the cisplatin–HA conjugates showed similar anti-tumor activity and were well tolerated in rodents compared to intravenous cisplatin [26]. The AUC of cisplatin in the axial lymph nodes after subcutaneous injection of cisplatin-HA increased 74% compared to free cisplatin. Therefore, the cisplatin–HA nanoparticles are suitable drug delivery vehicles for platinum chemotherapy in the lymphatics.

Several studies have reported lymphatic delivery of paclitaxel (PTX) using nano-/micro-spheres. Liggins *et al.* investigated PTX-loaded PLA microspheres for prevention or intraperitoneal carcinomatosis [28]. Empty microspheres ranging from 1 to 40 μm (mean 8.6 μm) were injected intraperitoneally into rats, after which 24 ± 9 μm (~ 30 μm) microspheres were detected in the mediastinal lymph nodes. Paclitaxel-loaded microspheres (100 mg of 30% loading) with size range of 30 – 120 μm were injected intraperitoneally into a rat model of a tumor cell spill after a cecotomy repair. Rats treated with PTX microspheres showed no evidence of tumors or nodules in the peritoneal cavity or in the surgical wound site, possibly owing to the sustained release of PTX from the microspheres. In comparison, the control group had a zero survival rate after 4 weeks. Using an oil-in-water emulsification, PTX was encapsulated into polylactic acid (PLA) nanoparticles of 200 nm with 70% encapsulation efficiency [29]. The antitumor activity of free PTX and PTX-loaded PLA nanoparticles was investigated in F344 rats with NuTu19 ovarian cancer in the peritoneal cavity. Rats were injected intraperitoneally weekly for 5 weeks with either free PTX (5 mg/kg), PTX-PLA nanoparticles (5 mg/kg PTX-equivalent), empty PLA nanoparticles or saline. The PTX-PLA group had superior antitumor activity with 45% less tumor weight and 12% ascites volume compared to the PTX group. In the PTX-PLA group, PTX was found to be mainly concentrated in the pelvic lymph nodes and tumor (peaking at 48 h) and then the liver, whereas the free PTX groups had the highest drug concentration in the heart tissue (more toxicity). In the PTX-PLA group, PTX was detectable in the plasma from 8 h and until 48 h post injection (T_{max} 24 h). In contrast, the free PTX group had a peak plasma concentration after 12 h and was undetectable

after 24 h. These results indicated that sustained release of PTX in the PTX-PLA group increased accumulation in the tumor and lymph nodes, and longer circulation in blood, may have increased the antitumor activity of PTX-PLA compared to intravenous PAX.

In conclusion, with the advantages of particle engineering and a better understanding of the lymphatic anatomy and the drug transporting mechanism by the lymphatic system, lymphatic delivery via polymeric nano-/microparticles may improve the efficacy of current cytotoxic chemotherapeutics.

4. Immunotherapy

Early detection of lymphatic invasion and tumor metastasis is critical for the success of cancer treatment. However, it is almost impractical to distinguish between the blood and lymphatic vessel systems *in vivo* owing to the fact that these two systems are so interrelated. Recent development of lymphatic-specific markers, including the lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1), prox-1, podoplanin, VEGFR-3 and D2-40 monoclonal antibody [34], have made it possible to visualize lymphatic vessel invasion, tumor metastasis and even dynamic tumor cell trafficking *in vivo*. Lymphatic vessel endothelial hyaluronan receptor-1 is one of the most important lymphatic-specific markers currently used for detecting lymphangiogenesis and tumor metastasis [35]. In 67 human breast cancer patients who underwent breast cancer surgery, tumor tissue samples were immunohistochemically stained with hematoxylin and eosin (H&E), the pan-endothelial marker factor VIII, rabbit polyclonal antibody against LYVE-1 (LYVE-1/PCAB) or mouse nomoclonal antibody against LYVE-1 (LYVE-1/MCAB) [34]. Lymphatic vessels were found in the extralobular stroma but mostly not in the intralobular stroma or the tumor body, while blood vessels were localized in both the intra- and extralobular stroma. In detecting tumor lymphatic vessels and lymphatic vessel invasion (LVI), LYVE-1/PCAB was found to be more sensitive than the other immunostains. The LVI quantified by LYVE-1/PCAB was detected in 25 out of 67 patients, and LVI was statistically related with both lymph node metastasis ($p = 0.0248$) and unfavorable overall survival ($p = 0.0453$). Lymphatic vessel endothelial hyaluronan receptor-1 has been used for the detection of the lymphatic invasion of regional lymph node metastasis in early gastric cancer [36]. Lymphatic and blood vessel invasion were differentiated by using the LYVE-1 antibody specific to lymphatic vessels and the von Willebrand factor (vWF) antibody specific to blood vessels. Tumor tissues from 66 node-positive and 66 node-negative patients with early gastric cancer were immunostained with antibodies against LYVE-1 and vWF. The lymphatic invasion was found mostly at the cancer periphery and rarely in the central part of the cancer; in contrast, blood vessel invasion was seen in the central and peripheral portions of the primary tumor. In the multivariate logistic regression analysis of node-positive early gastric cancers, the results indicated that lymphatic invasion was a significant predictor for regional lymph node metastasis ($p = 0.0094$), while blood vessel invasion was not an independent predictor.

Fluorescently labeled LYVE-1 antibody has made *in vivo* lymphatic imaging and real-time trafficking of cancer cells possible [37]. AlexaFluor-conjugated LYVE-1 was injected into the tissues surrounding the inguinal node in athymic nude mice. Whole body *in vivo* fluorescent imaging revealed that the clearest signal was detected at 4 h post injection and was still visible at 48 h in the draining lymphatics. Both AlexaFluor-conjugated IgG and FITC-dextran provided minimal fluorescent signal in the draining lymphatics at 4 h, which decayed rapidly resulting in no detectable signal at 12 h post injection. To study real-time tumor cell trafficking, the human pancreatic cancer cell line, XPA-1 expressing red fluorescent protein (RFP), was locally injected into and around the inguinal lymph node 4 h after the AlexaFluor-conjugated LYVE-1 was administered. The *in vivo* imaging clearly showed the trafficking of the tumor cells from the inguinal node to the draining axillary node, where the tumor cells accumulated.

Antibody–drug conjugates are a type of immunoconjugate with bifunctional molecules, consisting of a targeting domain (antibody) that localizes the conjugate to tumors and a therapeutic moiety (antitumor drug). The first report of using monoclonal antibody–drug conjugates to prevent the local recurrence of colorectal cancer was published in 1992 [38]. The murine monoclonal antibody A7 (Mab A7) against human colorectal cancer was conjugated to neocarzinostatin (NCS) through a disulfide bond. As indicated in one of their previous studies, A7-NCS was stable *in vivo* and had similar pharmacokinetic properties as that of the parent Mab A7 when systemically administrated [39]. In tumor-free Balb/c mice, the retention of Mab A7 was much higher when administrated locally to the pelvis and thigh than when given intravenously, which might be explained by the prevention of Mab A7 (similar size of IgG) transport into the blood capillaries by the tight vascular endothelium and by the presence of a continuous basement membrane. A significantly higher tumor localization of ^{125}I-Mab A7, injected intratumorally or peritumorally, was observed in comparison to intravenous injection. The ipsilateral regional lymph node had extensive accumulation of the ^{125}I-Mab A7 but not the contralateral regional lymph node. Meanwhile, accumulation of Mab A7 was lower in the blood, liver, spleen, kidneys and lungs of the locally injected group than in the intravenously injected group. Intratumoral injection of A7-NCS led to the complete remission of established tumors in 5 out of 6 antigen-positive xenograft-bearing mice but in only 1 out of 6 antigen-negative xenograft-bearing mice. A single dose of A7-NCS was effective in inhibiting the tumor development in 12 out of 16 antigen-positive tumor-bearing mice and 5 of 15 antigen-negative tumor-bearing mice. Neither A7-NCS nor NCS injected systemically and

NCS or saline injected locally significantly inhibited tumor growth. The systemic toxicity of NCS decreased significantly when it was conjugated to the A7 antibody and was administered locally.

Lymphatic imaging and drug delivery using antibody-based immunotherapy has its own advantages, including more specific targeting to the lymphatic system and tumor cells, compared to the passive targeting achieved by liposomes or nano-/microparticles. However, this benefit must be weighed against the high cost and difficult production of antibodies compared to relatively inexpensive natural and synthetic polymer carriers.

5. Expert opinion and conclusion

The lymphatics have the potential to play a large role in anticancer treatment as lymphatic spread is recognized to precede hematological spread in many cancers including melanoma, breast, colon, lung and prostate cancers. Sentinel node mapping is a crucial tool in cancer staging and treatment, and new lymphatic imaging agents that enhance magnetic resonance and deep tissue fluorescent imaging of the lymphatics will greatly increase the diagnostic power of lymphatic imaging in cancer treatment. Lymphatic drug delivery is in its infancy, but localized treatments of the lymphatics will decrease systemic toxicities associated with cytotoxic chemotherapy and reduce recurrence owing to residual local disease. By increasing our understanding of lymphatic transport and uptake and the role of lymphatics in cancer spread, we can design new therapeutics that may one day supplement or even replace radiotherapy for local disease control.

In summary, we believe a future direction in cancer therapy will involve combining emerging nanocarrier technologies with locoregional therapy to the lymphatics. This combination has the potential to both reduce non-specific organ toxicities and increase the chemotherapeutic dose to the most likely sites of locoregional cancer metastasis. In addition, advanced lymphatic imaging tools will improve cancer staging and reduce the need for destructive nodal dissections.

Declaration of interest

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