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EXPERT REVIEW

Nanoparticles: A Promising Modality in the Treatment of Sarcomas

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ABSTRACT Improvements in surgical technique, chemotherapy, and radiotherapy have enhanced the prognosis of sarcoma patients, but have since reached a plateau in recent years. Novel approaches have been sought but with limited results. Nanomedicine offers solutions in diverse areas of sarcoma therapy including diagnosis and treatment. Several varieties of nanoparticles, including multifunctional nanoparticles, are available that localize the biodistribution of conventional chemotherapeutics to the tumor site. Also, nanoparticles loaded with chemotherapeutic drugs have the ability to overcome drug resistance which is a major obstacle impeding the progress of the treatment. Multifunctional nanoparticles, which have the potential to further augment the bioavailability of drugs, are being actively investigated. In this review, we will discuss the application of nanoparticles for improving the treatment of sarcoma patients.

KEY WORDS nanoparticle · sarcoma · tyrosine kinase · drug resistance · cancer-initiating cell

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INTRODUCTION

Bone and soft tissue sarcomas comprise a diverse group of malignant mesenchymal tumors, affecting patients of all ages. Sarcomas account for approximately 1% of all adult and 12% of pediatric malignancies (1,2). They represent more than 40 histological subtypes and display different biological characteristics and clinical behavior, treatment of which requires different (sub-type-specific) therapeutic strategies. Each year, there are approximately 11,000 new cases of sarcomas in the United States, which includes both soft tissue and bone. Metastatic disease is common, and as a result, there are approximately 5,500 deaths annually (3). The majority of these patients are young; thus, sarcomas are significant public health problems despite their low incidence. Current-day multimodality therapy including surgery, chemotherapy, and radiotherapy has improved the prognosis of sarcoma patients, but overall prognosis of patients has since plateaued in the past decade. While doxorubicin and ifosfamide are the two most widely used agents for the treatment of several sarcomas, they provide only a narrow therapeutic window except for a few responsive subtypes. Many sarcomas are chemoresistant and/or radioresistant, and recurrent tumors are often progressive, with further chemotherapy often being palliative and toxic. The choice between combination therapy and sequential single agents or variations in dose and dose intensity has not yielded definitive improvements (4–6).

Although sarcomas are generally divided into either arising from the bone or the soft tissue, some bone sarcomas arise in the soft tissue only and *vice versa*. Therefore, it is sometimes more amenable to divide sarcomas into two types based on molecular genetics, cytogenetics, and expression profiling. There is one group, comprising about one-third of the population (7), with clear

diagnostic molecular events (Table I), and another two-thirds have multiple histological and genetic changes. Targeting underlying molecular events in specific sarcomas can provide striking effects, as has been demonstrated in gastrointestinal stromal tumors treated with imatinib mesylate (Gleevec, Novartis Pharmaceuticals Corp.) (8). Although there seems to be considerable cross talks between different signaling events, understanding the biology of sarcomas and identifying critical signaling pathways involved in the etiology and progression of these tumors could lead to the discovery of novel therapeutic agents.

Nevertheless, there are currently limited amounts of antitumor agents that are effective against sarcomas, and even those sarcomas that show sensitivity to drugs sometimes experience recurrent disease which is drug resistant. It is important to note that because of the diverse variety of sarcomas, conventional and ongoing studies for improving the treatment of the disease have to be tailored to a particular pathology type. Nanotechnology holds promising potential by increasing the biodistribution of the chemotherapeutic drugs to the tumor site and overcoming the drug-resistant sarcomas. The delivery of RNAi could also be further improved by nanoparticles. The requirement for pathology-specific treatment is why therapeutic progress has been slow for sarcomas, even though the prevalence of sarcoma is low relative to other forms of cancer. Nanoparticles could partially unify the treatment of different sarcomas by offering a customizable delivery platform, thus facilitating the progress.

Nanoparticles have been constructed from a wide variety of materials and are used to solubilize and encapsulate biologically active agents for improved delivery or to provide unique optical, magnetic and electrical properties for imaging and therapy. There have been several applications of nanoparticles in the treatment of sarcoma, including polymeric nanoparticles (9–16), micelles (17), lipid nanoparticles (18–20), dendrimers (21), quantum dots (22,23), and metallic nanoparticles (24,25). Although there have been numerous studies using various nanoparticles, each with its own advantages and disadvantages, they need to share a common goal—they have to be able to reach the tumor by overcoming various barriers without losing their content, and they have to release the maximum dose of their content in the tumor environment efficiently. Ideally, nanoparticles should be inert, free of leachable impurities, and biodegradable. The potential of nanoparticles to cause systemic side effects has been well recognized, but most of the toxicity stems from the use of materials that are not designed for *in vivo* use. One of the advantages of using nanoparticles is that their sizes are adjustable and their surface characteristics can be modified by adding hydrophilic polymers, enabling them to escape opsonization by macrophages. In order to increase the efficiency of the contents' release, many novel ideas have been reported. Modulation of the tumor environment through physical manipulation can enhance drug delivery and efficacy. A variety of combination therapies using ultrasound (26,27), photodynamics (28,29) and hyperthermia (30,31) have been

Table I Cytogenetic Aberrations in Sarcomas

Tumor type	Cytogenetic changes	Gene rearrangement
Ewing's sarcoma/primitive neuroectodermal tumour	t(11;22)(q24;q12)	*FLI1-EWS* fusion
	t(21;22)(q22;q12)	*ERG-EWS* fusion
	t(7;22)(p22;q12)	*ETV1-EWS* fusion
	t(17;22)(q12;q12)	*EIAF-EWS* fusion
	t(2;22)(q33;q12)	*FEV-EWS* fusion
Alveolar rhabdomyosarcoma	t(2;13)(q35;q14)	*PAX3-FKHR* fusion
	t(1;13)(p36;q14)	*PAX7-FKHR* fusion
Myxoid/round-cell liposarcoma	t(12:16)(q13;q11)	*CHOP-TLS* fusion
	t(12;22)(q13;q11-12)	*CHOP-EWS* fusion
Desmoplastic small round cell tumor	t(11;22)(p13;q12)	*WT1-EWS* fusion
Synovial sarcoma	t(X;18)(p11.2;q11.2)	*SSX1-SYT* fusion
		SSX2-SYT fusion
Clear-cell sarcoma	t(12;22)(q13;q12)	*ATF1-EWS* fusion
Extraskeletal myxoid chondrosarcoma	t(9;22)(q22;q12)	*TEC-EWS* fusion
Dermatofibrosarcoma protuberans/giant-cell fibroblastoma	t(17;22)(q22;q13)	*PDGFB-COL1A1* fusion
Infantile fibrosarcoma	t(12;15)(p13;q25)	*ETV6-NTRK3* fusion
Alveolar soft-part sarcoma	t(x;17)(p11;q25)	*PAX3-TEF3* fusion
Low grade fibromyxoid sarcoma	t(7;16)(q32;p11)	*FUS-CREB3L2* fusion
Endometrial stromal sarcoma	t(7;17)(p15;q21)	*JAZF1-SUX12* fusion

reported that increase the efficiency and potency of the nanoparticulate system. Also, nanoparticles could be adjusted to be pH sensitive, such as pH-sensitive liposomes (32), so that the content of the nanoparticles will be released only in the acidic nature of the tumor environment. Inclusion of a histidine-lysine peptide in the nanoparticulate complex has been reported to enhance endosomal release resulting in increased effect (33,34). These novel characteristics make nanoparticles an ideal system for the improved delivery of chemotherapeutic drugs and genes. The purpose of this review is to highlight the biological, therapeutic, and clinical role of nanoparticles in overcoming the barriers in the current treatment of sarcomas.

NANOPARTICLES FOR TUMOR IMAGING

Tumor imaging has greatly improved the prognosis of sarcoma patients. Imaging is a key in detecting the tumor mass and recurrence and determining the therapeutic response after adjuvant therapies. In surgery, MRI has greatly aided in pre-operative planning. We are now able to resect the tumor with enough margins to avoid local or distant metastasis. Recently, there has been a great improvement in visualizing the tumor's biological events with the introduction of positron emission tomography (PET), single photon emission computed tomography (SPECT), and optical imaging including fluorescence-mediated tomography and near-infrared fluorescence reflectance (NIRF) imaging (35–37). Now commonly used, PET utilizes 18F-labeled fluorodeoxyglucose (FDG), which is up-taken by cells showing increased glucose metabolism. It has been implemented in some sarcomas, but is not suitable for sarcomas with no increase in glucose uptake.

Nanoparticles have the potential to be an effective diagnostic tool because contrast agents and/or tracking agents can be encapsulated in the nanoparticles, or alternatively, functional groups can be conjugated to the surface of the particles to allow coupling with various imaging probes. The surface of the particles can be further modified with ligands that actively target tumor cells. These nanoparticles can be used to image specific sarcomas with high accuracy and monitor how these sarcomas react with different treatments. Recently, tumor-targeted optical, radioactive, and magnetic probes have been produced and investigated in *in vivo* models and several clinical trials (38–41). Nanocrystals such as quantum dots have been utilized to simultaneously target and image prostate tumors in living animal models (42). Quantum dot producing NIRF signals has also been reported (43). Metallic nanoparticles such as magnetic iron oxide nanoparticles have been an attractive tool in the development of target-specific MRI contrast agents (44). Nanoparticles of specific sizes can be synthesized under special conditions to obtain the desired optical and magnetic properties to further improve the diagnosis and early detection of the tumor, and consequently the prognosis of sarcoma patients.

NANOPARTICLES FOR DRUG AND siRNA DELIVERY

One reason that anticancer drugs fail to eradicate cancer cells is because they are administered systemically; this results in variations in the biodistribution, absorption, and metabolism of the drugs. Tumors are often localized in regions that are difficult for chemotherapeutic drugs to penetrate; additionally, tumors are protected by the local microenvironment due to increased tissue hydrostatic pressure and altered tumor vasculature (45,46). Nanoparticles are able to accumulate at the tumor site by way of passive targeting and/or active targeting (Fig. 1). Passive targeting is a conventional pathway that relies on the unique properties of tumor vasculature such as the enhanced permeability and retention (EPR) effect (47). Nanoparticles, taking advantage of their inherent size (<100 nm), are able to pass through the leaky vasculature of a tumor (600~800 nm) and remain in the tumor microenvironment due to poor lymphatic drainage. Dramatic increases in drug accumulation have been reported by as many as 10-fold compared to free drugs (48). It has been reported that accumulation of nanoparticle-delivered drugs is 45–250 times higher at the site of tumors compared to other vital organs such as the liver, kidney, lung, spleen, or heart (49). In spite of these results showing increased accumulation of chemotherapeutic drugs at the tumor site via passive targeting, residual accumulation, especially in the reticuloendothelial system, still exists, and increased tumor specificity is desirable to decrease off-target effects.

Targeted Delivery of Nanoparticles to Sarcomas

An alternative strategy is to actively target nanoparticles by conjugating an antibody or a ligand specific to the tumor on the surface of nanoparticles. The objective of attaching a targeting molecule to nanoparticles is to increase the bioavailability of chemotherapeutic drugs within the tumor microenvironment, subsequently decreasing the residual toxicity of the drugs. For a ligand to be effective, it needs to have a high affinity for its receptor and stimulate internalization of the nanoparticles and their conjugates. Currently, several targeted nanoparticle formulations have been investigated in preclinical studies for other types of cancers (50,51).

In sarcomas, there are groups of tumors characterized by specific genetic changes and overexpression of certain

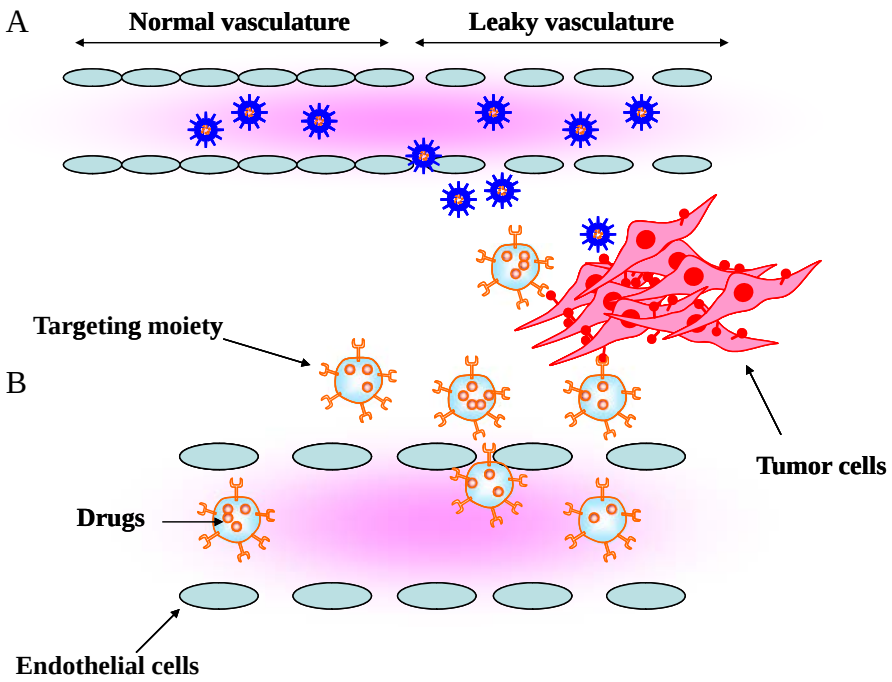

Fig. 1 Passive targeting and active targeting. **A.** Pictorial representation of passive targeting. Tumor tissue vasculature is hyperpermeable compared to the normal vasculature, and nanoparticles are able to accumulate preferentially in the tumor environment due to the enhanced permeability and retention effect. **B.** Targeting ligand or antibody is conjugated to the nanoparticle, thereby allowing increased accumulation of the chemotherapeutic drugs or genes to the tumor site.

proteins. These profile markers that are preferentially expressed in subsets of sarcomas could be used as molecular targets for the delivery of drug-loaded nanoparticles. In a report by Morizono *et al.*, a mouse melanoma model engineered to express the human ABCB1 gene was used to show that metastatic cells can be effectively targeted with lentiviral vectors linked to an anti-P-gp monoclonal antibody (52). Furthermore, a dual-ligand targeting approach with nanoparticles has been reported to improve the specificity over single-ligand targeting due to the fact that many tumor cells overexpress multiple types of surface receptors (53).

In general, a nanoparticulate complex will be taken into a cell via receptor-mediated endocytosis. Once inside the cells, drugs will be dissociated from the nanoparticles by lysosomal enzymes, and the receptors will be recycled back to the cell surface. Molecular targets, although present in the tumor environment, may also exist in normal tissues. For example, P-gp, which is overexpressed in drug-resistant sarcomas, is also an important constituent of various normal tissues such as peripheral blood cells and hematopoietic progenitors found in normal human bone marrow and the blood brain barrier (54–58). In these physiologically normal tissues, P-gp plays an important role in the transport of steroids, the efflux of toxic molecules, and the production of bile, and is an important component of cellular defense and protection (59,60). Although P-gp expression in these tissues is relatively low, P-gp may be important in protecting rapidly dividing cells from toxicity after exposure to anticancer drugs (61). Consequently, treatment-related morbidity, mortality, and increased marrow toxicity associated with chemotherapeutics and biological agents that target P-gp have to be considered carefully. Folate receptors are another example of molecular targets that are highly expressed in tumor cells of ovary, brain, kidney, breast, and lung cancers. Several nanoparticles with high affinity for folate receptors are currently under development. The first clinical study with actively targeted multifunctional nanoparticles involved doxorubicin and galactosamine conjugated to HPMA (51). The authors reported that the targeted nanoparticulate complex accumulated effectively in hepatocellular carcinoma compared to non-targeted polymer complexes. However, the formulation had high affinity to normal cells as well as cancer cells. Unfortunately, to date, the attachment of targeting moieties such as antibodies has not been very successful in animal studies. Selecting an appropriate molecular target that is specific to sarcomas will be the key in future developments of novel multifunctional nanoparticles for the treatment of the disease.

Targeting Tyrosine Kinases

One of the most well studied areas in sarcoma treatment has come from targeting tyrosine kinases. More than 500 members of these cellular proteins encoded by the human genome are expressed ubiquitously and mediate a wide variety of cellular functions, including proliferation, migration, and apoptosis. A receptor kinase that has an "oncogenic addiction" (62) for a particular signal could be blocked by certain agents, leading to growth arrest or apoptosis. To date, there have been at least 30 different

kinase inhibitors that have been developed to the level of phase 1 clinical trial for various types of cancer; new inhibitors are expected to emerge as high-throughput technologies and bioinformatics continue to advance. By combining targeted therapeutics with nanoparticles or using these markers for active targeting, it may be possible to modulate the various oncogenic pathways for the treatment of cancers. For sarcomas, there have been some promising but partial responses using several kinase inhibitors. The IGF 1R pathway has been implicated in osteosarcoma (63,64), Ewing sarcoma (65,66), and rhabdomyosarcoma (66,67). Other targets include the ERBB family (68,69), Met (70), Src (71), the MAPK cascade (72), Raf (73), c-KIT (74), PDGFR (75), AKT (76), and signaling pathways such as WNT/β-catenin (77), Hedgehog (78), and Notch (79). Recently, we have identified PLK1 (80)and MIRK (81) as potential targets of osteosarcoma using a lentiviral shRNA library. Overexpession of PLK1 correlated with worse prognosis in osteosarcoma patients; the PLK1 inhibitor, scytonemin, showed dose-dependent antiproliferative effects and induced apoptosis. MIRK also correlated with worse prognosis and inhibition of the kinase using siRNA inhibited cell growth and induced apoptosis.

Alternatively, other targets, such as VEGF, that do not target the tumor directly, but the niche that tumors grow in, have also been extensively studied in sarcomas (82,83). Various alterations in the tumor-host environment such as local metabolism of a drug by the stromal cells, angiogenesis, and vasculogenesis could affect the transit time of drugs within sarcomas and the way in which cells in a sarcoma interact with each other and with interstitial cells of the host. Although kinase inhibitors have been studied as therapeutic drugs either as monotherapy or in combination for the treatment of various cancers and have shown tremendous results in some instances, these same kinase targets have the potential to modify and improve the therapeutic potency of nanoparticles for the treatment of various sarcomas. Recently, multifunctional nanoparticles, with deslorein, a luteinizing hormone-releasing hormone (LHRH) agonist, and Arg-Gly-Asp (RGD) peptide conjugated nanoparticles encapsulating anti-vascular endothelial growth factor (VEGF) intraceptor (Flt23k; RGD-Flt23k-NP) have been reported to show enhanced efficacy against H1299 lung cancer cells (84).

Although the potential of combining targeted therapeutics with nanoparticulate delivery systems seems limitless, there have been only few reports of this combination (85,86). This novel combination may be able to overcome some of the problems that single targeted therapies face, such as drug resistance after prolonged therapy and high off-target effects from the inhibitors. Nonetheless, to optimize the application of these molecules as targets, it is clear that a more profound knowledge regarding cross talk between each kinase is necessary to achieve a higher specificity and affinity for target tissues.

Illustrative Examples for siRNA Delivery

Although many studies have focused on small molecules inhibiting certain signaling cascades of sarcomas, RNAi has emerged as a potential and promising method for inhibiting these genetic signals. RNAi has gained attention in the last 10 years and has become a promising tool in the development of cancer therapy. SiRNA is pursued because of its high specificity, high efficiency, and low toxicity. Theoretically, when appropriate siRNAs are used, they could silence nearly any gene in the body, giving them a broader therapeutic potential than typical small molecules.

RNAi is a fundamental pathway in eukaryotic cells by which sequence-specific siRNA is able to target and cleave complementary mRNA. The phenomenon of gene silencing via RNAi was originally identified in *Caenorhabditis elegans* as a response to the administration of double-strand RNA (dsRNA) in 1998 (87). When dsRNAs are introduced into the cytoplasm, they are cleaved by the RNase III enzyme Dicer into siRNAs which are 19–21 nucleotide duplexes. SiRNA is then taken up by RNA-induced silencing complex (RISC) where the sense passenger strand is cleaved, and the antisense strand, whose 5′-end is less tightly bound, is incorporated as the active guide strand. Mature RISC, which contains the "splicing" protein (Argonaute 2), finally recognizes and cleaves the target mRNA (88,89).

Sarcomas show two types of genetic alterations where one group includes reciprocal translocation resulting in fusion genes and oncogene or tumor-suppressor mutations such as c-kit and p53. Although another group of sarcomas characterized by considerable genetic heterogeneity is impractical to treat by RNAi alone, sarcomas characterized by fusion oncogenes and specific mutations are good candidates for treatment with RNAi. Onyx-0115, a type 2/5 chimeric adenovirus that has been modified by attenuation of the E1B-55 kDa gene has been used in clinical trials to treat advanced sarcomas (90). E1B-55 kDa in complex with other proteins inactivates the p53 tumor suppressor gene. Toub *et al.* developed polyisobutylcyanoacrylate aqueous core nanocapsules that encapsulate siRNA targeting the EWS-Fli1 transcript (at the junction point type 1) to treat Ewing sarcoma. These biodegradable siRNA-loaded nanocapsules effectively inhibited EWS-Fli1 and showed dose-dependant inhibition of tumor growth in mice xenografted with EWS-Fli1-expressing tumors (91).

Currently, application of RNAi in the clinical setting hinges on the low transfection efficiency, rapid degradation by serum nucleases, poor tissue penetration, and non-specific immune stimulation. Another problem when

targeting fusion genes is that most fusion genes are transcription factors that are located in the nucleus, which makes it extremely difficult to introduce therapeutic tools to the destination. Nanoparticles have the potential to become the vehicle for stable, efficient, and tumor-specific delivery of RNAi. Currently, a phase 1 clinical trial is recruiting patients to test an intravenous nanoparticle-based siRNA treatment for solid tumors. The nanoparticles used are formulated using the three-part RONDEL technology, which combines a cyclodextrin polymer, an adamantine-modified stabilizer, and an adamantine-modified ligand targeted to the transferrin receptor on tumor cells. These nanoparticles were tested in human primates and shown to be safe (92). Many other research projects are ongoing to improve the delivery of siRNA to the tumor site, but factors such as non-specific immune response by the host and off-target effects of the siRNA also need to be addressed to improve the quality of RNAi. Novel chemical modification of the siRNA and smart nanoparticulate complexes are needed to ensure an efficient and safe application of RNAi.

DRUG RESISTANCE IN SARCOMAS

Another possibility in improving the prognosis of sarcoma patients is by overcoming drug-resistant sarcoma cells which are refractory to conventional therapeutic drugs. Though there are many factors influencing the poor outcome of sarcoma treatment, drug resistance to chemotherapeutic drugs is one of the major obstacles during therapy. Mechanisms of drug resistance include increased recognition and repairing of DNA damage induced by the drug or ionizing radiation, altered cell cycle checkpoint control, impaired function of apoptotic pathways, and reduced drug accumulation because of increased expression of ATP-binding cassette (ABC) transporters that efflux drugs (93). In most cases, application of a chemotherapeutic drug results in resistance not only to the drug that was given, but also to other structurally and functionally unrelated drugs; this is known as multidrug resistance (MDR). The MDR phenotype is believed to be caused by a combination of the above-mentioned mechanisms.

One of the major causes and well studied mechanisms of drug resistance is the overexpression of ABC transporters, which are able to efflux drugs out of tumor cells. There are at least 48 structurally related transporters, known collectively as ABC family transporters (94). Among the ABC transporters, P-glycoprotein (P-gp) has been implicated as the major mechanism of MDR in various cancers and sarcomas. Acute induction of P-gp has been observed in osteosarcoma (16) and other human tumors following exposure to doxorubicin (95). In fact, P-gp overexpression has been implicated as the primary mechanism of MDR before malignant transformation (96). P-gp overexpression has been associated with poor prognosis in many types of tumors (93), and in one study, P-gp was found to be expressed in as many as 61% of pre-treatment soft tissue sarcomas, and that proportion was even higher after treatment with doxorubicin (95).

Another point of importance is that due to the increasingly popular treatment option with kinase inhibitors, drug resistance to specific kinase inhibitors will have a major impact on the development of sarcoma treatment. For example, most imatinib-resistant GISTs continue to express activated c-KIT due to secondary resistance mutations within the c-KIT kinase domain (97). Additionally, multiple distinct resistance mechanisms have been observed within the same patient sample, and this prompts us to search for a more accurate and robust genotype-guided treatment analysis. Multiple mechanisms of MDR continue to be extensively studied and validated, but like antibiotic resistance observed in bacteria, it is likely that tumor cells will develop new mechanisms to evade novel therapeutics.

Overcoming Drug Resistance

There has been extensive research to discover agents to reverse MDR with high efficiency and low toxicity, but to no avail. Phase 3 trials of these agents have been largely disappointing due to high toxicity (98–100). This failure may be partially explained by the redundancy of the individual transporters within the MDR phenotype together with several other resistance-related proteins expressed in solid tumors (eg, glutathione S-transferase, metallothionin, O6-alkylguanine-DNA-alkyltransferase, thymidylate synthase, dihydrofolate reductase, heat shock proteins) (93). P-gp polymorphisms that result in a synonymous mutation (C3435T) have also been implicated as the cause for altered expression and subsequent changes in drug disposition (101). Consequently, it is imperative to develop alternative, less toxic, and more efficient strategies to overcome MDR such as using nanoparticulate delivery systems (Fig. 2). Nanoparticles are known to bypass P-gp and deliver chemotherapeutic drugs effectively even in drug-resistant cells over-expressing P-gp (16). *In vitro* studies using polymeric nanoparticles have been reported to increase the accumulation of doxorubicin in several osteosarcoma cell lines (16). By evading P-gp, which is highly expressed in these cell lines, we observed higher accumulation of doxorubicin in the nucleus of drug-resistant cell lines. Interestingly, the accumulation of doxorubicin was compatible to the drug-sensitive cell lines as assessed by fluorescence microscope and flow cytometry.

Other possible ways to circumvent MDR include hammerhead ribozymes against the MDR1 mRNA (102),

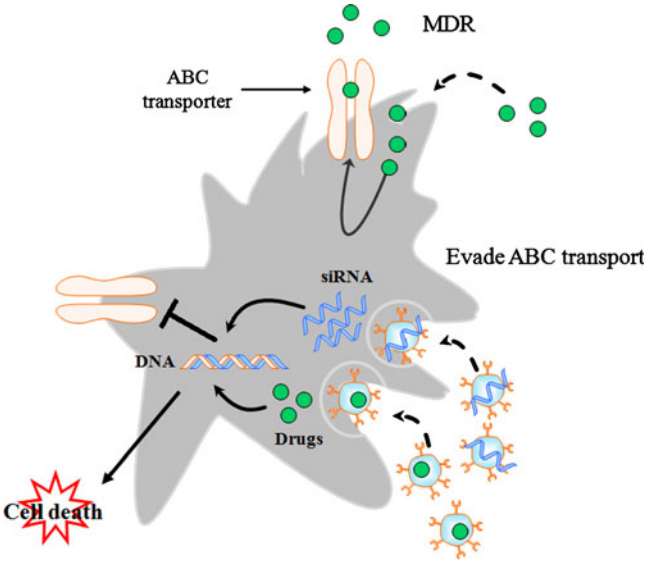

Fig. 2 Potential mechanism of overcoming multidrug resistance using nanoparticles. ABC transporters extrude chemotherapeutic drugs resulting in the survival of sarcoma cells. Conjugation of drugs and genes to nanoparticles results in increased accumulation of the drugs via non-specific endocytosis which could result in cell death. Drugs could be cytotoxic chemotherapeutic drugs, agents that could suppress the activity of ABC transporters or novel kinase inhibitors. Also, specific siRNA to the ABC transporters expressed in each sarcoma will result in the suppression of the ABC transporter.

antisense oligonucleotides (103), and RNAi. The potent knockdown of a gene of interest, such as ABCB1, with high sequence specificity makes RNAi an especially promising tool, albeit with mixed results *in vivo*. Sufficient down-regulation of P-gp has proved difficult to attain due to the lack of an efficient delivery system. Wu *et al.* showed that their siRNA was specific for P-gp, but the maximum inhibition was 65% using a commercial transfection agent. In addition, the maximum decrease of MDR1 mRNA was only observed after 24 h, and mRNA had returned to baseline values within 72 h (104). Several other nanoparticles have been used as a delivery vehicle for MDR1 siRNA (105,106). Patil *et al.* used poly (D, L-lactide-co-glycolide) nanoparticles to encapsulate paclitaxel and P-gp-targeted siRNA (106). They also functionalized the surface with biotin for active targeting and showed that their nanoparticulate complexes significantly inhibited tumor growth *in vivo* using a drug-resistant mammary adenocarcinoma JC cell line. We are currently using polymeric nanoparticles to stably suppress P-gp and re-sensitize MDR osteosarcoma cell lines to a level equal to drug-sensitive cell lines (unpublished data). While the inhibition of tumor growth by RNAi is still incomplete, nanoparticles hold great promise as a vehicle for efficient delivery of siRNA to overcome the problem of drug resistance.

Because there are multiple mechanisms in relation to multidrug resistance, overcoming one form of MDR using small molecules may affect only a proportion of the total MDR population. Other novel methods, which include restoration of tumor apoptotic threshold using a combination of ceramide, paclitaxel and nanoparticle (107) and modulating tumor microenvironment or increasing drug delivery using heat, light and mechanical disruption (26–31), have produced promising results. It is still not clear whether inhibition of individual pathways could lead to inhibition of proliferation or if inhibition of multiple pathways is necessary to achieve this effect. Nevertheless, nanoparticles hold great potential as a delivery vehicle for both the chemotherapeutic drugs and genes in overcoming MDR. Although only speculative, nanoparticulate systems may become a standard platform for the combination of conventional chemotherapeutic drugs with kinase inhibitors or with RNAi for the treatment of various sarcomas.

Targeting Cancer-Initiating Cells

In parallel to the acquired multidrug resistance by cells post chemotherapy, another important aspect of the disease is the correlation between cancer-initiating cells and the intrinsic drug resistance of sarcoma cells. Cancer stem cell theory states that acquired MDR that occurs in more differentiated cells, characterized by gene amplification or rearrangements, may contribute to an aggressive phenotype, but it is not the primary reason for cancer recurrence or spread after therapy. The theory implies that a sarcoma has innate drug resistance by virtue of its resting stem cell phenotype (Fig. 3). It is currently not known whether all sarcomas contain cancer-initiating cells. Recently, however, several sarcomas, including osteosarcoma (108), chondrosarcoma (109), and Ewing sarcoma (110), were shown to possess cancer-initiating cells which are refractory to conventional chemotherapeutic drugs. Although the original concept regarding cancer-initiating cells was first proposed in 1968 by Fiala (111), Dick *et al.* were the first to demonstrate the existence of tumor-initiating cells in acute myeloid leukemia (AML) using the non-obese diabetic severe combined immunodeficient (NOD-SCID) mouse model (112). In the cancer stem cell hypothesis, there is a small subset of cancer cells, the cancer-initiating cells, which constitute a reservoir of self-sustaining cells with the ability to self-renew and maintain the whole population. These cancer-initiating cells have the capacity to both divide and expand the cancer-initiating cell pool and to differentiate into the heterogeneous cancer cell types that constitute the bulk of the tumor. If cancer-initiating cells are relatively refractory to therapies that have been developed to eradicate the rapidly dividing cells that constitute the majority of a differentiated tumor, then these therapies are unlikely to be curative, and relapses would be expected. Although still controversial, the cancer stem cell hypothesis

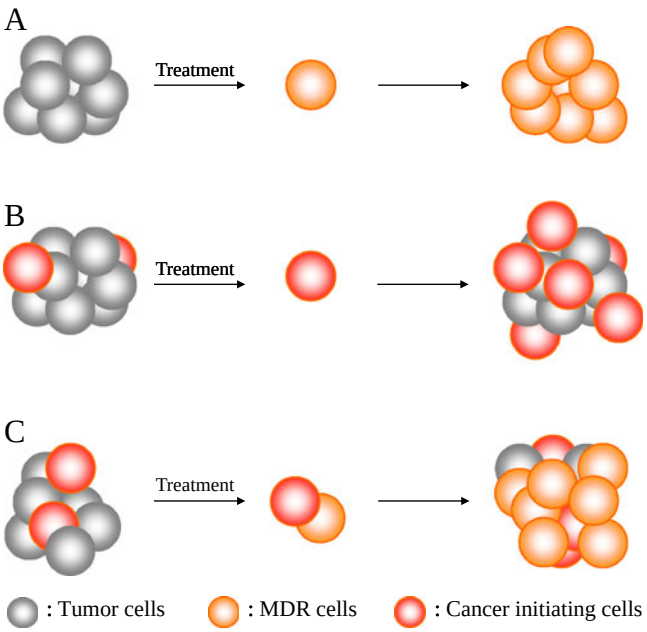

Fig. 3 Models of sarcoma drug resistance. **A.** In the acquired resistance model, sarcoma cells with genetic alterations that confer multidrug resistance (MDR) survive and proliferate to form the recurrent tumor. **B.** In the cancer stem cell model, the initial sarcoma contains a rare population of tumor-initiating cells, which will survive the chemotherapeutic treatment and regrow. **C.** Both models of tumor maintenance could be involved in the tumorigenesis. Initially, sarcoma growth will be controlled by the cancer-initiating cells. After chemotherapy, genetic alterations may lead to formation of new MDR cells which will become the dominant population for proliferation of the sarcomas.

would require that we reanalyze the way we diagnose and treat sarcomas, as our objective would have to shift from eliminating the bulk proliferative cell population and be refocused on the minority stem cell population that fuels tumor growth. On a population level, this hypothesis may explain the reason why different malignancies may appear to be heterogeneous with respect to drug responsiveness.

Cancer-initiating cells have been shown to highly express ABC transporters (113), suggesting that the application of nanoparticles would be beneficial in this population, as nanoparticle use would lead to higher accumulation of chemotherapeutic drugs through endocytosis. The expression of ABC transporters is most likely not the sole reason for the MDR of cancer-initiating cells but one of the mechanisms along with increased capacity for DNA repair and anti-apoptotic properties, such as overexpression of NF-κB and bcl-2. Cancer-initiating cells are generally considered quiescent and non-dividing and are refractory to chemotherapeutic drugs that target either the cell cycle or rapidly dividing cells.

There is growing evidence that Wnt, Hedgehog and Notch signaling along with additional signals such as growth factors converge to generate the distinctive features of cancer-initiating cells, including self-renewal, proliferation, survival and differentiation. Ewing sarcoma (110) and osteosarcoma (108) cancer-initiating cells have been identified through a single molecular marker, CD133. Molecular markers which are preferentially expressed in cancer-initiating cells are potential candidates for actively targeted nanoparticle-based therapies.

In addition, the possibility that differentiation of more primitive cells within a malignancy may lead to tumor degeneration and increased susceptibility to conventional chemotherapeutic drugs has been recognized for some time (114). Therefore, differentiation therapy holds promise as another approach to target cancer-initiating cells. Potential strategies that induce quiescent cancer-initiating cells to differentiate into more mature tumor cells include activation of distinct signaling pathways, such as morphogen-driven signaling cascades (115), alteration of gene expression profiles using microRNAs (miRNAs) (116), and epigenetic differentiation therapy (117,118). It has been reported that bone morphogenetic proteins could induce the differentiation in $CD133^+$ glioblastoma tumor-initiating cells into astrocyte-like cells, which markedly attenuated their tumor-forming ability in a preclinical model (115). Nanoparticle therapy could dramatically aid in the delivery of differentiating agents and therapeutics to the cancer-initiating cell population by exploiting the EPR effect and relying on the lack of lymphatic drainage to increase tumor residence of the therapeutics and to ensure that the therapeutics reach these persitor cells.

RADIO-RESISTANCE

In regard to radio-resistance, tumors that are sensitive to radiation therapy such as Ewing's tumor pose a challenge for the treatment. Repopulation during the treatment period and recovery from radiation damage between fractions have been shown to increase tumor resistance against fractionated radiotherapy (119,120). Recent experimental evidence has suggested that a higher proportion of cancer-initiating cells correlate with higher radio-resistance (121,122). Increased response to DNA damage after radiotherapy in cancer-initiating cells has been observed in several studies. The radio-resistance of $CD133^+$ glioblastoma tumor-initiating cells can be reversed with a specific inhibitor of checkpoint kinase 1 (CHK1) and CHK2 (123). In breast cancer, certain tumor-initiating cells have lower levels of reactive oxygen species (ROS) than corresponding non-tumorigenic cells. Pharmacological depletion of ROS scavengers in cancer-initiating cells markedly increased DNA damage and resulted in radiosensitization (124). Additionally, cell cycle restriction through the expression of cyclin-dependant kinase inhibitor 1A (CDNK1A) limits DNA damage and maintains the self-

renewal of leukemia-initiating cells (125). These studies show the potential of inhibiting DNA damage responses to overcome radio-resistance in the treatment of sarcoma. Nanoparticles have been used in studies to overcome radio-resistance in melanoma (126). The authors used gadopentetic acid-chitosan complex nanoparticles to apply gadolinium neutron-capture therapy. Gadolinium neutron-capture therapy is a cancer therapy that uses the Gadolinium-157 neutron-capture reaction by thermal neutron irradiation. Radio-resistant B16F10 melanoma xenografted mice were used, and after irradiation, tumor growth in the nanoparticle-administered group was significantly suppressed compared to that in the gadopentetate solution-administered group. The same antiproliferative effect of neutron-capture therapy with gadolimium has been shown *in vitro* in malignant fibrous histiocytoma cell line (127).

IMMUNOTHERAPY

Tumor cells exist in a dynamic balance of progression or regression, and host immune mechanisms have been implicated as one of the factors in controlling tumor fate. It is now clear that immunodeficiency not only leads to virally associated cancers, but also to solid tumors including colon cancer, lung cancer, renal cancer, bladder cancer and melanoma (128). Thus, there have been many studies trying to implement immune-based therapy for a variety of tumors.

Dendritic cells (DCs) are the master cells that regulate immune responses to foreign antigens in mammals. They are potent antigen-presenting cells capable of initiating a primary immune response and possess the ability to activate T cells and stimulate the growth and differentiation of B cells. DCs provide a direct connection between innate and adaptive immune responses and arise from bone marrow precursors that are present in immature forms in peripheral tissues, where they are prepared to capture antigens. DCs migrate from the peripheral tissues to the closest lymph nodes through afferent lymphatic vessels to present foreign antigens, stimulating T-cell activation and initiating a cellular immune response. Currently, DCs are known to play a prominent role in various diseases, particularly in cancer. The development of genetic changes in tumor cells can lead to potentially cytotoxic T-lymphocyte responses (129), and lymphocytic infiltration by $CD8^+$ lymphocytes has been associated with improved patient survival in a variety of cancers (130,131). Proteins such as HMGB1, a high mobility group protein associated with chromatin, that are released from stressed or dying cells can interact with macrophages and dendritic cells to induce potentially cytotoxic responses against a tumor (132). Thus, targeting nanoparticles to DCs provides a promising strategy for developing an efficient and balanced immune response. Nanoparticles, by increasing the efficacy of tumor antigens that enter DCs, can modulate the immune response and may be potentially useful as effective vaccine adjuvants for sarcoma therapy, inducing both T-cell and antibody responses.

There have been several reports of immunotherapy for various sarcomas including osteosarcoma (133), Ewing sarcoma (134) and synovial sarcoma (135), but with limited results. The primary problem of vaccine therapy is the lack of production for enough cytotoxic T cells in the vaccinated patients. It is rare to see more than 0.5% T cells in the peripheral circulation specific for a particular tumor antigen in the majority of the trials. This compares with the 20–25% cytotoxic T cells observed after inoculation with vaccines for infectious diseases. Although many studies are ongoing to increase the efficacy by various methods, nanoparticles could be an option where tumor-specific antigens could be delivered to DCs with higher efficiency. Introduction of nanoparticles to immunotherapy has the potential to control sarcomas without the side effects that are associated with current chemotherapy-based methods.

FUTURE DIRECTION

Sarcomas are a diverse group of tumors showing unique tumor-associated events even in the same categorical group. Recent advances in the understanding of molecular signaling, biology, and genetics have shown characteristics of tumor-associated biomarkers which imply very complex mechanisms of the disease rather than a single factor. There are many ways to address improving sarcoma treatment, but nanoparticulate systems offer a flexible platform that can be used to tailor different therapeutic strategies to suit individual sarcoma patients. Nanoparticles have a huge advantage because of their large surface area, the possibility

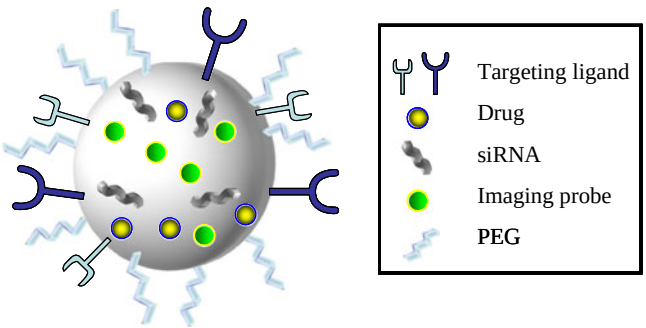

Fig. 4 Multifunctional nanoparticle. Nanoparticles are able to evade the reticuloendothelial system clearance with poly (ethylene glycol) (PEG) modifications while carrying a variety of drugs and genes. Various biomolecular targeting through ligands or antibodies will result in increased accumulation of the encapsulated contents to the tumor site. In addition, novel imaging probe conjugation could result in visualization of sarcomas during treatment which could be utilized to monitor the treatment effects.

of surface modifications for further conjugation, and encapsulation of large amounts of therapeutic agents. Additionally, the benefits of conjugating contrast agents to nanoparticles for the early detection of tumors and for assessing treatment efficacy is being actively explored. There is no doubt that the next generation of nanoparticulate systems will be multifunctional nanoparticles capable of simultaneously achieving many goals (Fig. 4). Multifunctional nanoparticles using siRNA, conventional chemotherapeutics, and active targeting hold great promise in the advancement of the sarcoma treatment. Nanotechnology will certainly lead to the development of more selective, efficacious, and safe nanoparticulate systems that could lead to a paradigm shift for the treatment of sarcoma.

CONCLUSION

Due to the continuing progress in the development of nanotherapeutics and their application in tumor imaging, there is an increasing demand to bridge the gap between the benchtop and bedside for a nanotechnology-based sarcoma treatment. There has been significant progress in the basic research of sarcoma, unraveling multiple signaling pathways involved in development of the disease. Therapeutic thresholds could be overcome by the reversal of acquired MDR, ablating cancer-initiating cells by using target-specific markers or indirectly by inhibiting angiogenesis, disrupting tumor-niche interactions, and immuno-modulation. Nanoparticles could be implemented in a variety of ways in the above strategies; for example, several limitations regarding RNAi and drug delivery to drug-resistant sarcoma could be overcome by using nanosystems. In the future, nanotechnology will undoubtedly contribute to the advancement of sarcoma therapy.

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