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Eugenie S. Kleinerman
Richard Gorlick *Editors*

Current Advances in Osteosarcoma

Clinical Perspectives: Past, Present and Future

Second Edition

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Editors
Eugenie S. Kleinerman
Department of Pediatric Research,
Department of Cancer Biology
University of Texas MD Anderson
Cancer Center
Houston, TX, USA

Richard Gorlick
Division of Pediatrics
University of Texas MD Anderson
Cancer Center
Houston, TX, USA

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To my father, Jerome I. Kleinerman, M.D., who was Chair of Pathology at Mt. Sinai Medical School in New York City, and later MetroHealth Medical Center, Case Western Reserve University School of Medicine. He inspired me to love the discipline of medicine and laboratory research and the importance of education. During his distinguished career as a pulmonary pathologist, his research integrated clinical medicine and experimental models of lung disease in an effort to improve the health of the people with occupational and chronic obstructive lung disease and lung cancer. He was my first role model, a man of ethics and conviction. His memory and words of wisdom continue to guide and inspire me.

This book is also dedicated to my mother, Seretta Miller Kleinerman, who preached and fought for equal opportunity for women in the workplace – a woman way ahead of her time. She pounded into my head that career and family were not mutually exclusive. Yet, she also stressed the importance of maintaining a lady-like decorum, insisting on perfect manners and gracious behavior.

My sisters and I often said we would write a book entitled "Seretta Says."

Finally, I thank my husband, Dr. Leonard Zwelling, for supporting, encouraging, and believing in me. He gave me the strength I needed to push through the insecurities that stem from being a professional woman and mother.

Eugenie S. Kleinerman, M.D.

Preface

Osteosarcoma: The State of Affairs Dictates a Change in Clinical Practice and Clinical Trial Design

We have made many new discoveries with regard to osteosarcoma biology and uncovered potential new targets for therapy. The challenge for us moving forward is: Can we apply these discoveries and alter clinical research practices to achieve success?

Osteosarcoma continues to claim the lives of too many children, adolescents, and young adults. Being both a rare and a pediatric cancer, the resources allocated to finding a cure and improving outcomes have been and will continue to be sparse. This is why, as we move forward, we must be judicious and strategic in the selection of which new agents we incorporate into our clinical treatment regimens and the clinical trial design constructed to assess the activity of these new agents. Experience and multiple clinical trials have defined an accepted three-drug chemotherapy regimen that results in a 65–70% overall survival at 5 years. However, clinical trial after clinical trial adding additional chemotherapeutic agents to this three-drug backbone failed to have an impact with no improvement in outcome since 1987. This is an unacceptable statistic. We need to recognize that we have achieved what we can with combination chemotherapy and move on.

The era of "targeted therapy" based on genomics and proteomics of the tumor cells has emerged. Genomic analysis of tumor tissue has identified potential targets for other solid tumors. However, the genetic signatures from individual osteosarcoma patient samples and even different metastatic tumor nodules in the same patient are not consistent, showing diverse genetic mutations and alterations. Furthermore, tumor cells do not grow in isolation. In my opinion, this approach will fail therapeutically unless we also understand (a) the interactions between the osteosarcoma cells and the lung microenvironment (the most common site of metastases), (b) which molecular pathways are altered epigenetically that permit bone cells to grow in the lung, and (c) how the osteosarcoma cells circumvent the immune response. We also need to understand how the osteosarcoma cells adapt to the lung microenvironment.

Recognizing the success of using chemotherapy to treat newly diagnosed osteosarcoma patients but also admitting that we have reached a plateau using this approach dictates that we must incorporate non-chemotherapy agents

into our current three-drug regimen to improve patient outcomes. Such new agents can include those that target the dysregulated pathways that have been identified in the tumor cells, the tumor microenvironment, and the immune response.

How best to combine the new agent with chemotherapy and how to interdigitate it into the treatment schema based on our knowledge of the agent's target and whether chemotherapy can help or interfere must be a primary focus. These two books (the first focused on clinical practice and novel therapeutic discoveries and the second on laboratory research that will hopefully inspire new therapeutic ideas) have been compiled to bring the latest findings in regard to these three areas. National and international authorities have summarized the historical perspectives and their own clinical, translational, and laboratory research in an effort to provide a single resource to serve as the starting point for discussions as we move forward in designing novel therapeutic strategies. We cannot continue to merely add one new agent and measure success by evaluating response in the setting of bulky, visible relapsed disease. This has been our approach for the last 40 years. While it was successful in identifying the active chemotherapy agents, it is not appropriate for assessing the activity of immunotherapies, agents that target the tumor microenvironment or even agents that target specific pathways. In addition, we cannot continue to assess therapy activity by tumor shrinkage. Agents that activate an immune response resulting in immune cell infiltration into the tumor may be interpreted as tumor progression if response is judged by radiographic measurements. Without histologic evaluation, we cannot decipher whether an enlarged mass is a growing tumor or the result of immune cell infiltration, dead amorphous tissue, and edema. We must incorporate histologic evaluation and biologic measures that confirm that the target of the chosen agent is being affected. Proper resources must be devoted, and carefully designed clinical trials must be implemented. It is imperative that we use the discoveries made by the authors in this book to design our clinical trials, keeping in mind the biology of both the tumor and the organ microenvironment. If we do not implement such changes in our clinical research practice, we will continue to struggle and fail.

In this spirit, I express my gratitude to all of my distinguished colleagues for their willingness to contribute to this book. Without their assistance and their expertise, this project would not have been possible. It is my hope that the information in this book will provide inspiration, data, and the rationale needed to change the way we practice clinical research and design our clinical trials for patients with newly diagnosed and relapsed osteosarcoma.

Houston, TX, USA Eugenie S. Kleinerman, M.D.

Contents

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Contributors

Peter M. Anderson Cleveland Clinic, Pediatric and Taussig Cancer Institutes, Department Pediatric Hematology/Oncology and BMT, Cleveland, OH, USA

Case Comprehensive Cancer Center, Cleveland, OH, USA

Izhar S. Batth The University of Texas MD Anderson Cancer Center, Department of Pediatrics – Research, Houston, TX, USA

Robert S. Benjamin Sarcoma Medical Oncology, The University of Texas MD Anderson Cancer Center, Houston, TX, USA

Anchit Bhagat Department of Pediatrics, MD Anderson Cancer Center, Houston, TX, USA

Brian D. Crompton Pediatric Hematology/Oncology, Dana-Farber/Boston Children's Cancer and Blood Disorders Center, Boston, MA, USA

Najat C. Daw Department of Pediatrics – Patient Care, MD Anderson Cancer Center, Houston, TX, USA

Christopher DeRenzo St. Jude Children's Research Hospital, Department of Bone Marrow Transplantation and Cellular Therapy, Memphis, TN, USA

Kumar Felix Department of Pediatrics Research, MD Anderson Cancer Center, Houston, TX, USA

Miriam B. Garcia Department of Pediatric Research, MD Anderson Cancer Center, Houston, TX, USA

Jonathan Gill Division of Pediatrics, University of Texas MD Anderson Cancer Center, Houston, TX, USA

Nancy Gordon Department of Pediatrics Research, MD Anderson Cancer Center, Houston, TX, USA

Richard Gorlick Division of Pediatrics, University of Texas MD Anderson Cancer Center, Houston, TX, USA

Stephen Gottschalk St. Jude Children's Research Hospital, Department of Bone Marrow Transplantation and Cellular Therapy, Memphis, TN, USA

Samuel Z. Grinberg Medical Student, University of Pennsylvania, Perelman School of Medicine, Philadelphia, PA, USA

Kenneth R. Gundle Oregon Health & Science University, Department of Orthopaedics & Rehabilitation, Portland, OR, USA

Portland VA Medical Center, Operative Care Division, Portland, OR, USA

Douglas Harrison Department of Pediatrics – Patient Care, MD Anderson Cancer Center, Houston, TX, USA

Pooja Hingorani Division of Pediatrics, University of Texas MD Anderson Cancer Center, Houston, TX, USA

Simin Kiany Department of Pediatrics Research, MD Anderson Cancer Center, Houston, TX, USA

Eugenie S. Kleinerman Department of Pediatrics, MD Anderson Cancer Center, Houston, TX, USA

Ryan A. Lacinski West Virginia University School of Medicine, Department of Orthopaedics, Morgantown, WV, USA

Dean A. Lee Department of Hematology, Oncology, and BMT, Nationwide Children's Hospital, Columbus, OH, USA

Brock A. Lindsey West Virginia University School of Medicine, Department of Orthopaedics, Morgantown, WV, USA

Shulin Li The University of Texas MD Anderson Cancer Center, Department of Pediatrics – Research, Houston, TX, USA

Justin E. Markel West Virginia University School of Medicine, Department of Orthopaedics, Morgantown, WV, USA

Paul A. Meyers Memorial Sloan Kettering Cancer Center, New York, NY, USA

Kirsten K. Ness Department of Epidemiology and Cancer Control, St. Jude Children's Research Hospital, Memphis, TN, USA

Abigail Posta Undergraduate Student, Swarthmore College, Swarthmore, PA, USA

Duncan C. Ramsey Oregon Health & Science University, Department of Orthopaedics & Rehabilitation, Portland, OR, USA

Damon R. Reed Cancer Biology and Evolution Program, Adolescent and Young Adult Oncology Program, Department of Interdisciplinary Cancer Management and Sarcoma Department, H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL, USA

Michael Roth Division of Pediatrics, University of Texas MD Anderson Cancer Center, Houston, TX, USA

Keri L. Schadler Department of Pediatric Research, MD Anderson Cancer Center, Houston, TX, USA

Buhvana A. Setty Department of Hematology, Oncology, and BMT, Nationwide Children's Hospital, Columbus, OH, USA

David S. Shulman Pediatric Hematology/Oncology, Dana-Farber/Boston Children's Cancer and Blood Disorders Center, Boston, MA, USA

Brian P. Tullius Department of Hematology, Oncology, and BMT, Nationwide Children's Hospital, Columbus, OH, USA

Kristy L. Weber Penn Medicine Department of Orthopaedic Surgery, Philadelphia, PA, USA

Robert J. Wilson Penn Medicine Department of Orthopaedic Surgery, Philadelphia, PA, USA

1 Adjuvant and Neoadjuvant Chemotherapy for Osteosarcoma: A Historical Perspective

Robert S. Benjamin

Abstract

Osteosarcoma was initially resistant to chemotherapy that worked for Ewing sarcoma and rhabdomyosarcoma as well as other chemotherapeutic agents available in the 1960s. In the early 1970s, responses of osteosarcoma to adriamycin were reported, and at about the same time, so were responses of osteosarcoma to high-dose methotrexate. These agents were introduced into adjuvant therapy due to the dire prognosis associated with apparently localized osteosarcoma. After initial questions regarding the role of chemotherapy delayed its uniform acceptance, there is now general agreement that chemotherapy is primarily responsible for the cure of patients with osteosarcoma when combined with surgical elimination of the primary tumor. Advances with combination chemotherapy later adding cisplatin and ifosfamide have improved ultimate survival. The history of the development of effective chemotherapy combinations at Memorial Sloan Kettering Cancer Center, UT MD Anderson Cancer Center, and the Rizzoli Institute are highlighted, and recent large cooperative group studies are reviewed in the context of those findings.

Keywords

Osteosarcoma · Adjuvant chemotherapy · Neoadjuvant chemotherapy · Adriamycin · Methotrexate · Cisplatin · Ifosfamide

History

Osteosarcoma was initially resistant to chemotherapy that worked for Ewing sarcoma and rhabdomyosarcoma as well as other chemotherapeutic agents available in the 1960s. In the early 1970s, Wang, Cortes, and Holland reported responses of osteosarcoma to adriamycin (before the name doxorubicin was invented) [70]; and at about the same time, Jaffe reported responses of osteosarcoma to high-dose methotrexate [36]. That was the beginning of the modern era of osteosarcoma chemotherapy. It was also recognized at that time that the vast majority of patients with apparently localized osteosarcoma would die of their disease despite radical amputation, one joint above the level of the tumor [37, 44]. There had even been attempts to delay amputation with radiation of the primary tumor, so that when it was obvious that the patients' lungs were filled with metastases, mutilating surgery could be avoided [18, 42, 55]. With that background, it is easy to see why Jaffe and Cortes pushed the active chemotherapeutic agents that they had discovered into adju-

R. S. Benjamin (✉)
Sarcoma Medical Oncology, The University of Texas MD Anderson Cancer Center, Houston, TX, USA
e-mail: rbenjami@mdanderson.org

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vant therapy for patients with localized disease. Their back-to-back publications in the New England Journal of Medicine indicated remarkable improvements in survival and disease-free survival compared with well-established historical control series [23, 37].

So why was there so much controversy regarding the use of adjuvant chemotherapy in the two decades that followed? Several issues contributed. First, as my former mentor, John Murray, frequently said "the worst enemy of a good outcome is long-term follow-up." The initial series of Jaffe and Cortes were published with less than 1 year of median follow-up in the rush to notify the world of a major breakthrough in the treatment of a previously deadly disease. At that time, the vast majority of patients treated with amputation alone had developed metastases and many had died. Further follow-up on the treated patients, however, showed that although the time to the development of metastatic disease was prolonged, the majority of patients ultimately relapsed and died. At the last update of these series, disease-free survival had dropped from 60 to 85% at 1 year to about 40% with 5-years of follow-up [24, 38]. Second, chemotherapy was toxic. High-dose methotrexate was difficult to manage. It involved giving a lethal dose of chemotherapy and then following with an antidote to protect normal cells. Initially, methotrexate levels were not available to monitor drug clearance, and particularly in adults, clearance was not so rapid and predictable as in children. Some patients died. For adriamycin, too, there were infectious complications (there were no hematopoietic growth factors, and antibiotics had limited spectrum), mucositis, and great fear of late congestive heart failure. Third, the statisticians from the Mayo Clinic, a chemotherapeutically conservative institution at that time, showed evidence that their patients treated only with surgery were doing much better than previously and suggested that the improvements claimed by others using chemotherapy were simply due to a change in the natural history of the disease [67, 68]. Fourth, the medical profession, taught to be skeptical and not to believe the results of studies that do not have concurrent randomized controls, believed the illogical assertions of the Mayo Clinic statisticians. Why should the natural history of a cancer change? Was there evidence of that happening in any other cancer? Did the use of plain tomography eliminate such a high proportion of patients with metastatic disease on presentation who would not have been detected with X-rays that the remainder of patients had such a better outcome? Is it not more likely that the referral bias of patients traveling to the Mayo Clinic accounted for their changes? And even if the natural history had improved such that almost 50% of patients were cured with surgery alone as initial therapy, in fact, two-thirds of patients in Taylor's reports relapsed [67, 68]. That some were salvaged by subsequent therapy does not negate the fact that initial surgery was curative in only one-third of patients, and what logical reason is there not to try to improve the lot of those who relapsed despite amputation? How could omitting potentially helpful systemic treatment do that? Were not patients at greater risk of dying from not doing something than from doing too much?

Nonetheless, the medical community was divided. Some, most notably Dr. Gerald Rosen from Memorial Sloan Kettering Cancer Center, chose to build on the activity of high-dose methotrexate and adriamycin by developing combination regimens to increase the cure rate [59–63]. So did both the pediatric [65, 66] and adult groups at MD Anderson [51, 58]. Others chose a more conservative interpretation of the data claiming that until there was a randomized study demonstrating conclusively that adjuvant chemotherapy was beneficial, its use should be considered unproven and experimental. Their view was strengthened by publication of a randomized pilot study from the Mayo Clinic that demonstrated no difference in disease-free survival between patients treated with adjuvant high-dose methotrexate and those treated solely with surgery [28]. A careful examination of that study reveals several issues of concern. First, the population, with a median age of over 21, is not representative of the overall population of patients with osteosarcoma where the peak incidence is in the second decade. Second, and most important, 7 of the 20 patients in the treatment arm never

reached the target therapeutic dose of 7.5 g/m^2, due either to delayed drug excretion or very early disease progression (and few today would ever consider a methotrexate dose as low as 7.5 g/m^2 to be adequate).

Clearly, the most influential studies for the medical community as a whole were the two randomized controlled studies that compared the outcomes of patients treated with adjuvant chemotherapy with those treated by surgery alone[29, 43]. These studies put to rest the controversy as to whether chemotherapy added to the cure of localized osteosarcoma. The answer was a resounding yes. In Link's multi-institutional study [43], 77 of 113 eligible patients declined randomization leaving only 36 patients randomized to receive a complex multidrug adjuvant regimen utilizing high-dose methotrexate at 12 g/m^2, adriamycin, a combination of drugs (now felt not to have much activity) called BCD [50], and the combination of adriamycin and cisplatin (modified from Rosen's T-10 protocol) [59], versus amputation alone. The chemotherapy group had a 2-year disease-free survival of 66% compared with 17% in the control group. Of interest, the 59 patients refusing randomization and selecting to receive chemotherapy had a 67% disease-free survival compared with 9% in the 18 patients selecting amputation. Thus, one might argue that no more was learned from the patients who were randomized than from those studied and observed.

In Eilber's study [29], patients all received one cycle of preoperative chemoradiation therapy and were randomized postoperatively to receive a similar regimen to that used in Link's study with somewhat lower doses and the omission of the four cycles of the adriamycin-cisplatin combination (modified from Rosen's T-10A protocol) [59]. The 32 patients randomized to adjuvant chemotherapy had a 55% 2-year disease-free survival compared with 20% for the 27 patients randomized not to receive adjuvant therapy.

Since both Link and Eilber's studies were based on therapy developed by Rosen, it is worth reviewing the existing data from Rosen's studies at the time of the initiation of those two randomized trials. After studying the sequential use of high-dose methotrexate and adriamycin in patients with metastatic osteosarcoma [62], his group embarked on a series of studies in patients with primary tumors. For chemotherapy, they first utilized high-dose methotrexate and adriamycin, later adding high-dose cyclophosphamide, their T-4 and T-5 protocols [60, 61]. With these protocols, they noted late relapses between 12 and 33 months, so they then substituted the combination of bleomycin, cyclophosphamide, and dactinomycin (BCD)[50] for high-dose cyclophosphamide, but they increased the frequency of high-dose methotrexate administration to weekly, resulting in 18 rather than 6 doses of methotrexate, their T-7 protocol [60].

Rosen's most important contribution was not the regimens he developed but rather the concept of neoadjuvant chemotherapy. During the time it took to develop a custom endoprosthesis so that a tumor involving a portion of a weight-bearing bone could be widely resected while preserving the neurovascular bundle and permitting limb salvage rather than amputation, he gave preoperative chemotherapy [60, 61]. He rightfully observed that tumor shrinkage in a tumor with a bony matrix was not a good indicator of the response to therapy. Since tumors were removed and analyzed histologically, however, it was possible to estimate the effects of chemotherapy by histologic response. Huvos first described the histologic findings in the patients that Rosen treated [35]. He described four grades of response ranging from I, essentially no response, to IV, complete disappearance of tumor. Rosen observed that patients whose tumor was completely or almost completely killed by neoadjuvant chemotherapy (Huvos grade III-IV) had improved disease-free survival compared with those whose tumors demonstrated lesser degrees of tumor kill. That observation added further support to the conclusion that the improved disease-free survival of those treated in the adjuvant situation was a direct result of the chemotherapy administered [60].

Rosen also noted in treating patients with established disease that some patients responded only after escalation of the methotrexate dose above 8 g/m^2. In the T-10 protocol, preoperative therapy was heavily weighted toward methotrexate

and consisted of 4 weeks of high-dose methotrexate at 8–12 g/m^2, one course of BCD, 2 more weeks of methotrexate, one course of adriamycin, and 2 more weeks of methotrexate. Postoperative therapy for good responders was repeating the second portion of the preoperative regimen three times. Poor responders had chemotherapy changed to the combination of adriamycin and cisplatin for two courses followed by BCD and repeating that sequence two more times. By modifying postoperative chemotherapy in poor responders, he converted their prognosis to that of good responders [59].

Rosen's emphasis on escalation of the methotrexate dose in order to obtain a response was studied in terms of peak plasma concentration of methotrexate by Delepine and colleagues in a modified T-10 protocol [25–27]. Patients whose methotrexate dose was adjusted to reach a peak level of ≥1000 μM had a higher rate of good histologic response and better disease-free survival than those whose peak levels were <1000 μM [25]. A subsequent report by Bacci from the Rizzoli Institute (IOR) using multivariate analysis in 336 patients showed no correlation between methotrexate levels and histologic response [7]. It must be emphasized, however, that the protocols at the IOR utilized only two preoperative doses of methotrexate as well as two of adriamycin and cisplatin preoperatively, whereas the T-10 protocol utilized eight doses of methotrexate, one of adriamycin, and one of BCD. Adequate methotrexate levels are critical to the activity of methotrexate, but if much of the preoperative response rate is due to adriamycin and cisplatin, the methotrexate level is irrelevant; as is, perhaps, the administration of methotrexate at all in that regimen. It is clear, however, if one wants methotrexate to work, adequate levels (≥1000 μM) are important.

The activity of cisplatin against osteosarcoma was discovered during phase I clinical trials [21, 41] and confirmed in additional phase II studies [12, 52, 69]. It was put into adjuvant therapy in a regimen alternating with adriamycin by Ettinger and colleagues from Roswell Park [30, 31]. After 3-year median follow-up time, 64% of patients were continuously free of disease [31]. After noting at MD Anderson that cisplatin could be used by intra-arterial infusion in patients with melanoma [57], we expanded our studies to include patients with osteosarcoma [13, 19, 22, 47]. The response rate seen in patients with primary bone tumors (8/15) was substantially higher than the 21% reported in the earlier phase I-II studies of intravenous cisplatin. We also noted in our pharmacologic observations that systemic exposure to cisplatin was the same with intravenous or intra-arterial administration, but the concentration in the vein draining the tumor was 1.5–4 times higher with intra-arterial administration [64]. Thus, intra-arterial cisplatin delivers a full systemic dose plus a boost to the primary tumor.

Jaffe extended those studies to children, confirming the activity [39]. He subsequently compared the activity of intra-arterial cisplatin with intravenous high-dose methotrexate in a randomized study [40]. In the methotrexate arm, 4 of 15 patients responded (3 CR, 1 PR), but in the intra-arterial cisplatin arm, 9 of 15 patients responded (7 CR, 2 PR). In addition, two patients randomized to methotrexate were subsequently treated with and responded to intra-arterial cisplatin. Responses were defined by pathology using the criteria of Ayala, who modified the Huvos grading by quantifying the degree of tumor necrosis [2, 3]. Ayala noted that some degree of tumor necrosis could be seen in the absence of any chemotherapy, but necrosis in excess of 60% represented a definite chemotherapy effect. Most subsequent papers have simply used the 90% necrosis cutoff as a good response and anything less as a poor response. Raymond described in detail the procedures for processing the tumor to get the best estimate of the percent necrosis [58].

While Jaffe was refining the use of intra-arterial cisplatin in pediatric patients, we on the adult sarcoma service at MD Anderson studied the effects of combining systemic adriamycin and intra-arterial cisplatin as preoperative chemotherapy for patients with localized osteosarcoma [14, 58]. Since the dose-limiting toxicities of adriamycin are myelosuppression and mucositis and those of cisplatin are nephrotoxicity and ototoxicity, we reasoned that the two drugs could be given in combination at full single-agent

doses. It is harder, particularly in adults, to add methotrexate to that combination since mucositis and nephrotoxicity overlap the toxicities of the other two drugs. Our studies using different drugs confirmed the observations of Rosen in methotrexate-weighted T-7 and T-10 protocols. Continuous disease-free survival was 58% for the entire group of 40 patients, but it was 91% in those with tumor necrosis ≥90% and only 14% for those with necrosis <90%. Subsequent modification of the postoperative adjuvant regimen in patients with poor necrosis with the addition of high-dose methotrexate and BCD improved disease-free survival to 34%, and with high-dose methotrexate and ifosfamide to 67% [16]. I will return to this subject later as recent studies question the very basic concepts of neoadjuvant therapy.

The group most influenced by our experience with intra-arterial cisplatin, and the group that best developed neoadjuvant and adjuvant therapy for osteosarcoma in the ensuing years was the group from the Rizzoli Institute. Drs. Bacci and Picci spent several months visiting MD Anderson before returning to the IOR where their sequential protocols with large numbers of patients treated at a single institution are landmarks in the history of osteosarcoma therapy. The great advantage of the IOR is that it serves as the referral center for the entire country of Italy for complex orthopedic procedures and thus captures the vast majority of patients with osteosarcoma.

The first adjuvant studies used adriamycin and then added low-intermediate doses of methotrexate. Disease-free survival at 5 years was 45% compared with 10% in their historical control [5, 20]. They then initiated their first neoadjuvant study with intra-arterial cisplatin, initially given 1 week after intermediate- (750 mg/m^2) or high-dose methotrexate (7.5 g/m^2). Patients with good response to initial chemotherapy were randomized to receive only one more cycle of methotrexate and cisplatin versus 24 weeks of therapy that added also adriamycin [10]. Only 5 of 15 patients in the first group remained continuously disease-free compared with 19 of 19 who had the longer treatment with the addition of adriamycin. In the report of the entire series of 127 patients with the same primary chemotherapy, they observed a higher rate of good response (62% vs 42%) in the patients receiving high-dose methotrexate rather than intermediate-dose methotrexate [9]. They also observed superior disease-free survival in the good responders who received prolonged postoperative chemotherapy (62%) to that of those with intermediate response (42%) or poor response (10%). Overall 5-year disease-free survival was 49%. Another conclusion that can be drawn from the study is that five cycles of alternating full-dose adriamycin and BCD were inadequate therapy for patients with truly poor response (< 60% necrosis).

The second neoadjuvant study from the IOR added systemic adriamycin to intra-arterial cisplatin 1 week after high-dose methotrexate for two courses preoperatively and continued the same drugs for three courses postoperatively in good responders [8]. Poor responders (<90% tumor necrosis) received a complex, prolonged postoperative regimen that added three courses of ifosfamide at 10 g/m^2 and substituted three courses of cisplatin plus etoposide for single-agent cisplatin. The regimen was continued for 30 weeks compared with 21 weeks for the good responders [8]. The rate of good necrosis increased to 71% with the addition of preoperative adriamycin (compared with 62% in their previous study). Continuous disease-free survival at 5 years was 63% (compared with 49% in their prior study). Long-term follow-up on these patients confirms disease-free survival of 61% at more than 10 years and no difference between good and poor responders [6]. Importantly, disease-free survival of good responders was 71% and for poor responders was 57% (73% vs. 72% when those with major protocol violations were excluded). This is another study that demonstrates that the addition of an active agent in a prolonged course of postoperative therapy can alter poor prognosis of poor responders.

In the next study from IOR, patients were randomized preoperatively to receive cisplatin intra-arterially or intravenously [11]. This study was prompted in part by the findings of the German Cooperative Osteosarcoma Study Group (COSS) that compared intra-arterial and intravenous

administration of cisplatin in the combination with ifosfamide in the context of a four-drug preoperative protocol and found no difference in the rate of good tumor necrosis between the two routes of administration [71]. In contrast to the COSS study, the IOR group found a higher rate of good necrosis in patients who received intra-arterial cisplatin (78%) than in those who were treated intravenously (46%) [11]. There was no difference in disease-free survival between the groups but fewer local recurrences in the group receiving intra-arterial therapy. Another advantage of intra-arterial cisplatin is the rapidity of the response. Symptomatic improvement is noted usually after the first course of therapy, sometimes in only a few days. Systemic therapy does not usually work so rapidly. So how are we to interpret the COSS study? The more agents that are used in neoadjuvant therapy, the less important optimization of any one is. The COSS study used all of the active agents neoadjuvantly, Bacci used three, MD Anderson uses two. It is not surprising that there is no effect on the ultimate outcome between intra-arterial therapy and intravenous therapy. The ultimate outcome is based on the systemic effects of the drugs, not a local effect. Local control of the tumor is determined by surgery, not chemotherapy, so most groups now use intravenous cisplatin because intra-arterial administration is more complex, expensive, and time-consuming. For patients where limb-salvage surgery can be performed only with marginal margins, however, there may still be a role for intra-arterial therapy, especially if the number of drugs used in the neoadjuvant setting is limited, since there is a high correlation between failure to obtain a good response to initial therapy and risk of local recurrence unless surgery is truly radical [34, 54].

The subsequent study from the IOR modified the preoperative regimen introducing a cycle of ifosfamide-cisplatin and ifosfamide-adriamycin but did not improve overall results from previous studies[4]. Subsequent studies expanded participation to the Italian Sarcoma Group (ISG) and collaborated in one with the Scandinavian Sarcoma Group (SSG). Their study with the SSG added high-dose ifosfamide (15 g/m^2 over 5 days by continuous infusion) in the preoperative phase but did not improve on their prior results [33]. The next study limited to the ISG looked at the addition of ifosfamide either to the preoperative regimen or limiting its use to postoperative therapy only in poor responders [32]. There was no improvement with the addition of ifosfamide preoperatively, but there was increased myelosuppression.

The most controversial drug in the treatment of osteosarcoma is ifosfamide. The activity of ifosfamide against advanced osteosarcoma was noted in the mid-1980s [1, 46, 56]. Further studies suggested not only dose response [15] but also schedule dependency [53]. With that background, its addition to adjuvant and neoadjuvant studies has been extensive. As noted previously, studies from MD Anderson [16] and the IOR [6, 8] demonstrated superior disease-free survival when ifosfamide was added to the postoperative therapy in poor responders. In contrast, the addition of preoperative ifosfamide did not improve disease-free survival [32]. Cooperative group studies with more patients have reached very different conclusions.

A large study from the Children's Oncology Group (COG) studied 662 patients with osteosarcoma and randomized them to receive induction therapy with either methotrexate, adriamycin, and cisplatin (MAP) as their standard regimen or methotrexate, adriamycin, and ifosfamide. Patients were also randomized to receive or not receive mifamurtide (liposomal muramyl tripeptide, MTPPE) [48, 49]. The study showed improved survival and improved (although not statistically significant at the $p < 0.05$ level) event-free survival in the patients randomized to receive mifamurtide, but no advantage to the addition of ifosfamide. On the other hand, there was no difference in the rate of good response (modified Huvos grade III and IV) between MAP and MAI. One could argue that the data from the study suggest that ifosfamide is as active as cisplatin in primary therapy and cisplatin may well be the single most active agent against osteosarcoma.

An even larger cooperative study, the EURAMOS trial, accrued 2260 patients [17, 45].

Good responders were randomized to receive or not receive pegylated interferon alfa-2b after completion of chemotherapy (although poor adherence to randomization and dropout due to toxicity make interpretation of the data difficult) [17], and poor responders were randomized to receive a postoperative regimen containing ifosfamide (at good doses and schedule) or to continue on the same regimen used preoperatively, MAP (including two doses of methotrexate at 12 g/m^{2}per course for two courses) [45]. Only 618 of the 1060 poor responders participated in the randomization. There was no statistically significant benefit from the addition of ifosfamide, but there was a clear separation of the event-free survival curves during the first 2 years. The investigators speculate that some of this difference was an artifact of delayed post-treatment imaging since the patients randomized to ifosfamide finished their therapy after 40 weeks while those who got MAP ended at 29 weeks. An alternate explanation is that there was a guaranteed time while chemotherapy was continued, regardless of when post-treatment imaging started. Another explanation is that ifosfamide delayed but did not eliminate the development of metastases. Either of these last interpretations would suggest that a longer course of postoperative therapy for poor responders would be beneficial. On the other hand, the patients randomized to ifosfamide actually received fewer of their planned doses with a smaller percentage receiving at least 80% of their planned dose than those randomized to the shorter postoperative MAP regimen, so maybe just giving the therapy written into the protocol might have improved the potential cure rate of the poor responders. Nobody will ever know. The study represents real-world experience, but one wonders whether the poor dose intensity reported in the study was observed to the same degree in centers with more experience treating osteosarcoma.

So how should one interpret the data from the large randomized EURAMOS study (or other large cooperative group studies) in the context of much smaller studies from Memorial Sloan Kettering, MD Anderson, and the IOR with regard to modification of postoperative chemotherapy in poor responders? If the induction regimen is MAP as given in EURAMOS, benefit from adding ifosfamide in postoperative therapy of poor responders is questionable at best. If the preoperative regimen uses MAP with less than half the dose intensity of methotrexate than that used in EURAMOS, adding ifosfamide and cisplatin-etoposide postoperatively is beneficial. If preoperative therapy contains mostly methotrexate, adding additional active agents (adriamycin and cisplatin) postoperatively is beneficial. If the preoperative regimen is adriamycin and cisplatin, a postoperative regimen adding methotrexate and ifosfamide is beneficial. The fact that the EURAMOS study failed to show benefit from postoperative ifosfamide with their induction regimen does not mean that it has no value with other induction regimens, despite the size of the study, and since EURAMOS does not show improved disease-free survival to other studies, its size alone does not make it the new standard.

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Part I

Surgical Considerations and Outcomes

Limb Salvage and Reconstruction Options in Osteosarcoma

2

Samuel Z. Grinberg, Abigail Posta, Kristy L. Weber, and Robert J. Wilson

Abstract

Advances in chemotherapy, sophisticated imaging, and surgical techniques over the last few decades have allowed limb-salvage surgery (LSS) to become the preferred surgical treatment for bone sarcomas of the extremities. The goal of LLS is to maximize limb functionality to allow for the maintenance of quality of life without compromising overall survival and tumor local recurrence rates. Today, limb-salvage procedures are performed on 80–95% of patients with extremity osteosarcoma, and the 5-year survival rate in extremity osteosarcoma patients is now 60–75%.

This chapter will focus on LSS for extremity osteosarcoma. Common types of surgical reconstruction techniques including endoprostheses, intercalary or osteoarticular allografts, vascularized fibular autografts, and allograft prosthetic composites (APC), and their complications such as infection, local recurrence, graft fracture, implant failure, and nonunion will be discussed in detail. Anatomic locations of lesions discussed include the proximal femur, distal femur, proximal tibia, distal tibia, proximal humerus, distal humerus, and forearm bones.

Keywords

Limb salvage surgery · Osteosarcoma · Allograft · Endoprosthesis · Allograft prosthesis composite · Infection · Loosening · Wear · Femur · Local recurrence · Nonunion

Introduction

Advances in chemotherapy, imaging, and surgical techniques over several decades have allowed limb-salvage surgery (LSS) to become the preferred surgical treatment for bone sarcomas of the extremities [1, 2]. The goal of LSS is to maximize limb functionality to allow for the maintenance of quality of life without compromising overall survival and tumor local recurrence rates. Today, limb-salvage procedures are performed on 80–95% of patients with extremity osteosarcoma [1, 3]. Data suggest local

S. Z. Grinberg
Medical Student, University of Pennsylvania, Perelman School of Medicine, Philadelphia, PA, USA
e-mail: Samuel.Grinberg@Pennmedicine.upenn.edu

A. Posta
Undergraduate Student, Swarthmore College, Swarthmore, PA, USA
e-mail: aposta1@swarthmore.edu

K. L. Weber · R. J. Wilson (✉)
Penn Medicine Department of Orthopaedic Surgery, Philadelphia, PA, USA
e-mail: Kristy.Weber@uphs.upenn.edu; Robert.wilson3@uphs.upenn.edu

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recurrence rates and overall survival are equivalent when comparing LSS to amputation, and LSS may have better function [4]. The five-year survival rate in extremity osteosarcoma patients is now 60–75%.

This chapter will focus on LSS for extremity osteosarcoma. As it pertains to this chapter, limb salvage is defined as the "successful resection of a tumor and reconstruction of a viable, functional extremity" [5]. Common types of surgical reconstruction techniques including endoprostheses, intercalary or osteoarticular allografts, vascularized fibular autografts and allograft prosthetic composites (APC), and their complications such as infection, local recurrence, graft fracture, implant failure, and nonunion will be discussed in detail [6]. Anatomic locations of lesions discussed include the proximal femur, distal femur, proximal tibia, distal tibia, proximal humerus, distal humerus, and forearm bones**.**

Types of Reconstruction

This review will focus on common types of reconstruction. Materials used include composite metals, cadaveric allograft, or biologic options. An endoprosthesis is a metal implant used to replace resected bone and joints that is secured to the remaining bone with a cemented or press-fit stem. Alternatively, an osteoarticular or intercalary allograft can be used to reconstruct the limb with a matched bone from a cadaver, which is commonly attached to the remaining bone with an intramedullary nail or a plate/screw construct [7]. An intercalary allograft can be used with or without a vascularized fibular graft to replace resected tumors in the diaphysis while sparing the joints. Osteoarticular allografts are an option when joint preservation is not possible; however, they are not used as commonly as endoprostheses in the United States. The remaining soft-tissue connections on the allograft allow for some functional advantages, especially when reconstructing the extensor mechanism for proximal tibia tumors [3]. However, there is often instability at the joint with increased risk for cartilaginous wear, and there are fewer size-appropriate allograft bones for pediatric patients compared to adult patients [3].

Finally, an APC is also a valid reconstruction option. An APC combines a cadaveric allograft with a hinged prosthesis to replace the resected bone and joint.

Each reconstructive method has advantages and disadvantages after a tumor resection. Endoprostheses with cemented stems often allow for weight-bearing immediately following surgery; however, there is a risk for long-term device loosening and wear [7]. A key advantage of many allografts is that tendons and ligaments remain attached to the graft bone for host soft tissue attachment. Disadvantages of osteoarticular allografts are allograft fracture risk, nonunion, joint instability, and osteoarthritis of the reconstructed joint [7]. Intercalary allografts share the nonjoint-related concerns. Lastly, APCs have combined advantages of endoprostheses and allografts. There is avoidance of the osteoarticular allograft joint and instability problems, restoration of bone stock, and tendon to tendon reconstruction of the soft tissues (rather than tendon to prosthesis). However, the risk for allograft fracture nonunion at the host graft junction remains [7].

The decision as to which type of reconstruction to use depends on multiple factors. The anatomic location of the osteosarcoma, age of the patient, and what specific type of reconstruction are the most effective issues to consider. Furthermore, with primarily retrospective clinical data and a lack of consensus among surgeons, the type of reconstruction is also based on surgeon preference, experience, and patient- and tumor-specific factors. These factors will be further explored.

Endoprosthetic Failure Classification

In 2011, Henderson et al. [8] published a literature review of failure mechanisms for endoprostheses used in tumor surgery. They also provided a classification of different failure modes. Failures were classified as: Type 1, soft-tissue

failure; Type 2, aseptic loosening; Type 3, structural failure; Type 4, infection; and Type 5, tumor progression. They reported 534 failures following primary reconstructions in 2174 patients (24.5%). Of these failures, 12% were Type 1, 19% were Type 2, 17% were Type 3, 34% were Type 4, and 17% were Type 5. Throughout this chapter, the failure results from multiple studies of endoprostheses will be reported according to this classification system.

Anatomic Locations

The anatomic location of an osteosarcoma is a crucial factor in determining the feasibility and success of limb salvage surgery as well as the type of reconstruction. Along with the specific location, it is important to consider the advantages and disadvantages of each type of reconstruction as well as patient age, anticipated function, activity levels, and projected overall survival.

Proximal Femur

Reconstruction of the proximal femur is most commonly performed using an endoprosthesis or APC. Compared to the pelvis, functional outcomes tend to be better and patients frequently resume a higher level of function after reconstruction. However, functional outcomes generally are not as good as they are with distal femoral reconstruction.

In a literature review comparing endoprosthesis to APC in reconstructions of the proximal femur, Janssen et al. [9] found similar functional outcomes for both, which were described as reasonable to good, although they noted high revision surgery rates for both groups. The APC group experienced higher rates of Type 3 and Type 4 failure. Biau et al. [10] studied 32 patients who underwent reconstruction with APC and noted that, when compared to historical controls, there was no improvement over megaprostheses. Without successful union of the host bone/graft junction, the theoretical mechanical benefits of APC (improved abductor strength) are not realized when compared to an endoprosthesis.

Focusing on functional results following endoprosthetic reconstruction of the proximal femur, Hobusch et al. [11] looked at activity level and participation in sports after surgery. Of the 16 patients included, 14 participated in sports for an average of 5 hours/week before surgery. After surgery, 11 of these patients participated for an average of 2 hours/week. Additionally, there was a significant decrease in the UCLA and modified Weighted Activity Score levels from preoperative levels. While some patients were involved in higher impact sports preoperatively, following surgery patients engaged in lower impact sports such as hiking, biking, swimming, and golf.

The literature thus far appears to favor endoprosthetic reconstruction over APC for the proximal femur as APC reconstruction had higher levels of complications without offering improved functionality. Infection, aseptic loosening, and prosthetic dislocation are the primary complications encountered in this location.

Femoral Diaphysis

For osteosarcoma of the femoral diaphysis, the most common option is an intercalary allograft which maintains the native hip joint above and the native knee joint below. Aponte-Tinao et al. [12] performed 83 femoral reconstructions with intercalary allograft. The overall allograft survival rate was 85% and 76% at 5 and 10 years, respectively. Of the 83 patients, 38 experienced complications that required a follow-up surgery, and the allograft was removed in 15 of these patients. Complications included 1 infection, 14 fractures, and 20 nonunions. Of the 20 patients with nonunions, 3 received adjuvant radiation and 15 received preoperative chemotherapy. The average Musculoskeletal Tumor Society (MSTS) score was 27 of 30 for the 68 patients who retained their allograft. Ogura et al. [13] used free vascularized fibula autografts in addition to intercalary autografts in 11 patients. The mean MSTS score was 81%, and there were four complications in three patients. Complications included

two infections, one implant failure and one fracture. The graft was removed in the two patients with infections. Bone union occurred in 10 of the 11 patients.

For rare osteosarcomas that involve the majority of the femur, it may be necessary to use a total femoral replacement. Sevelda et al. [14] reviewed the results of 44 patients treated with a total femoral replacement of which 10 received an expandable prosthesis. They found overall implant survival rates of 97% for conventional prosthesis and 100% for the expandable prosthesis. There were 25 complications among the group receiving conventional implants, most commonly Type 1 and Type 4 failures. Unplanned revision rates were 50% for the conventional implant and 90% for the expandable. Overall, MSTS scores were 70% for the conventional group and 88% for the expandable group.

For osteosarcomas of the femoral diaphysis, the primary treatment is reconstruction with intercalary allograft. One important complication is nonunion at the host bone-allograft junction, and there is an increased risk for nonunion with chemotherapy and radiation. A free vascularized fibula graft can be used in conjunction with an intercalary graft to improve bone union. In rare circumstances, a total femoral replacement can be performed when reconstruction with an intercalary allograft is not possible.

Distal Femur

The distal femur is the most common location for osteosarcoma. Endoprosthesis and APC are primarily used to reconstruct the distal femur and knee. Simon et al. [15] compared amputation to limb salvage treatment and saw similar rates of overall survival and disease-free survival for patients receiving limb salvage, above the knee amputation, and hip disarticulation. Of note, endoprosthesis, APC, and osteoarticular allograft reconstruction were pooled together in the limb salvage group. In a follow-up, they found significantly improved functionality with limb salvage when compared to the two amputation groups [4].

While endoprostheses are most commonly used, results of osteoarticular allografts and APCs have still been described in the literature. Puerta-GarciaSandoval et al. [16] compared APC reconstruction in the distal femur or the proximal tibia. For the distal femur group, they saw no fractures, complete bone healing in 79% of patients, a mean MSTS score of approximately 79%, and prosthesis survival of 94% at 10 years with few complications. In 32 patients receiving an APC for tumors of the distal femur, Wang et al. [17] reported a mean MSTS score of 94% after an average follow-up of 54 months. Two patients had nonunion that healed following refixation. Wunder et al. [18] compared the results from 11 patients treated with allograft reconstruction and 64 patients treated with prosthetic reconstruction. Allografts failed 55% of the time, while prostheses failed 16% of the time. Additionally, allografts were successful in saving the limb 64% of the time compared to 95% for prosthesis. Prostheses also had better MSTS scores, 75% compared to 57%. In a retrospective review of 83 patients receiving massive distal femoral osteoarticular allografts, Mnaymneh et al. [19] saw poor functional results in 5 patients and excellent or good results 53 patients. However, complication rates were 36% and included nonunion, allograft fracture, infection, knee instability, and arthritis of the knee.

Recently, most of the literature focuses on reconstructions using endoprostheses. Options for distal femur endoprosthesis include either cemented or uncemented implants as well as fixed hinge or rotating hinge mechanisms for reconstruction of the knee. Pala et al. [20] reviewed the results of 247 rotating-hinge modular endoprostheses for distal femoral and proximal tibial reconstruction with a minimum follow-up of 2 years. Of the 247 implants, 175 were used for the primary procedures and 72 were used to revise a previously failed reconstruction. For younger patients with primary bone cancer, implants were frequently uncemented. One hundred and eighty-seven replacements were used in the distal femur. Functionally, the mean MSTS score was approximately 85% with distal femur reconstruction. The total failure

rate in the distal femur group was 27%. Out of 187 distal femur implants, 7% experience Type 1 failure, 5% experienced Type 2 failure, 9% experienced Type 4 failure, and 6% experienced Type 5 failure. Of note, there were no structural failures. Overall implant survival was 60% at 8 years. Haijie et al. [21] performed a systematic review exploring implant survival and complications of endoprostheses used for distal femoral and proximal tibial replacement. For distal femoral replacements, mean implant survival rates at 5, 10, 15 and 20 years were 78%, 70%, 62% and 38%, respectively. Aseptic loosening (Type 2 failure) and infection (Type 4 failure) were the most frequent complications occurring 9% of the time each.

Based on the literature, endoprosthetic reconstruction is currently the most commonly used technique compared to APC and osteoarticular allograft. Aseptic loosening and infection continue to be the most common causes of complications with endoprosthetic reconstruction.

Case Example: Distal Femur

A 52-year-old female with Paget sarcoma of right distal femoral diaphysis treated with neoadjuvant chemotherapy followed by wide resection and cemented megaprosthesis reconstruction followed by adjuvant chemotherapy (Image 2.1).

Proximal Tibia

The proximal tibia is the second most common anatomic location for osteosarcoma after the distal femur. While reconstruction of the proximal tibia is similar anatomically to the distal femur, it tends to have higher complication rates and lower functional outcomes compared to other anatomical sites [22]. Particular challenges include limited soft tissue coverage, vascular abnormalities, and difficulty restoring the extensor mechanism. As a result, endoprosthetic survival rates are shortest while amputation rates and revision rates are highest for proximal tibia reconstruction when compared to other anatomic sites [7, 23].

Homlar et al. [7] performed an in-depth systematic review of the literature to compare postoperative complications, functional outcomes, success of limb salvage, and implant survival between endoprostheses, APCs, and osteoarticular allografts as reconstruction option in the proximal tibia. All included studies had at least 10 patients. The mean pooled MSTS score was 76% for the endoprosthesis group, 90% for the osteoarticular allograft group, and 77% for the APC group. Based on their results, each type of reconstruction had advantages and disadvantages. Endoprostheses had lower infection rates than osteoarticular allografts. Endoprostheses also had the highest rates of amputation. Osteoarticular allografts had a lower extensor mechanism failure rate than the other two reconstruction types. Local recurrence was similar among the three groups, and allograft fracture was significantly more common with osteoarticular allograft compared to APCs.

Puchner et al. [24] reviewed the results from 81 patients who underwent proximal tibia reconstruction with endoprostheses. The overall complication rate was 56%. Out of the total number of patients, 10% experienced Type 1 failure, 12% experienced Type 2 failure, 15% experienced Type 3 failure, 12% experienced Type 4 failure as their primary complication. The mean MSTS score was 83% and was not statistically different based on complication, fixed or rotating hinge prostheses, and extensor mechanism reconstructions.

Albergo et al. [25] compared the results of 88 patients who underwent reconstruction with an endoprosthesis and 44 patients who underwent reconstruction with an osteoarticular allograft. They found no difference in the probability of failure at 5 years (18% for endoprosthesis; 27% for osteoarticular allograft) and 10 years (44% for endoprosthesis; 32% for osteoarticular allograft). While there was no difference in MSTS scores between the groups, allograft reconstruction resulted in an improved range of motion and less extension lag than endoprosthetic reconstruction (13.56° for endoprosthesis; 2.41° for osteoarticular allograft). While osteoarticular allografts resulted in improved range of

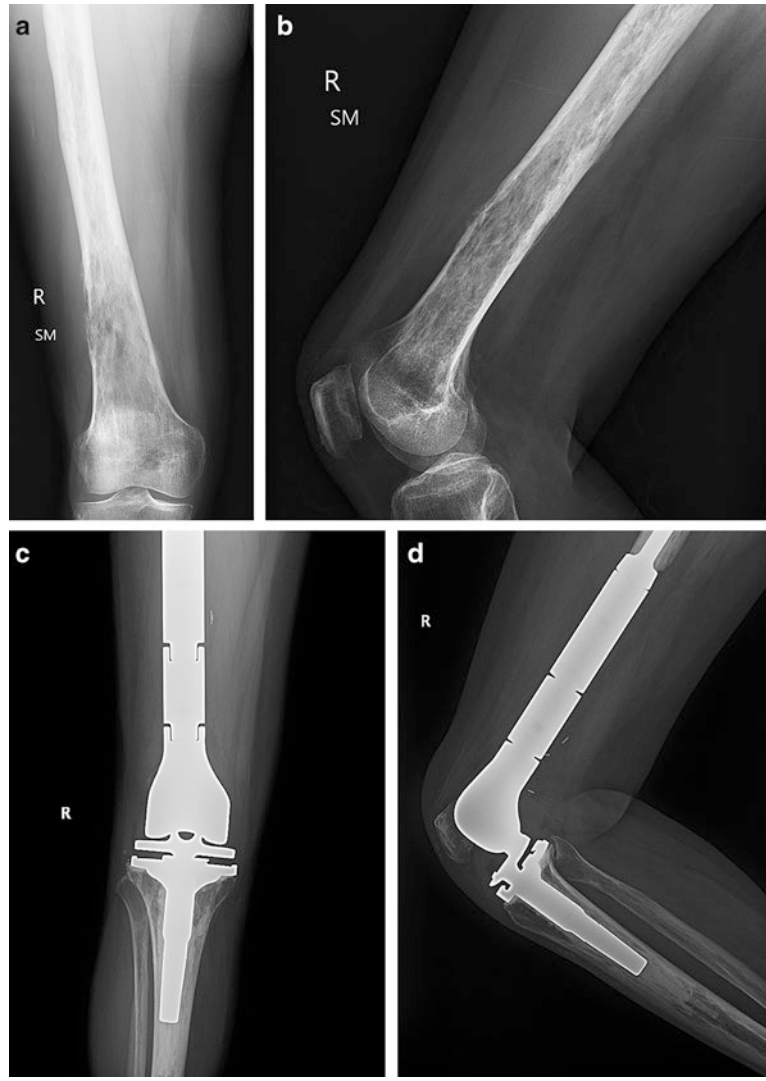

Image 2.1 AP and lateral radiographs reveal an osteolytic lesion on the right distal femur, which was diagnosed as Paget's Sarcoma (**a**, **b**). Postoperatively, AP and lateral radiographs show the reconstruction with a wide megaprosthesis following resection (**c**, **d**)

motion, there are significant technical difficulties in successfully reconstructing the joint allografts.

Müller et al. [26] compared APC to megaprosthesis for reconstruction of the proximal tibia. Of the 42 patients, 23 received a megaprosthesis and 19 patients received an APC. At an average follow-up of 62 months, five megaprosthesis patients and four APC patients experienced reconstruction failure. Ten-year implant survival rates were 79% and 94% for megaprosthesis and APC, respectively. Neither failure rate nor implant survival was significantly different between the two groups, and there were no functional differences between the groups. While the difference was not statistically significant, the APC group on average had less extensor lag (7.2°) than the megaprosthesis group (11.4°). Furthermore, two patients in the megaprosthesis group experienced extension lag of greater than 30°, whereas no patients in the APC group did. This led them to conclude that without other risk factors, APC can provide a better functional outcome.

The use of endoprostheses, osteoarticular allografts, and APCs is all supported in the literature. APC and osteoarticular allografts may provide better long-term functional outcomes through reconstruction of the extensor mecha-

nism. However, allograft use is associated with an increased risk of fracture and infection.

Case Example: Proximal Tibia

A 16-year-old male with proximal tibia osteosarcoma treated with neoadjuvant chemotherapy followed by wide resection and megaprosthesis reconstruction followed by adjuvant chemotherapy (Image 2.2).

Distal Tibia

Osteosarcomas of the distal tibia are rare and typically have a better prognosis compared to osteosarcomas in more proximal anatomic locations [27]. They are commonly treated with below-knee amputation, limiting the clinical data of limb-sparing procedures. Like the other anatomic sites, reconstruction techniques using endoprostheses, allografts, and APCs as well as ankle arthrodeses have allowed for limb salvage. Furthermore, depending on the type of reconstruction, limb preservation can result in improved functionality compared to amputation [28]. Zhao et al. [29] saw similar MSTS scores between autograft reconstruction and below-knee amputation, which were both superior to allograft reconstruction. Autograft reconstruction was performed with nonvascularized fibular grafts, pasteurized autograft, or a combination of the two. Both types of reconstruction had more complications than amputation. In another study, Zhao et al. [28] performed a literature review comparing endoprostheses to biological reconstruction with either allograft or autograft and found that autograft performed better than allograft functionally, and both performed better than endoprostheses. Intercalary allografts, fibular autografts, and treated resected autografts were used for arthrodesis. Osteoarticular allografts were used to reconstruct the ankle. A major limitation of the study is the lack of stratification between types of reconstructions with different allografts and autografts. Each type of reconstruction had advantages and disadvantages in congruence with those previously mentioned.

Kundu et al. [30] performed ankle arthrodeses in patients using the centralization of a free fibular graft alone after resecting distal tibia tumors, resulting in a mean MSTS score of approximately 76%. While the procedure resulted in a loss of ankle mobility and varying amounts of leg length discrepancies, these did not cause significant disability for the patients. Given the limited data, the choice of a reconstruction procedure versus a

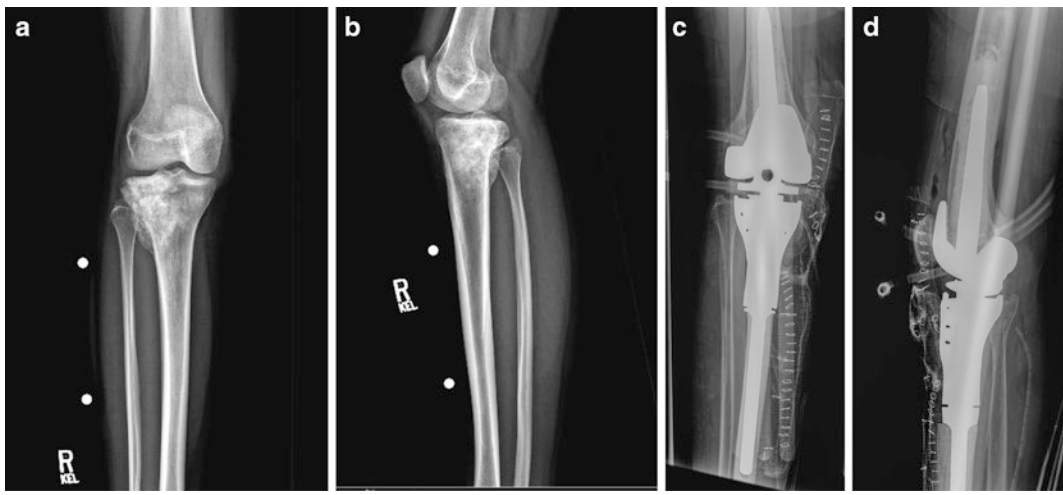

Image 2.2 AP and lateral radiographs reveal osteolytic and osteoblastic lesion in the right proximal tibia (**a**, **b**). Postoperative AP and lateral radiographs show the resection and reconstruction with a megaprosthesis (**c**, **d**)

below-knee amputation needs to be made based on the surgeon's experience and the functional needs of the patient without compromising a margin-negative resection of the tumor.

According to the literature, ankle fusions and reconstruction with either autograft or allograft are the primary methods for salvaging the distal tibia. However, below-knee amputation continues to be the primary method for treating tumors of the distal tibia and can often provide comparable functional outcomes with typically fewer complications.

Proximal Humerus

Successfully reconstructing the upper limb is important in maintaining a patient's function. Whereas a prosthetic for a lower limb amputation can allow for ambulation, upper extremity prosthetics are less able to restore normal or near-normal function. Choosing how to reconstruct the shoulder depends on the margins of the resection as well as the soft tissue structures that are preserved during surgery [31]. Ideally, shoulder, elbow, and hand functionality should be maintained with limb salvage surgery of the proximal humerus.

Historically, preserving shoulder function has been difficult. De Wilde et al. [32] showed that utilizing a reverse total shoulder prosthesis after tumor resection allowed for glenohumeral function with the deltoid compensating for the absence of the rotator cuff. In another study, they found that functionality was maintained after a mean follow-up of 7.7 years utilizing a reverse shoulder prosthesis with irradiation of the resected humerus before being used as an autograft [33]. Reverse shoulder arthroplasty is indicated when the deltoid, axillary nerve, and enough of the glenoid are spared and when resection of the rotator cuff is required [31, 34].

When the rotator cuff is also spared, it is possible to use an endoprostheses or APC [31]. In a systematic review, Teunis et al. found no difference in outcomes between endoprostheses and APC; however, both had worse outcomes than reverse shoulder arthroplasy [31, 35]. While osteoarticular allografts have been used to treat osteosarcomas of the proximal humerus, they have high failure rates and some discourage their use given the advances in endoprostheses [36]. One exception is skeletally immature patients, where there have been high rates of complications with expandable prostheses [37]. van de Sande et al. [38] retrospectively reviewed proximal humeral endoprostheses, APCs, and osteoarticular allografts. They determined that endoprosthetic reconstruction had better implant survival, fewer complications, and comparable functional outcomes to APC.

The literature supports the use of endoprostheses as the most common reconstruction of the proximal humerus. Depending on whether the rotator cuff is spared, either a reverse prosthetic total shoulder prosthesis or standard endoprosthesis can be used. Resection and reconstruction decrease shoulder stability, and painless endoprosthetic subluxation is common.

Case Example: Proximal Humerus

A 17-year-old female with osteosarcoma of the right proximal humerus treated with resection and reconstruction with an APC (Image 2.3).

Distal Humerus

Tumors of the distal humerus are rare and account for only 1% of primary bone tumors [39]. Similar to the tumors of the proximal tibia, reconstruction of the distal humerus presents a unique challenge. The successful reconstruction of the elbow is important for a well-functioning upper extremity. Poor soft tissue coverage and the proximity of the neurovascular bundle to the elbow joint makes reconstruction technically difficult [40]. Due to the small number of primary bone tumors in the distal humerus, studies often pool reconstruction patients presenting with either primary bone tumors or metastatic disease. In the literature, reconstruction techniques tend to be limited to either endoprosthetic reconstruction of the elbow or reconstruction with APC. Large defects

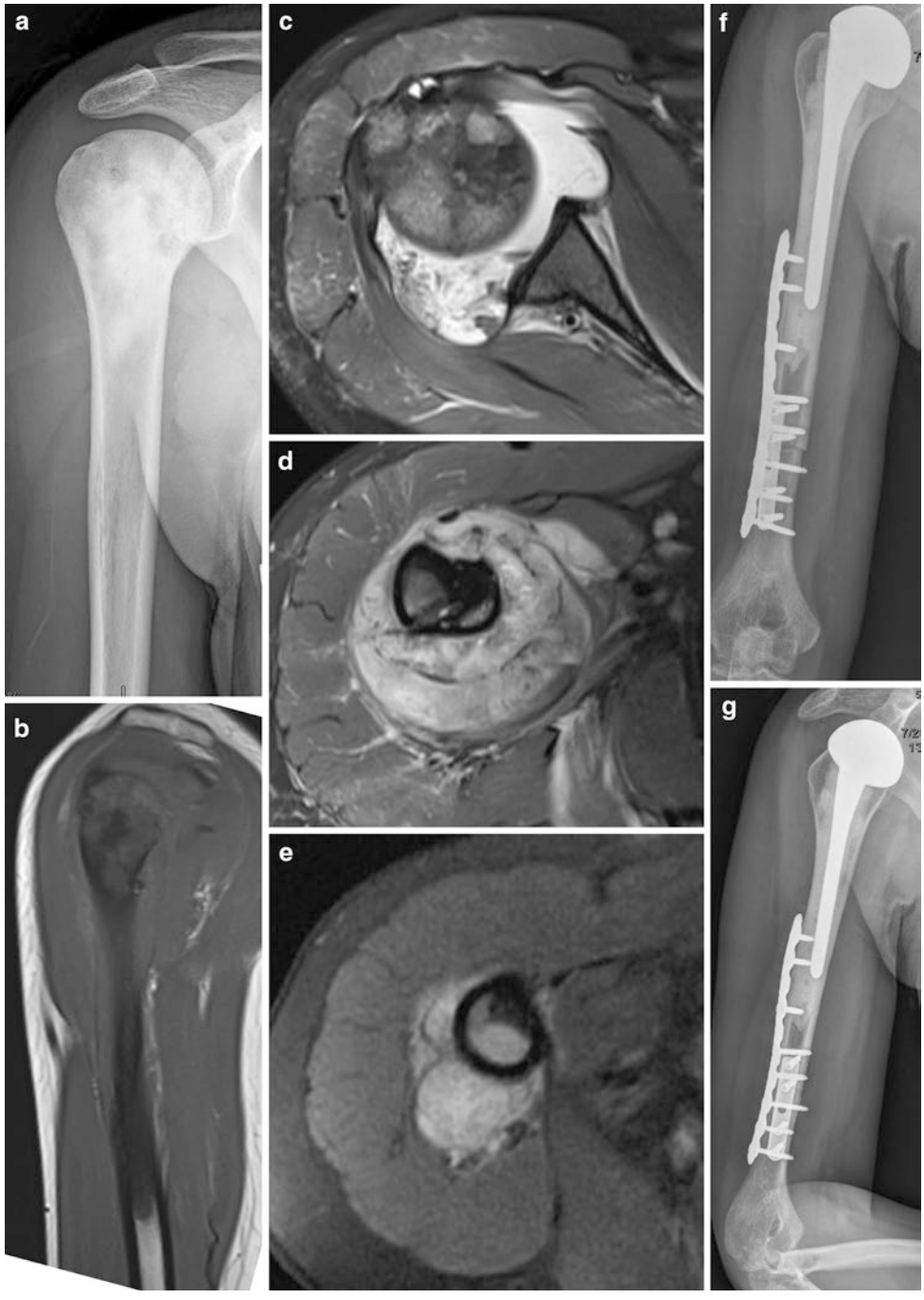

Image 2.3 An AP radiograph of the right proximal humerus in a 17-year-old girl reveals an osteoblastic lesion (**a**). T1-weighted coronal (**b**) and T2-weighted axial MR images (**c**–**e**) show a large circumferential soft tissue mass that extends into the glenohumeral joint (**b**–**e**). AP and lateral right humerus radiographs show the results 1 year after extra-articular resection of the osteosarcoma and reconstruction with an allograft-prosthetic composite and distal plate fixation (**f**, **g**)

and tumors extending to the proximal humerus may require total humeral replacement as a method of reconstruction.

Weber et al. [41] reviewed the results from 23 patients who underwent complex elbow reconstructions following tumor resection. Of the 23 patients, 18 patients had tumors in the distal humerus or humeral diaphysis. They also included patients with soft tissue tumors and multiple myeloma affecting the elbow. The types of reconstruction included total humeral replacement (12 patients), prosthesis (seven patients), allograft (five patients), and segmental elbow replacement (11 patients). Of the 12 living patients at final follow-up, the mean MSTS score was 77%. While all patients had some functional restrictions, 96% had improvement in pain and greater function when compared to an amputation. Total humeral and elbow reconstruction had a mean MSTS score of 70% compared to 80% with segmental elbow reconstruction. Early complications were seen in 35% of patients. Seventeen percent of patients experienced nerve palsies, 9% had infections, and 30% experienced prosthesis or allograft complications.

Most of the literature on tumors of the distal humerus focuses on endoprosthetic reconstruction of the humerus and elbow. Aseptic loosening is a common complication when using endoprostheses. Also, given the proximity of the neurovascular bundle, patients are at risk for nerve palsies following reconstruction of the distal humerus and elbow.

Forearm

Osteosarcomas of the radius and ulna are quite rare. Little exists in the literature describing the treatment of primary forearm osteosarcoma.

Case Example: Forearm

A 14-year-old female with osteosarcoma of distal radial diaphysis treated with neoadjuvant chemotherapy followed by wide resection and free vascularized fibula reconstruction followed by adjuvant chemotherapy. Her resection specimen is shown with the skin paddle from prior open biopsy included (Image 2.4).

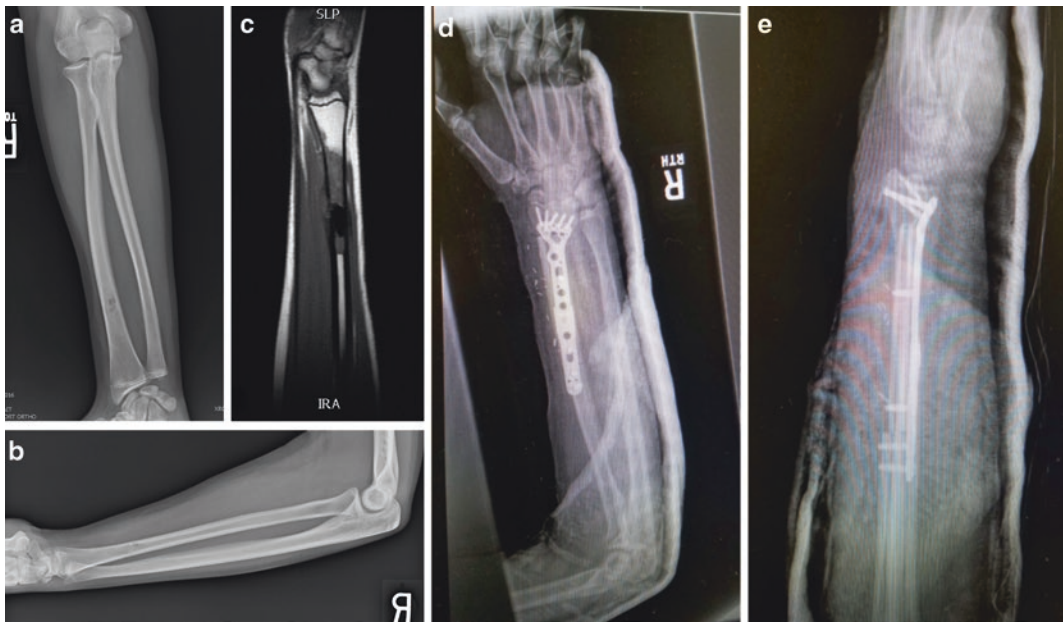

Image 2.4 AP and lateral radiographs show a lesion associated with osteosarcoma of the distal radius (**a**, **b**). T1-weighted MR image reveals the extent of the tumor within the distal radius (**c**). AP and lateral radiographs show the reconstruction with a free vascularized fibula graft (**d**, **e**)

Skeletally Immature Patients

Skeletally immature patients present a unique challenge for successful reconstruction and limb salvage. Osteosarcoma most often occurs in the metaphysis of long bones near the physeal plate in skeletally immature patients. Resection of the physeal plate before physeal closure prevents future growth of the remaining portion of the resected bone. Due to the necessity for wide surgical margins in treating osteosarcoma, resection and subsequent reconstruction can lead to significant limb length discrepancies (LLD) [42]. The functional effect of the resulting LLD is largely dependent on the amount of LLD, age of the patient, and the anatomic location of the resection and reconstruction. In the upper limb, differences in length between the two limbs may result in cosmetic problems but typically do not impact function as long as the joint function and motor function of the hand is spared [42]. The major difficulties with limb preservation of skeletally immature patients occur with tumors involving the metaphysis of the lower limb. The degree to which LLD will have a clinically and functionally important effect is a product of the final difference between the affected limb's length and that of the contralateral limb. It is important to properly estimate future growth before deciding on a specific method of reconstruction.

There are a number of methods to estimate limb growth, which can be aided by computer software. The anticipated LLD is estimated assuming a normal growth rate in the contralateral limb while factoring in the patient's skeletal age and the growth remaining of the resected growth plate [3]. Levin et al. [3] suggest that when the final LLD is <2 cm, surgical procedures to accommodate the discrepancy are not necessary. For 2–5 cm, they suggest halting the growth of the contralateral side, typically via contralateral epiphysiodesis. Finally, for estimated deficits greater than 5 cm, their recommendation is to use expandable prostheses or later limb lengthening procedures. For very large predicted discrepancies, it may be necessary to consider amputation or rotationplasty.

For tumors of the diaphysis that do not involve the metaphysis, resection is often possible while sparing the growth plate. Reconstruction with allograft, vascularized autograft, or a combination of the two is the standard of care [3]. While internal fixation with plate constructs that extend to the epiphysis is often necessary to provide stability after surgery, once host-graft fusion has occurred, the epiphyseal screws can be removed to allow for the resumption of growth [3].

When resection of the growth plate is unavoidable, there are a number of reconstruction options. To preserve the articular surface and joint, Cañadell et al. [43] described the use of physeal distraction, a technique typically used for bone lengthening. As long as the epiphyseal edge of the resected bone is tumor free, they utilize external fixation for stabilization and distraction while filling the defect with a bone graft. Out of 20 patients, no patients experienced subsequent tumors in the epiphysis. Two experienced infection, one had a dislocation of the graft, one had a peroneal nerve palsy, and one had an allograft fracture. They reported mostly excellent and fair outcomes depending on the anatomic location.

Most reconstructions involving a joint in the skeletally immature are performed using endoprostheses in the United States. Implants can either be fixed length implants such as those used in adults or expandable implants that allow for later lengthening and the prevention or minimalization of LLD. Endoprostheses enable early weight-bearing and provide a stable construct. In children, implants need to be durable to prolong the need for future replacement of an endoprosthesis in the years following surgery as patients return to activities. Utilizing a slightly longer implant or fusing the contralateral growth plate is an option for patients closer to skeletal maturity [3]. In younger patients, an expandable prosthesis is often the best choice in preventing a clinically significant LLD.

There are multiple types of expandable endoprostheses on the market. Some use noninvasive magnetic expansion, which allows for expansion of a shorter length over a greater number of expansions without the need for additional surgery [37]. Most expandable endoprostheses require surgical expansion to directly lengthen the device, which increases the overall risk to the patient as a number of surgical

extensions must be performed to reach the correct limb length. In a systematic literature review of the outcomes for limb-sparing surgery in pediatric patients, Groundland et al. [44] reported that patients receiving expandable implants had on average 2.95 expansions for a total expansion length of 29.9 mm in the proximal femur, 6.9 expansions for 84.8 mm for total femur, 4 expansions for 46.5 mm in the distal femur, and 5.7 expansion for 31.3 mm in the proximal tibia. They reported LLD in 24% of proximal femur patients, 0% of total femur patients and 13% of distal femur patients with no data for the proximal tibia. Additionally, a failure of the lengthening device occurred in 3.4% of patients at all locations. Futani et al. [45] reported the MSTS scores from three separate studies. The mean MSTS scores ranged from 74% to 81% with no difference based on the specific type of extendable prosthesis. However, complication rates tend be high, primarily arising from Type 4 and Type 2 failures [3, 45].

Treating skeletally immature patients requires special consideration for possible LLD following reconstruction of the lower limb. If the physeal plate is sacrificed with resection in a young patient, an extendable endoprosthesis and/or contralateral physeal ablation can be used to mitigate future LLD. However, it must be noted that some of these devices have had high rates of implant failure and may require an invasive procedure for lengthening.

Case Example: Expandable Prosthesis

A 9-year-old female with osteosarcoma of the left distal femur treated with neoadjuvant chemotherapy followed by wide resection and magnetic growing prosthesis reconstruction and adjuvant chemotherapy. Her resection specimen shows the extent of the intramedullary disease as well as the extra-osseous soft tissue component of the tumor (Image 2.5).

Case Example: Expandable Prosthesis

A 3-year-old girl who stopped using her right arm for 5–6 days due to acute onset of pain without trauma. Radiographs revealed a pathologic fracture through an osteoblastic and osteolytic lesion of the right proximal humerus consistent with an osteosarcoma. She was treated with resection and reconstruction with an expandable proximal humeral megaprosthesis (Image 2.6).

Case Example: Intercalary Graft

A 14-year-old boy with pain in the left knee after competitive biking was found to have an osteoblastic lesion in the left proximal tibia with ossification in the lateral soft tissues. He was diagnosed with osteosarcoma and was treated with resection and reconstruction with an intercalary allograft, plate fixation, and a supplemental onlay vascularized fibular graft since the tumor did not extend into the proximal tibial epiphysis. Metastasis of the vertebral body was found in the thoracic spine, and patient died despite chemotherapy and radiation (Image 2.7).

Conclusion

There are a variety of reconstruction options that can be utilized to successfully preserve affected limbs for patients with osteosarcoma. Endoprostheses, osteoarticular allografts, and APCs are common options for LSS. Each has its own advantages and disadvantages that differ among various anatomic locations. LSS has become the most common surgical treatment modality for extremity osteosarcoma. Large prospective trials comparing surgical techniques are generally not available and retrospective studies tend to have small sample sizes, limiting the evidence behind choosing one type of reconstruction over another. As a result, the type of reconstruction depends on the patient's functional needs and desires, surgeon proficiency in various techniques, the extent and anatomic location of the tumor, and the patient's age.

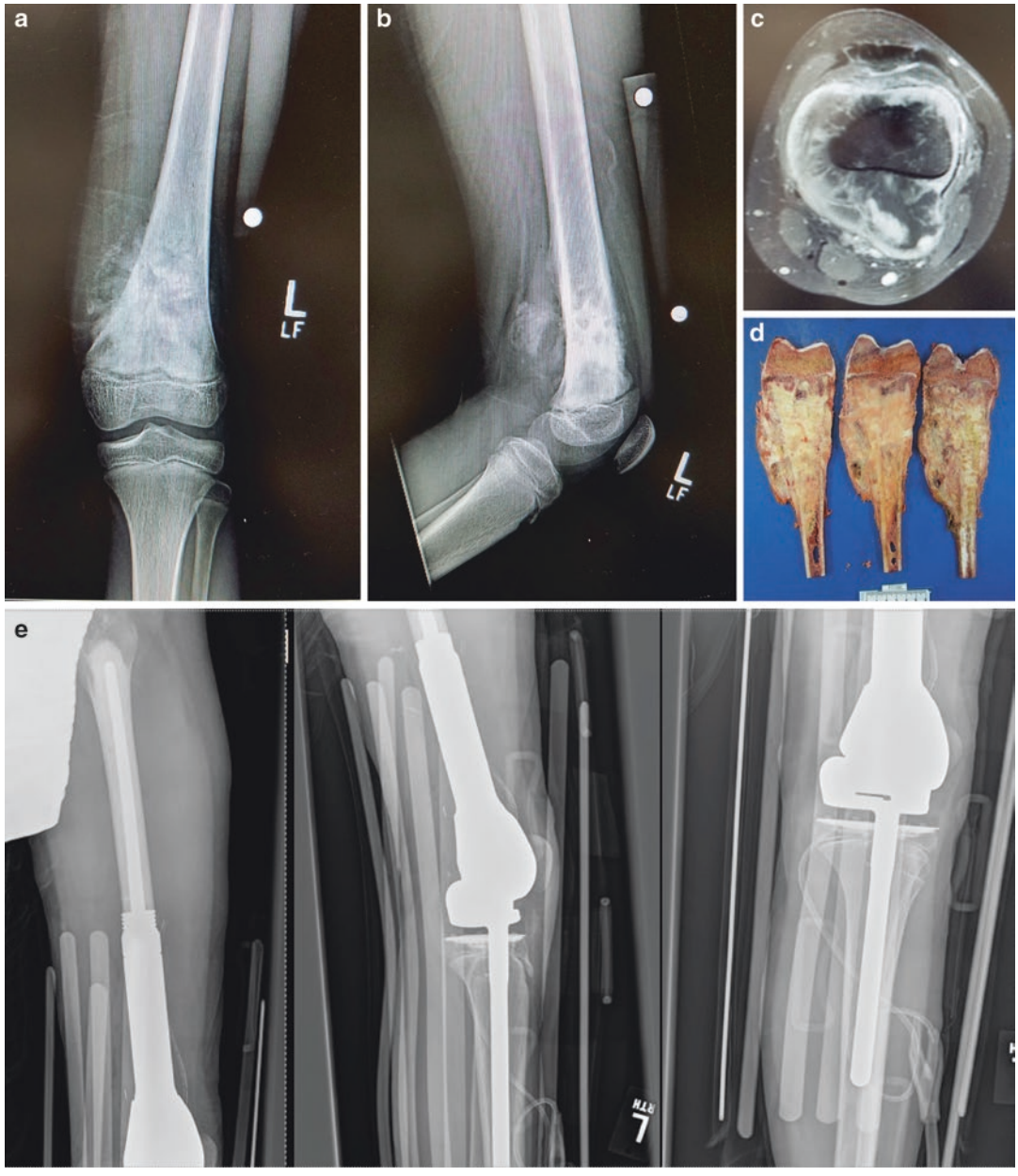

Image 2.5 AP and lateral radiographs show an osteoblastic and osteolytic lesion of the left distal femur consistent with osteosarcoma (**a**, **b**). The extent of the tumor can be seen on axial MR (**b**). The resected gross specimen can be seen in image **d**. Postoperative radiographs show the reconstruction performed with magnetic expandable prosthesis (**e**)

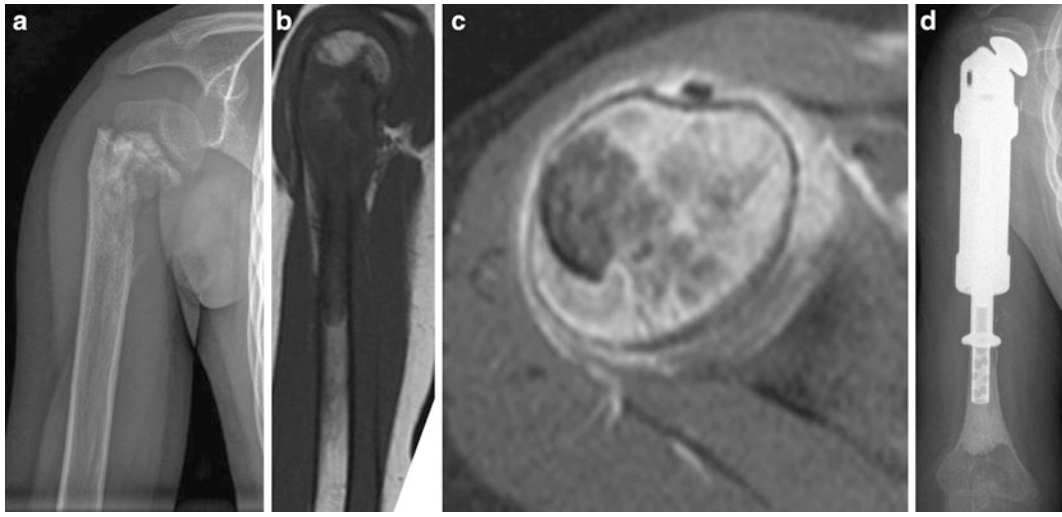

Image 2.6 The pathologic fracture through an osteoblastic and osteolytic lesion on an AP radiograph of the right proximal humerus consistent with an osteosarcoma. There is a Codman's triangle at the distal medial aspect of the lesion (**a**). T1-weighted coronal and T2-weighted fat sat MR imaging reveals the marrow and soft tissue extent of tumor (**b**, **c**). Five years after resection of the humeral osteosarcoma and reconstruction with an expandable proximal humeral megaprosthesis after several extensions (**d**)

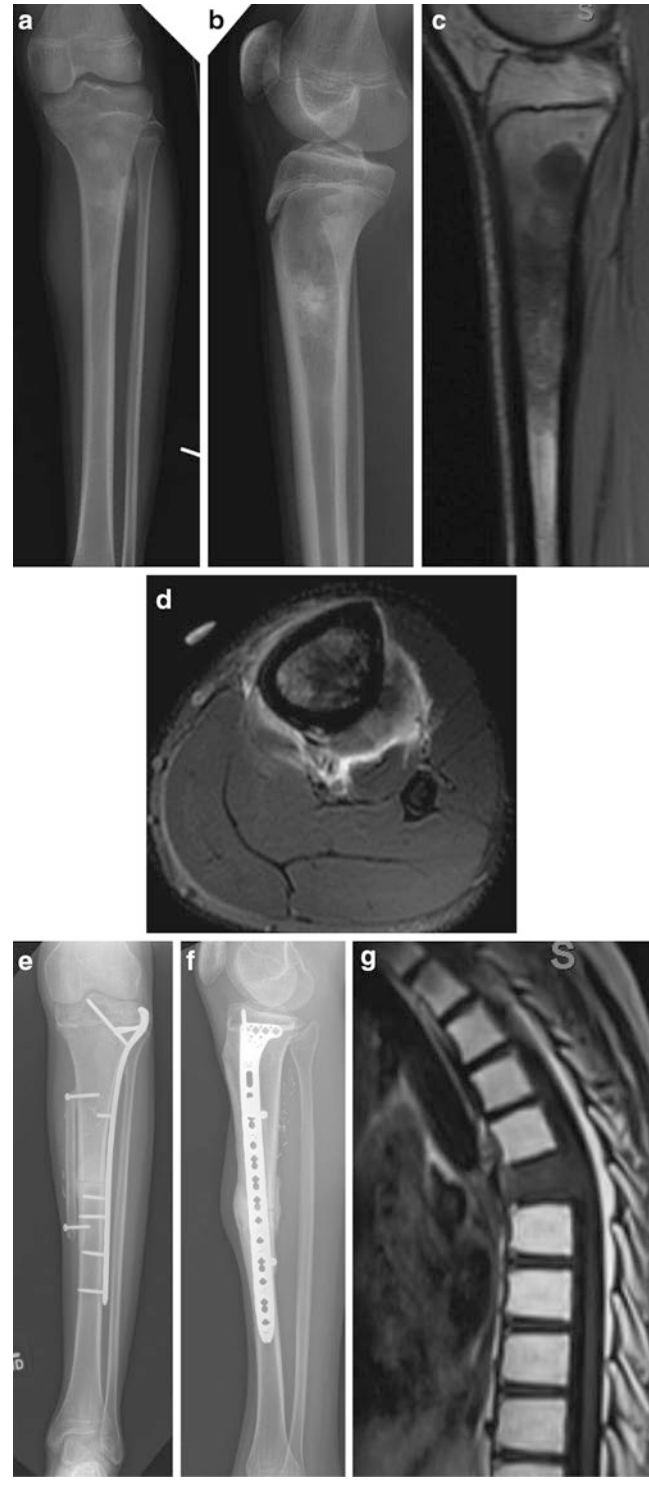

Image 2.7 AP and lateral radiographs of the left tibia show an osteoblastic lesion in the proximal tibia with ossification in the lateral soft tissues (**a**, **b**). Sagittal T1-weighted and Axial T2-weighted MR images revealing the extent of tumor within the marrow and soft tissues. The tumor does not extend into the proximal tibial epiphysis, allowing a resection that spares the knee joint (**c**, **d**). AP and lateral left tibial radiographs 2 years after transepiphyseal resection and a healed reconstruction with an intercalary allograft, plate fixation, and a supplemental onlay vascularized fibular graft (**e**, **f**). Sagittal T1 weight MR image of the thoracic spine revealing a vertebral body metastasis. Despite chemotherapy and radiation, the patient died 4 months later (**g**)

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3 Advances in the Functional Assessment of Patients with Sarcoma

Duncan C. Ramsey and Kenneth R. Gundle

Abstract

Functional assessment of patients with osteosarcoma may yield unique insights into the guide and advance treatment. A range of patient-reported outcomes has been validated, including general health and condition-specific measures as well as computer adaptive testing. Health state utility measures, which facilitate comparative-effectiveness research, are also available. Beyond these surveys, and laboratory-dependent gait analyses, is the potential for real-world evaluation through research-oriented and consumer-oriented accelerometers. Initial studies have shown promising validity of these activity trackers and may also have implications for traditional oncologic outcomes.

Keywords

Osteosarcoma · Sarcoma · Activity monitoring · Patient-reported outcomes · Health-related quality of life · Health state utilities · Computer adaptive testing · Accelerometers · Gait analysis

Introduction

Osteosarcoma is a malignant process of bone-forming mesenchymal cells. It is the most common primary bone malignancy, with an estimated annual incidence of approximately 4.7 per million persons in the 0- to 20-year-old age group [1].Five-year overall survival for osteosarcoma is approximately 68%, though this ranges from 40% to 80% depending on the stage at diagnosis [2]. The advent of neoadjuvant chemotherapy led to a large improvement in survival and potentiated local treatment with limb salvage and reconstruction [3].

Many questions remain in the management of osteosarcoma, including indications for limb salvage versus amputation, optimal chemotherapy regimens, and posttreatment surveillance protocols [4, 5].Patient's baseline health-related quality of life (HRQL) evaluation itself may provide oncologic prognostic information and/or necessitate inclusion as a variable in predictive models

D. C. Ramsey
Oregon Health & Science University, Department of Orthopaedics & Rehabilitation, Portland, OR, USA

K. R. Gundle (✉)
Oregon Health & Science University, Department of Orthopaedics & Rehabilitation, Portland, OR, USA

Portland VA Medical Center, Operative Care Division, Portland, OR, USA
e-mail: gundle@ohsu.edu

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[6]. Improved understanding of physical function measures and HRQL has the potential to answer ongoing questions and facilitate the evaluation of treatment strategies.

In this chapter, we provide an overview of functional assessment strategies. These include provider and patient completed surveys, questionnaires that are normalized by combining with preference data, gait lab testing, and directly tracking patients' free-living activity.

Quality of Life Measures

Measurement of health-related quality of life is paramount in the evaluation of disease states, treatment effectiveness, comparative effectiveness research, and economic analysis. Central to evaluating HRQL is a patient-reported outcome (PRO).

PROs are evaluations of a patient's health status obtained directly from the patient through self-reporting. This is in juxtaposition to more traditional physician- or other clinician-reported outcomes. PROs elucidate the patient's subjective experience of health without mediation or interpretation by a clinician. In oncology patients, they provide information on the patient's subjective experience separately from, though perhaps complementary to, oncological outcomes of survival and recurrence or objective physical examination findings. PROs are vital tools for estimating HRQL.

Any PRO instrument ideally meets several minimum requirements [7].Above all, it should show *validity*; that is, it should convey the most meaningful aspects of health that it seeks to address. It should be r*esponsive* (i.e., sensitive to change). Floor and ceiling effects should be avoided, as these indicate a lack of discriminating ability between patients at the extremes of health states. It should further be *reliable* and *reproducible*, meaning that random error is minimized. Finally, a survey should be as brief as possible to reduce "survey fatigue," which is burdensome for the patient and is known to deteriorate the statistical power and accuracy of surveys [8]. This is particularly a concern in situations where patients are asked to complete multiple questionnaires.

PROs can measure general health status or focus on a specific disease or anatomic location. There are myriad PROs available, and selecting the ideal measure(s) can be challenging. Ultimately, whether for research, quality improvement, or symptom reporting, it is the specific question that must drive instrument selection. Because PROs evaluating general health may lack sensitivity to change due to a specific disease, they should ideally be validated for the diseases in question. Translations of PRO instruments should also be validated in the target language and/or cultural subgroup to whom it is being applied [9].

A shortcoming of HRQL measures (PRO or otherwise) is that they do not take health state *preference* into account. How much value does a patient place in an improvement of x points on a given HRQL scale? Do three points of improvement in the mental component score of the SF-36 equate to three points in the physical component? Is a decline from 25 to 20 points on a 0–100 point scale as valuable as a decrease from 85 to 80? Without taking preference into account, separate health states are not directly comparable, limiting their use in comparative effectiveness research and economic analysis.

One way to resolve the problem of preference is through the use of health state utilities (HSUs). HSUs are PROs that incorporate a patient's or population's self-evaluation of their health state and the population's preference for this health state. HSUs are represented by a single numeric value, usually between 0 and 1.HSUs are generally validated in a population by asking respondents to imagine being in a particular health state. They may be arrived at by *direct* and *indirect* methods. Direct methods include the standard gamble approach and time trade-off approaches [10].In the standard gamble, a subject is presented with two options: the first is that they live out their remaining x years in a state of suboptimal health. Alternatively, they can gamble on a return to a state of perfect health for x years with probability p but risk immediate death with probability $(1 - p)$. The time trade-off method asks a

respondent how much of their lifetime they would sacrifice to trade their current state of health for a better one. For instance, if one had a remaining life expectancy of 5 years in their current suboptimal state of health (say, with daily severe arthritis pain), how many of those 5 years would they sacrifice to ensure that the remaining time was free of arthritic pain? Indirect methods of utility estimation require defining a function that maps a HRQL measure such as a PRO onto a utility instrument. An example of this is the EQ-5D, which is discussed below [11].

HSUs are HRQL measures in their own right, yet they are also suited for economic analysis. The resulting utility values can be used to compare health states within a single disease (such as osteosarcoma) or across multiple diseases, allowing both comparative effectiveness research and cost-utility analyses.

HRQL measures will have greater importance in the future. The Center for Medical Technology Policy issued a guidance document recommending, among other things, the use of PROs in all prospective comparative effectiveness research trials in oncology. It also recommended consideration of metrics amenable to cost-utility analysis [12].Of note, the Musculoskeletal Tumor Registry Pilot study will require PRO data as well, in the form of the Musculoskeletal Tumor Society score (MSTS) and the Toronto Extremity Salvage Score (TESS) [13].Corroborating the emphasis on this research is over $379 million awarded by the Patient-Centered Outcomes Research Institute in 2016 [14].

HRQL Measures

TESS

The Toronto Extremity Salvage Score (TESS) is a disease-specific PRO for assessing functional outcomes in bone and soft tissue sarcomas of the upper and lower extremity [15].It solicits information on the difficulty of various activities of daily living on a 5-point Likert scale and combines this with an evaluation of the importance of each activity to the patient. An aggregate final score from 0 to 100 is returned. There are separate upper and lower extremity activities, and it takes 12–15 minutes to complete [16]. It has been validated with high reproducibility in multiple languages and has excellent inter- and intrarelater reliability in lower extremity sarcoma [16, 17].

The TESS has been widely used in bone and soft tissue sarcomas, elucidating outcomes concerning a variety of clinical questions [18–20]. Notably, Robert et al. used the TESS in a comparison of limb salvage versus amputation in juveniles [21]. They showed that quality of life was related to limb functionality, regardless of whether they had undergone amputation or salvage. The TESS has also been used to demonstrate the similarity between primary osteosarcoma and radiation-induced bone sarcoma and demonstrated similar functional outcomes in osteosarcomas regardless of whether they presented with pathological fractures [22, 23].

The validity and reliability of the TESS are sufficiently high that it has been used to validate other HRQL scores for use in sarcomas [24]. It has also been used to evaluate and validate other functional assessments including wearable activity monitors, which will be discussed below [25].

MSTS

The Musculoskeletal Tumor Rating Scale (MSTS) is another disease-specific HRQL measure for bone and soft tissue sarcomas of the extremity as well as metastatic bone disease. In contrast to the TESS, however, it is a *provider-determined* score. While it has been completed by patients in some series, it was not designed as a PRO measure. It was initially developed by Enneking in 1987 and revised in 1993 [26, 27]. The revised version has six areas of evaluation (pain, function, emotional acceptance, general functional ability, gait handicap, and the use of gait aides), each scored on a 0–5 Likert scale.

Two significant disadvantages of the MSTS have been widely reported. The first, as noted, is its provider reporting. Discrepancies between

provider and patient-reported measures resulting from reporting bias have been widely reported across medical and surgical disciplines [28, 29]. In a comparison of patient and provider-rated MSTS scores in patients with bony metastatic disease, Janssen et al. found a statistically significant 8-point increase when scored by providers [30]. Furthermore, it has been noted to have significant ceiling effects, suggesting a lack of sensitivity to minor insults to health states [16]. It is convenient due to its brevity of only six questions and has shown validity in upper extremity bone tumors [31]. However, an analysis by Davis et al. evaluating multiple functional outcome scores in lower extremity sarcoma patients concluded that the MSTS "did not meet the standards of measurement." [16] Nevertheless, it continues to be widely reported.

SF-36

The 36-Item Short Form Health Survey (SF-36) is a proprietary PRO instrument created by the Rand Corporation [32]. It is a measure of general health and covers eight general categories. It is often partitioned into physical and mental components. The SF-36 is one of the most common PRO instruments for general health and is often reported in sarcoma research. The SF-36 has been used to study limb salvage versus amputation for bone sarcomas in multiple studies and generally has shown slightly improved scores for limb-sparing surgery in the physical (but not necessarily mental) scores [33, 34].

By virtue of being a general health measure, it may lack sensitivity to changes in health states due to a specific disease. Because it was created for a general, community population it may also have difficulty detecting differences between patients with a significant disability; indeed, there is some evidence for floor effects with the SF-36 [35]. For these reasons, the instrument comparison by Davis et al. [16] concluded that the TESS is superior to the SF-36 in sarcoma studies. A shorter 12-question version (the SF-12) exists, as well, that similarly returns physical and mental functional scores.

SF-6D

The Short Form-6D (SF-6D) is an HSU instrument based on the SF-36. It was made to transform full SF-36 data into HSUs, enabling the large swath of SF-36 datasets to take advantage of the benefits of utilities, such as economic analysis and more accurate comparative effectiveness research [36].The SF-6D utilizes 11 questions from the SF-36 and maps the responses to a six-dimensional health state that is then assigned a utility score between 0 and 1 based on a general population sample ranking health states using the standard gamble technique.

An examination of the SF-6D in bone and soft tissue sarcoma patients demonstrated a mean utility score of 0.59, similar to the morbidity of chronic obstructive pulmonary disease or chronic kidney disease in US populations. It further showed good convergent validity to the TESS without demonstration of floor or ceiling effects [24]. It has been used to evaluate wearable activity monitors in bone malignancies (see below) and cost-effectiveness examinations of radiation therapy and osteoarticular allograft in bone and soft tissue sarcoma [37, 38].

EQ-5D

The EuroQol-5D (EQ-5D) is an HSU instrument developed by a multi-national, multi-disciplinary team.

It has been translated into at least 130 languages and validated in many of these [11, 39]. It is one of the most widely-used HSU instruments available. The EQ-5D (also called EQ-5D-3 L) evaluates HRQL using a self-valuation in five dimensions: mobility, self-care, usual activities, pain/discomfort, and anxiety/depression. The respondent rates their health in each of these dimensions with a "1" (no problems), a "2" (some problems), or a "3" (extreme problems). This yields a five-digit number with 3^5 possible

values that is then mapped to a single utility value between 0 and 1.

It has been used extensively in solid cancers [40, 41].The first utility values reported in metastatic bone and soft tissue sarcomas used the EQ-5D instrument [42]. It provided baseline utility values for these patient populations and showed that the ED-5D was able to discriminate between certain subsets of patients based on disease progression. This set the stage for its use in later economic studies.

The EQ-5D has been shown to demonstrate substantial ceiling effects, questioning its sensitivity to detect changes in health status [35]. One study found profound ceiling effect in a cohort of breast, prostate, and colorectal CA patients, 13% of whom scored perfect states of health despite having end-stage cancer [41]. A new edition (EQ-5D-5 L) was created in 2009 to attempt to mitigate some of these ceiling effects [43]. Though more study is needed, there is evidence that the magnitude of the ceiling effects in cancer is somewhat decreased with the EQ-5D-5 L. [44]

PROMIS

The Patient-Reported Outcomes Measurement Information System (PROMIS) is a PRO instrument developed by the National Institutes of Health intended as a measure of general health over many domains of mental, physical, and social well-being [45]. It employs computer adaptive test (CAT) methods to generate the most informative next question based on previous answers. It then maps raw scores onto *T*-scores with a fixed mean and standard deviation that can then be used to make comparisons across domains, disease states, and the population at large. It is non-proprietary and free to the public to use.

PROMIS physical function scores have been shown to be reliable and valid in oncology populations [46, 47]. Because of the CAT methodology it employs, PROMIS questionnaires tend to be short; a comparison with upper and lower extremity TESS questionnaires in an orthopedic oncology population found that PROMIS questionnaires required a mean of 16.8 questions (versus 31 and 32 for the lower and upper extremity TESS questionnaires) [47].

PROMIS has been employed in many aspects of sarcoma research, including outcomes of planned versus unplanned sarcoma resections (which showed no difference in any PROMIS domains tested) [48]. It has also been used to examine limb salvage versus amputation, in which limb salvage outperformed amputation in physical function scores as well as showed higher emotion health scores than the US population at large (PMID 30958808). Another evaluation of postoperative non-metastatic sarcoma patients found improved depression domain scores than the general population, suggestive of a re-evaluation of goals and priorities with sarcoma diagnoses (PMID: 30799982).

Objective Functional Assessment

Despite the usefulness of PROs and other questionnaire-based HRQL scores, it has been asked whether true assessment of quality of life and functional status can be fully captured in surveys or questionnaires [49]. Health events unrelated to function may influence function PRO scores, as has been shown with depression and arthroplasty outcomes [50, 51]. Objective measures of physical activity have consistently been shown to have a modest correlation to PROs and other HQRL scores, suggesting significant functional information exists that is not being captured by them. A 2016 systematic review of objective measures of physical function in sarcoma patients noted a deficit in literature quantifying “balance, gait, and physical activity” in lower extremity sarcoma patients [52]. Hence, objective measures of real-world patient activity may helpfully elucidate the patient experience in terms of physical function.

Metabolic and Gait Analysis

Gait and ambulatory ability can be evaluated by metabolic measurements estimating energy effi-

ciency or by gait parameters such as velocity, stride, and strength. Energy efficiency is often estimated by oxygen consumption, via direct measurement of patient blood oxygenation or rebreather mask techniques.

Ambulation and gait efficiency can be significantly affected by amputation of limb salvage procedures. Gait efficiency is well-known to be altered by amputation level: Waters et al. classically showed a strong trend of decreased gait velocity, increased oxygen consumption, and increased metabolic cost with a higher level of amputation [53].

Bernthal et al. studied energy consumption strength in a cohort of 69 long-term survivors of endoprosthetic reconstruction for a lower extremity bone sarcoma [54]. Energy consumption was estimated by oxygen consumption using a breath-by-breath exchange unit. A comparison to healthy control subjects showed no difference in energy consumption or walking speed, although proximal tibia replacements showed reduced knee flexion and extension strength. Kawai et al. provided baseline data on stride velocity, cadence, and energy consumption for proximal and distal femoral replacements; they demonstrated less optimistic gait efficiency estimates and attributed some variation in consumption to the level of resection [55].

Rotationplasty has received particular attention in laboratory gait analysis, with multiple studies showing rotationplasty gait analysis and kinematics to be superior to above-knee amputation and similar to both endoprosthetic reconstruction and healthy controls [34, 56, 57].

A slightly more convenient method of measuring energy efficiency (albeit still requiring a laboratory) is the *physiological cost index*(PCI). PCI is calculated using only walking heart rate, resting heart rate, and distance walked as inputs [58, 59]. It has been used to compare gait efficiency in lower extremity bone cancer patients that underwent amputation versus limb-sparing surgeries; the latter showed superior PCI scores (though notably, TESS and SF-36 scores were similar) [60].

Though a useful comparative tool, laboratory analyses of gait and energy efficiency can be invasive and costly and require bulky equipment or labs, decreasing their usefulness for many treatment centers and patients. Furthermore, it is not clear that differences found in controlled laboratory settings correlate with real-world physical activity [54].

Real-World Functional Assessment

The impracticality of these physiological measurements has ushered innovation in real-world functional assessment across medical disciplines, including osteosarcoma and extremity sarcoma patients. Wearable activity monitors such as pedometers or accelerometers have shown promise in orthopedic patient functional evaluation [61]. Pedometers are electric or mechanical devices usually worn on the hip that count steps taken. They have proven to be accurate, low-cost alternatives to manually counting steps [62]. Accelerometers such as the Step Watch Activity Monitor (SAM, Modus Health, Washington, DC) are instruments that measure acceleration in space relative to a gravitational field. These devices have been used to evaluate activity in a variety of patient types including COPD [63], low back pain [64], hip and knee arthroplasty [65–67], and numerous others. Furthermore, baseline data for the general population are available in adult and pediatric populations [68, 69].

Unlike pedometers that estimate steps taken, accelerometers worn on the ankle are able to estimate the *intensity* of activity at any given time. Furthermore, steps may be underestimated by pedometers, especially in obese/heavy pts. [61, 67]. In a meta-analysis examining activity monitoring in arthroplasty patients, Naal et al. concluded that accelerometers were the most accurate and appropriate means of estimating activity when compared to oxygen consumption measurements, pedometers, PROs or other HRQL instruments, or activity logs [67].

A cross-sectional study of 29 lower extremity sarcoma patients validated the use of the SAM accelerometer and showed a significant positive correlation ($r = 0.56$) between daily steps taken and the TESS [70]. Interestingly, the osseous

tumor subgroup took fewer steps than the soft tissue subgroup did.

A prospective study following that validation examined 25 separate patients that underwent limb salvage for lower extremity osseous tumor [25]. This showed a strong correlation between steps taken and time from surgery, and moderate correlation between steps taken and the SF-6D and SF-36 physical (but not mental) scores, strengthening the validity of accelerometers as an instrument to evaluate physical function in sarcoma patients.

Rosenbaum et al. used a wearable accelerometer to evaluate 22 patients that had undergone lower extremity limb salvage with modular endoprostheses for sarcomas of bone [71]. Interestingly, no significant correlation was found between gait and locomotion parameters and either MSTS or TESS scores.

They noted activity of a similar magnitude as with successful (non-oncologic) hip arthroplasty patients.

They further warned that the higher than expected step counts could have significant implications for (endo)prosthesis design [71].

Ranft et al. evaluated functional activity of long-term survivors of Ewings sarcoma using the Step Watch Activity Monitor [72]. They similarly reported that total daily steps exceeded 10,000 [73]. They also demonstrated a low ($r < 0.30$) correlation between step and TESS and that pelvic tumors showed worse physical scores.

Real-Time, Real-World Monitoring

Wearable devices such as these have shown promise beyond passive data analysis for effectiveness or outcomes research; accelerometers have also shown promise in real-time activity monitoring in cancer patients. For instance, a pilot study was conducted in elderly adults with solid tumors who were monitored with accelerometer-equipped cell smartphones while receiving chemotherapy [74]. The method proved feasibility even with the elderly population; more importantly, results showed that patients were more likely to have experienced severe chemotherapeutic toxicity on days with substantial decline in daily steps. Many of these toxicities were managed over the phone, avoiding unnecessary hospital visits. A similar study that provided adult hematopoietic cell transplant recipients with pedometers showed a significant correlation between daily steps and worsening symptoms, pain, and PRO scores [75].

Wearable physical activity monitors are now low-cost and user-friendly. They show promise in objective functional assessment of sarcoma patients both as an adjunct to PROs and utility scores or as an objective evaluation in their own right, as they seem to convey information not contained in HRQL scores like ED-5Q and TESS. Though in its preliminary stages, the potential for proactive surveillance of activity through these devices as a surrogate for complications or impending poor outcomes shows significant potential.

Real-world tracked activity may have a significant role beyond the observational as described above; there is emerging evidence that physical activity may have an effect on *oncological* outcomes. Mouse animal models of Ewing sarcoma have found that the addition of an exercise regimen to doxorubicin therapy altered local vascular permeability, resulting in greater drug penetration and more efficiently inhibiting tumor growth [76]. The same mouse model showed that an exercise regimen was able to decrease acute and chronic cardiotoxic effects of doxorubicin-treated mice [77]. If these results prove consistent in human trials, tracking real-world activity in the peri-operative and chemotherapeutic period may become a vital adjuvant treatment from an oncologic perspective as well as from a functional one.

Conclusion

A firm understanding of HRQL measures is crucial to evaluating patients' quality of life, well-being, and disability beyond oncological outcomes. Soliciting health information directly from patients through PRO measures allows for improved patient counseling concerning both

their prognosis and the effect that their treatment may have on their health. They also enable comparative effectiveness research and economic and cost-utility analysis. Survey responses aggregate multiple streams of data, which may or may not be applicable to the specific question under consideration. Contrariwise, some functional information may not be captured properly by a questionnaire, urging evaluation of real-world activity. Free-living activity monitoring provides easily understandable data for assessing disability or advising patients. Real-time monitoring may even predict or alert clinicians of impending complications or poor outcomes.

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Part II

Novel Therapeutic Approaches Based on Biology

4 Radiopharmaceuticals for Treatment of Osteosarcoma

Peter M. Anderson

Abstract

Although trace amounts of radioactivity are routinely used to detect osteosarcoma, the use of larger therapeutic amounts of radiation is often an unrecognized opportunity to treat metastatic osteosarcoma. This chapter will review a number of approaches to use ionizing radiation in the form of injectable radiopharmaceuticals. Since bone metastases are a common pattern of metastatic spread of cancer in general, a number of bone-seeking radiopharmaceuticals have been developed and FDA approved for treatment of bone metastases. Although osteosarcoma, a bone-forming cancer, would seem ideally suited to be treated with bone seekers, patterns of relapse involving non-ossifying metastases remain a major problem to be overcome. Thus, this review will not only describe experience using a number of bone-seeking radiopharmaceuticals such as 153-samarium-EDTMP, 153-samarium-DOTMP, and 223-radium against osteosarcoma, but also approaches to identify patients who may benefit as well as some means to the improve overall efficacy including combination therapy with routine agents and using nuclear imaging to develop best strategy for use. These include imaging with not only ^{99m}Tc-MDP standard bone scans, but also ^{99m}Tc-MDP bone scans with SPECT CT, bone-specific sodium fluoride PET-CT ($Na^{18}F$), and ^{18}FDG-PET-CT. Accurate knowledge of oligometastatic active disease can facilitate more effective use of combination therapy, including radiosensitizers and local control measures, for example, stereotactic body radiotherapy (SBRT) and/or cryoablation to reduce disease burden as well as manage and prevent micrometastatic disease from growing and metastasizing. Finally, a new tumor-specific radiopharmaceutical, CLR 131, may also provide another radiopharmaceutical to treat both osteoblastic and non-ossifying areas of osteosarcoma.

Keywords

Strontium · Samarium · Radium · Radiosensitizer · Sodium fluoride PET · SPECT CT · Ifosfamide · Doxorubicin liposomes · Denosumab · Zolendronate · Pazopanib · Gemcitabine · Beta emitter · Alpha emitter · Stereotactic body radiotherapy (SBRT) · Cryoablation · Lipid raft-seeking radiopharmaceutical

P. M. Anderson (✉)
Cleveland Clinic Lerner College of Medicine, Case Western Reserve University, Pediatric and Taussig Cancer Institutes, Department of Pediatric Hematology, Oncology and BMT, Cleveland, OH, USA
e-mail: andersp@ccf.org

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Introduction

Osteosarcoma is a bone-forming tumor; alkaline phosphatase is a tumor marker associated with high osteoblastic activity. Metastatic osteosarcoma at diagnosis with high alkaline phosphatase in more than two organs (e.g., bone and lung) was associated with significantly inferior survival [1]. The initial bone-seeking radiopharmaceuticals, ^{89}SrCl and ^{32}P, were limited by a long half-life (50 days and 14 days, respectively) and nonspecific uptake of ^{32}P in other tissues. These were generally used for one and done palliation of bone pain [2]. The next era of radiopharmaceuticals with bone-seeking specificity used metal chelates to deliver a radioactive payload which tightly binds bone matrix (Table 4.1). 133-Ho-DOTMP development was halted because of renal toxicity which occurred when radiopharmaceutical that did not bind bone passed through the kidneys into the urine. 186-Re-HEDP and 188-HEDP were used for skeletal metastases in 1997–2007 [3–6] but are not currently available in North America.

Samarium

Goeckeler tested a number of chelates and ethylene diamine tetramethylene phosphonate (EDTMP) was shown to not only have very high bone specificity, but also very high retention in bone [7, 8]. Canine osteosarcoma studies with 153-Sm-EDTMP showed activity excellent against osteoblastic osteosarcoma [9]. 153-Sm-EDMP that does not bind bone is excreted into the urine unchanged [10]. Thus, when Anderson et al. dose escalated 153-Sm-EDTMP with stem cell rescue, the protocol used saline hydration, furosemide to increase urine output, and instructions to void frequently for 6 hours to reduce renal and bladder exposure to unbound radiopharmaceutical [11]. In this study, hypocalcemia from carrier EDTMP was found to be the dose-limiting toxicity when 153-Sm-EDTMP was escalated 30-fold from a standard dose of 1 mCi/kg to 30 mCi/kg. Others have successfully used high-dose samarium for osteosarcoma [12–14]. Loeb et al. also showed tandem dosing was possible in osteosarcoma [15].

Use of gemcitabine as a radiosensitizer after the highly bone-specific binding of high-dose 153-Sm-EDTMP resulted in improved imaging responses [16]. Total body measurements after 153-Sm-EDTMP then gemcitabine were 1.08+/−0.4 mCi (<3.6 mCi for safe infusion of stem cells) after 6–7 half-lives (12–14 days) [16] and all patients recovered hematologic function within 2 weeks after getting the stem cells (Fig. 4.1).

Standard dose 153-Sm-ETMP usefulness in osteosarcoma has been reviewed previously [17, 18]; the dose-limiting toxicity of 153-Sm-EDTP is delayed thrombocytopenia. This generally occurs 3–4 weeks after administration and resolves within 4–6 weeks. To date there are no reports of use of TPO agonists such as eltrombopag or romiplostim after 153-Sm-EDTMP to limit duration and/or severity of this side effect. Although 153-Sm decays to stable 153-Eu by beta decay, trace quantities of 154-Eu are produces during synthesis of 153-Sm via neutron capture. Thus, although not associated with any clinical effects, patients need a letter about prior 153-

Table 4.1 Bone-seeking radiopharmaceuticals for osteosarcoma

Radioisotope	$T_{1/2}$ (days)	Particle	Range (mm)	Bone tumor-seeking ligand
89-Sr	50.6	Beta	7	Alkaline earth metal (like calcium)
32-P	14.3	Beta	9	Metabolized into hydroxyapatite
133-Ho	1.2	Beta	9	DOTMP
186-Re	3.7	Beta	5	HEDP
188-Re	0.7	Beta	10	HEDP
153-Sm	1.9	Beta	4	EDTMP or DOTMP
223-Ra	11.4	Alpha	0.001	Alkaline earth metal (like calcium)

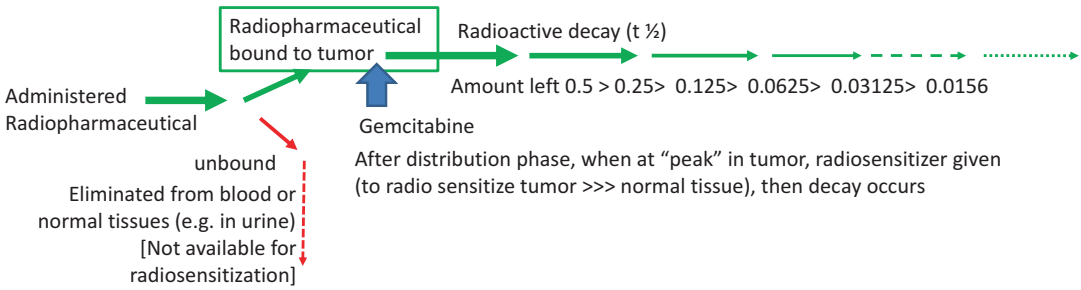

Fig. 4.1 The "Double Tap" for increased tumor-specific lethality. After elimination of unbound agent (e.g., unbound 153-Sm-EDTMP or 153-Sm-DOTMP is eliminated in the urine within 3- 6 hours), only bone bound agent remains when a radiosensitizer (e.g., gemcitabine, ifosfamide, or doxorubicin liposomes) is given later. Specifically bound radiopharmaceutical then decays; this leaves ½ the amount of radioactivity in the tumor after each half-life. Thus, after 7 half-lives 1/128th of the initial radiation is present

Sm therapy when traveling because of the extremely sensitive radiation detectors in airports will detect emissions form 145-Eu [18]. Loeb also described detection of 152-Eu in treated patients, too [19].

One approach to the saturation effect and excess EDTMP at high doses of 153-Sm-EDTMP is to synthesize a different chelate with higher purity and specific activity such as 153-Sm-DOTMP [20, 21]. This preparation has been termed "CycloSam". With high doses it may be avoid hyopcalcemia and 153-Sm-DOTA may become useful for both osteosarcoma and total skeletal irradiation.

Even high-dose samarium patients seem to have only temporary benefit. Isolated limb perfusion (ILP) of 153-Sm-EDTMP of dogs with osteosarcoma at provided some insights about potential reasons for osteosarcoma relapses after bone-seeking radiopharmaceutical administration. Autoradiography showed heterogeneous bone tumor distribution despite achieving a high dose for a short time using ILP. Lung metastases are often another pattern of osteosarcoma relapse or progression after bone-seeking radiopharmaceuticals because some lung metastases have very low amount of bone formation compared to bone metastases. Finally, the mass energy of a beta emitter is much less than alpha emitters which readily cause double-strand breaks.

Radium

Alpha-emitting radiopharmaceuticals have some advantages compared to beta-emitting agents. These include not only very high linear energy transfer (LET) because of high mass (an alpha particle has 2 protons and 2 neutrons), but also safer handling and lower radiation exposure of nontarget tissues [22, 23]. 226-radium was used >100 years ago but the major naturally occurring 226-radium isotope has not only an extremely long half-life but also long-lived radon daughters and thus was abandoned because of safety concerns [22]. Larsen, Henriksen, Nilsson, and Bruland were responsible for early development of 223-radium as a safe and effective agent for bone metastases [23–28]. Preclinical and early clinical trials work established an extremely favorable safety profile including low marrow toxicity and few side effects [27, 28]. Phase 2 studies showed safety, improved pain, and better survival in prostate cancer patients [27–30]. A subsequent randomized, placebo, double-blind phase 3 clinical trial showed improved pain and was stopped early because of a significantly improved survival benefit; this resulted in FDA and EMEA approval [31, 32]. Since prostate cancer causes osteoblastic reactive bone around the neoplastic cells, the 223-Ra may act to kill and contain the viable rim of a bone metastasis.

223-radium was first used for recurrent, progressive, metastatic osteosarcoma using the FDA compassionate access IND mechanism. In these patients not only pain but also the tumor marker, alkaline phosphatase, improved [22, 33]. Subsequently 223-radium has become part of the NCCN guidelines for relapsed osteosarcoma. Subbiah et al. showed safety of 1.5–3.0 microCi/kg [34] and blood-brain barrier penetration of 223-radium in osteosarcoma [35]. This group also demonstrated usefulness of $Na^{18}F$ PET for screening and monitoring of response [36]. The next step was combination therapy using radiotherapy (RT) and stereotactic body radiotherapy (SBRT) with other agents as detailed in Table 4.2.

Denosumab is an agent useful in the treatment of giant cell tumor and osteosarcoma [37], reducing osteopenia, and preventing complications of skeletal metastases. Since I have observed that denosumab also causes increased ossification of osteoblastic osteosarcoma tumors, the agent can be used improve the therapeutic index of 223-radium by facilitating increase 223-radium uptake. At Cleveland Clinic, 14 of 15 recent patients have also had denosumab as part of the 223-radium treatment regimen. It is possible that zolendronate may also be active in this respect and if osteosarcoma cells are like giant cell tumor zolendronate may also have an antiapoptotic effect [38]. Since zolendronate is now generic and has become inexpensive future use would be expected to increase in the treatment of osteosarcoma skeletal metastases, especially in combination with 223-radium. Figure 4.2 shows activity of combined use of continuous infusion 14-day ifosfamide/mesna and 223-radium.

Table 4.2 Agents that have been used with 223-radium (Cleveland Clinic)

Agent	Class of agent	Dose/route/frequency
Denosumab	Rank ligand antibody	120 mg sc monthly
Zolendronate	Bisphosphonate	4 mg iv monthly
Ifosfamide/mesna	Alkylating agent	1 gm/m2/d iv (CI[a]) × 14 days q month
Cyclophosphamide	Alkylating agent	25–50 mg po daily
Pazopanib	TKI[a]	400–600 mg po daily
Sorafenib	TKI[a]	400 mg po twice/day
Sirolimus	mTOR inhibitor	2–4 mg po daily
Everolimus	mTOR inhibitor	5 mg po daily
Nivolumab	Anti-PD1 antibody	480 mg iv monthly
Doxorubicin liposomes	Anthracycline	30 mg/m2 iv monthly

[a]TKI-tyrosine kinase inhibitor (mostly anti-VEGF)

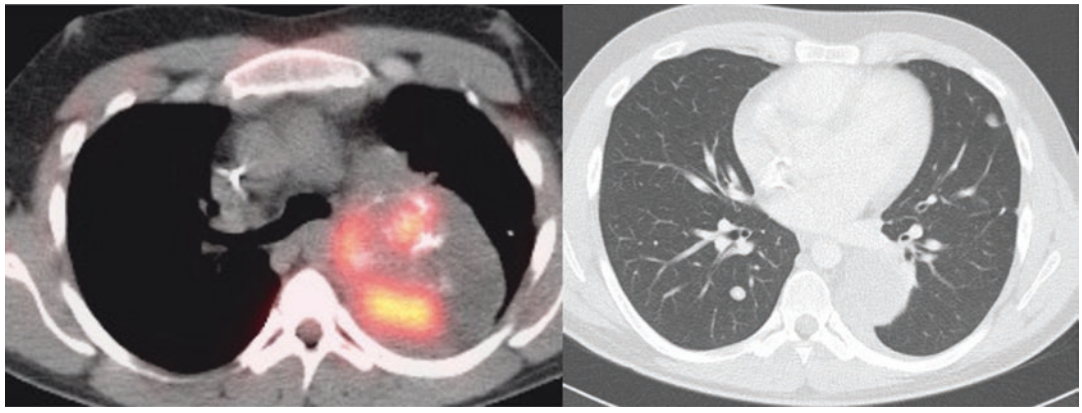

Fig. 4.2 Ifosfamide +223-radium combination therapy. Heterogeneous osteoblastic activity of an osteosarcoma lung metastasis using ^{99m}Tc-MDP bone scan/SPECT CT. This patient had an excellent response to the combination of denosumab+14-day continuous infusion ifosfamide/mesna and monthly 223-radium after two cycles. This allowed thoracic surgery to be done to remove the large mass

Choice of cytotoxic agents to combine with 223-radium was driven by agents and combinations with low marrow toxicity so as not to delay monthly 223-radium infusions. For example, oral cyclophosphamide can be adjusted to keep ANC > 1000–1500, and anemia and thrombocytopenia are rarely problematic. Although high-dose ifosfamide has high activity against relapsed osteosarcoma [39] including bone metastases [40], the 5-day regimen results in pancytopenia and would not be suitable for use with 223-radium. However, high-dose ifosfamide/mesna (14 gm/cycle but given as a continuous at 1 gm/m^2/day) has very low potential to cause thrombocytopenia; neutropenia can be overcome using PEG-GCSF [41–44].

Another means to attempt to overcome the problem of heterogeneous biodistribution of 223-radium is to use additional external beam radiation as either SBRT or RT if normal structures (e.g., trachea, carina, heart, mediastinum, stomach) do not permit SBRT to be safely given. In 15 patients treated with 223-radium treated at Cleveland Clinic >50 sites of osteosarcoma metastases have had SBRT or RT to improve both pain and/or durability of responses. Figure 4.3 shows an example to SBRT to the sacrum.

Other means of improving 223-radium efficacy have included use of TKI agents such as pazopanib, sorafenib, and regorafenib to provide radiosensitization and antiproliferative effects [45–47]. Although pazopanib, sorafenib, and regorafenib have activity against metastatic osteosarcoma [47–51], side effect profile for each is different. Since pazopanib seems to have fewer problems with rash and GI toxicity, this has been used in more of our 223-radium patients than other TKI agents at our institution. Finally, doxo-

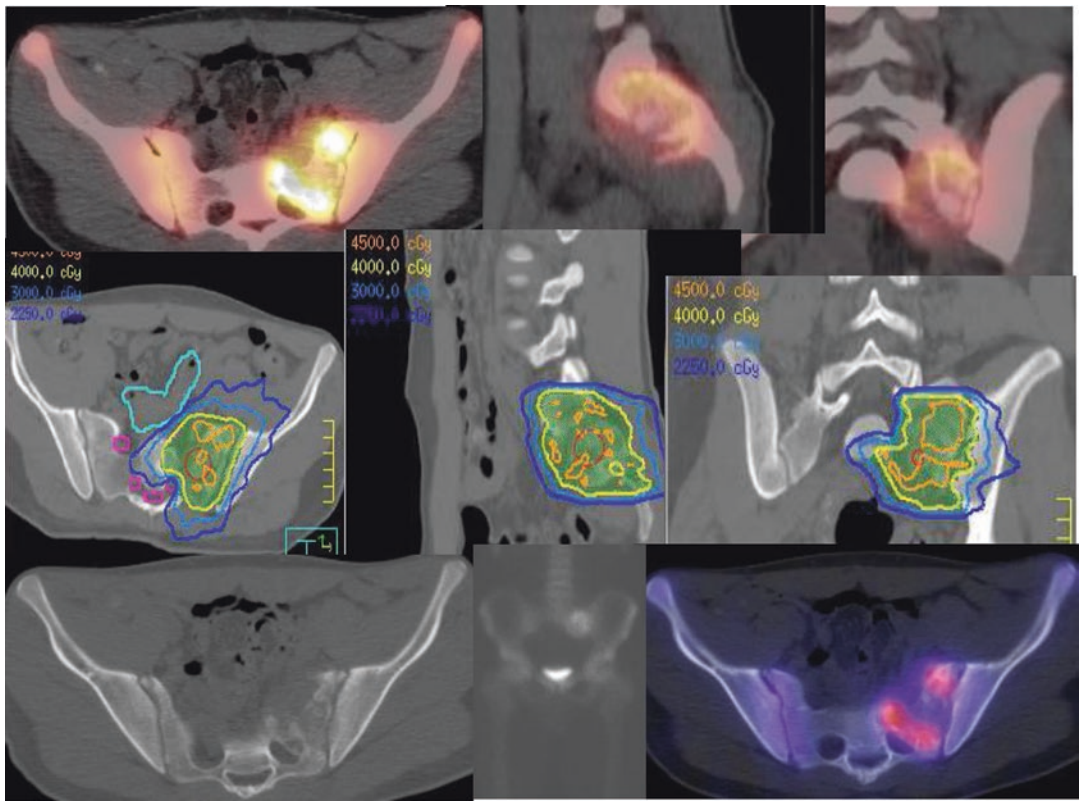

Fig. 4.3 Scan images and SBRT plans of osteosarcoma involving sacrum treated with denosumab, pazopanib, and 223-radium. Top: PET-CT showing ^{18}FDG activity; middle: SBRT plan (8 Gy × 5 = 40Gy; bottom: CT, planar ^{99m}Tc-MDP bone scan, and SPECT CT of lesion. This patient had a durable response in this location to the combination therapy and was able to participate fully in activities including climbing again and attending college

rubicin liposomes have been used with 223-radium because this agent is outpatient and well tolerated (Table 4.2). The anthracycline liposomal formulation, unlike the parent drug, has very low hematologic and heart toxicity [52] and may also have an effect on sarcoma stem cells in combination with mTOR inhibition [53, 54]. Nevertheless, relapse of metastatic osteosarcoma after 223-radium in non-osseous sites is common. In our series of patients with osteosarcoma osteoblastic metastases, 6/15 alive after 1 year and 3/15 > 2 years.

Another Radiopharmaceutical for Osteosarcoma: CLR 131

A new radiopharmaceutical with other tumor-specific properties is CLR 131. This agent has specificity for tumors via [36] lipid rafts which are highly expressed on tumor cells but not normal tissues [55].Thus, CLR 131 can deliver a nuclear payload containing iodine to osteosarcoma tumor deposits, even when these do not make bone. Preclinical models also show synergy with external bean radiation in vivo [56]. Preclinical work with pediatric cancers including neuroblastoma, rhabdomyosarcoma, Ewing sarcoma, and osteosarcoma demonstrated in vivo concentration ~6× in tumors as well as antitumor efficacy [57, 58]. The University of Wisconsin has a clinical trial testing this agent in children and college-aged young adults with solid tumors including osteosarcoma (NCT03478462). Escalation using stem cells (like MIBG) and/or gemcitabine radiosensitization should also be possible with the CLR 131 agent.

Patient Selection for Radiopharmaceuticals for Osteosarcoma: Practical Considerations

Table 4.3 reviews some aspects of how specific nuclear medicine scans can help make plans and/or decide on suitability (or not) as well as following response(s).

Table 4.3 Scans for plans: Imaging of osteosarcoma for control of oligometastatic disease

Imaging modality	Principle	Comment
^{99m}Tc-MDP SPECT CT	Three-dimensional imaging of bone formation	223-Ra or 153-Sm-DOTMP screening and/or dosimetry
Na^{18}F PET-CT	More sensitive than ^{99m}Tc-MDP	Follow response
^{18}FDG PET-CT	Shows metabolic activity	Follow response RT plans
CT	Sensitive detection of lung metastases (lung and bone windows) CT guidance into tumors CT guidance into tumors	Follow response RT plans Biopsy + cryoablation
MRI	Axial (head and neck, spine, and pelvis)	RT plans

Although planar ^{99m}Tc-MDP bone scan can give a yes or no about lesion being osteoblastic (avid) and 223-radium suitability, combining this imaging with SPECT CT can help one know more about location and heterogeneity of uptake as well and to develop plans for other local control measures (e.g., brachytherapy, RT, SBRT, or cryoablation) [59–61]. Sodium fluoride PET is perhaps the most sensitive means to follow osteoblastic lesions after 223-radium [36].

Table 4.4 shows an example of multiple osteoblastic lesions responding using Na^{18}F PET-CT as a means to show improvement. ^{18}FDG is the best means to follow non-osteoblastic bone or visceral lesions since these may not change much in size and/or be detected by the bone-specific ^{99m}Tc-MDP or Na^{18}F bone scans. Sometimes CT done with PET scans is not of diagnostic quality and a dedicated chest CT with and without contrast is the most specific and sensitive means to follow lung metastases. Instead of relying on tumor specificity of radiopharmaceuticals, treatment of oligometastatic disease using SBRT or cryoablation using CT guidance [59–61], may offer additional modalities to reduce osteosarcoma disease burden.

Table 4.4 Decrease in $Na^{18}F$ bone PET uptake of osteoblastic metastases after 223-radium

	SUV pre 223-radium	SUV post 223-radium × 2	Difference	
Bone lesion location			SUV	Percent less
Skull base (clivus)	9.3	5.1	−4.2	−46%
C-spine (C3)	21.2	8.2	−13.0	−61%
T-spine (T2)	26.9	7.8	−19.1	−71%
T-spine (T12)	30.1	25.3	−4.8	−16%
L-spine (L4)	24.9	10.6	−14.3	−57%
Sacrum	26.8	18.8	−8.0	−30%
Pelvis (femoral head)	24.7	6.9	−17.8	−72%
Ribs (post left 6th)	18.5	6.0	−12.5	−68%
Humerus (proximal right)	35.8	19.5	−16.3	−46%
Ankle (left distal tibia)	32.6	16.0	−16.6	−51%
Median	25.8	9.4	−15.8	−54%
Mean	25.1	12.4	−12.7	−51.8%

Summary and Obtaining Access to Radiopharmaceuticals for Osteosarcoma

Bone-seeking radiopharmaceuticals 153-Sm-EDTMP and 223-radium may improve pain and provide an underutilized means to treat osteoblastic metastases of osteosarcoma. Although dose escalation of 153-Sm-EDTMP and 153-Sm-DOTA is possible, osteoblastic heterogeneity may limit long-term effectiveness (cannot hit the target if there is no uptake). Because of low marrow toxicity and ease of administration, 223-radium can be used in combination with other agents. Nevertheless, other control strategies (e.g., SBRT, cryoablation) then immune therapy such as Cincinnati Children's trial of pembrolizumab, decitabine, and SBRT (NCT 03445858) or CLR 131 at the University of Wisconsin (NCT03478462) may be other options to consider.

Radiopharmaceuticals can provide benefit to osteosarcoma patients. This is an evolving field. The author uses virtual visits to help patients and caregivers understand what options are not only feasible but with a likelihood of benefit and also how to get access to these remarkable agents [62].

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HER2-Targeted Therapy in Osteosarcoma

5

Jonathan Gill, Pooja Hingorani, Michael Roth, and Richard Gorlick

Abstract

In this chapter, we will review studies of HER2 in osteosarcoma and discuss the controversies that have existed in this field. Our present understanding of HER2 in the context of osteosarcoma is that it is expressed on a subset of patient samples, but that expression is not prognostic. We will review the two trials that have been conducted in osteosarcoma which have targeted HER2. Use of an antibody, trastuzumab, did not suggest activity, but a smaller study using HER2-targeted CAR T cells suggested activity may be present. A trial of an antibody–drug conjugate targeting HER2 for recurrent osteosarcoma is under consideration. Trials targeting other surface proteins for the treatment of osteosarcoma have occurred or are in development. Indeed, this leads us to discuss in a broader fashion therapeutic approaches to targeting surface proteins. It is hoped that some of these approaches will lead to new effective therapies for patients with osteosarcoma.

Keywords

HER2 · Targeted therapy · Antibody–drug conjugates · CAR T cells · Trastuzumab · Pathogenesis · Prognostic markers

Introduction

The first studies of HER2 expression in osteosarcoma date back to the 1990s, recognizing a proportion of osteosarcoma samples express the protein. Early studies produced discordant results with the factors underlying variability in immunohistochemical staining in osteosarcoma not fully appreciated and genomic amplification of HER2, providing an alternative testing approach, not being present. The current understanding is expression is present on a subset of tumors from osteosarcoma patients, but this is not prognostic. Treating patients whose osteosarcoma samples express HER2 with trastuzumab did not demonstrate clinical activity, but a clinical trial utilizing HER2-directed CAR T cells suggested some clinical efficacy. The more recently developed approach for targeting low HER2-expressing malignancies is the use of antibody–drug

J. Gill · P. Hingorani · M. Roth · R. Gorlick (✉)
Division of Pediatrics, University of Texas MD Anderson Cancer Center, Houston, TX, USA
e-mail: rgorlick@mdanderson.org

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conjugates, which will likely be pursued in clinical trials for osteosarcoma. Beyond HER2, antibody–drug conjugates that do not necessarily rely on the target protein being an oncogenic driver may be an alternative path forward for osteosarcoma treatment.

HER2 as an Oncogene in Osteosarcoma

HER2 Biology

HER2 was first described by multiple groups in the 1980s, which has led to its multiple names in the literature [1] . Like its homolog, EGFR, HER2 is a transmembrane tyrosine kinase receptor [2]. During fetal development, HER2 is widely expressed in tissues including placenta, liver, kidney, lung, and brain. Lower levels of expression are also seen in adult tissues: kidney, liver, skin, lung, jejunum, uterus, stomach, and colon. The HER2 null mouse is embryonic lethal due to complete absence of cardiac trabeculae [3]. There are four members of the family of epidermal growth factor receptor tyrosine kinases: ErbB1 (EGFR), ErbB2 (HER2), ErbB3 (HER3), and ErbB4 (HER4). All of these receptors need to dimerize to initiate the signaling cascade and frequently form heterodimers. HER2 is unique in that it is the only member of this family for which there is no known ligand. However, it has been shown to be the preferred partner for the other members to form heterodimers. HER2 overexpression has been shown to be tumorigenic. The transfection of NIH3T3 cells with HER2 transforms the cells and leads to tumor formation in mice. The tumorigenicity is associated with level of expression of HER2 within the transformed cells [4, 5]. Transgenic mice expressing HER2 under the control of a mouse mammary cell-specific promoter form mammary tumors consistent with adenocarcinomas at 4 months of age. Ultimately most of the mice develop lung metastases as well [6].

HER2 in Osteosarcoma Cell Lines

Unlike in breast cancer cells, in osteosarcoma cell lines, HER2 displays primarily cytoplasmic or mixed membranous and cytoplasmic staining. Compared to EGFR, HER2 demonstrated less intense staining by immunohistochemistry. The expression levels by immunohistochemistry correlate with the levels of messenger RNA detected by PCR and protein by Western blots. In primary osteosarcoma cell lines, despite the lack of detection of HER2 on the membrane by immunohistochemistry, flow cytometry reveals higher quantities of HER2 than EGFR on the surface [7].

Two other studies have corroborated the cell surface expression of HER2 by flow cytometry in osteosarcoma cell lines. Hassan et al. demonstrated in primary as well as established osteosarcoma cell lines that HER2 is detectable in greater quantities than EGFR [8]. Scotlandi et al. found that 62% of the primary and established osteosarcoma cell lines demonstrate HER2 expression by flow cytometry, albeit at lower levels than the breast and ovarian cancer cell lines used as positive controls. None of the osteosarcoma cell lines demonstrated amplification of the *HER2* gene by fluorescence in situ hybridization [9]. Unlike the data in cell lines, the studies in patient samples have described conflicting results regarding whether HER2 is expressed in osteosarcoma and its role in defining prognosis.

HER2 Is a Negative Prognostic Indicator in Osteosarcoma

Six studies have demonstrated that HER2 expression in osteosarcoma portends a poor outcome. Onda et al. in 1996 first described HER2 expression in osteosarcoma. They found that 42% of tissues demonstrated various levels of expression by immunoblotting, which was scored from 0 to 3+ (no staining, weak, moderate, and high, respectively). This was corroborated by immunohistochemistry, revealing a primarily membranous pattern of staining. Southern blot analysis

did not reveal any amplification of the *HER2* gene. Patients whose tumors expressed HER2 (1–3+) had a significantly worse response to preoperative chemotherapy and survival. In this series, patients who had no HER2 expression demonstrated a 1-year survival rate of 100% and 3-year survival rate of 84%. In contrast, those with weak to high expression of HER2 had significantly worse outcomes with 1- and 3-year survival rates of 61% and 14%, respectively [10].

Gorlick et al. evaluated 53 patients treated on the T12 protocol. This randomized trial found no survival benefit to dose intensification of the preoperative chemotherapy, allowing all the samples to be treated as a single cohort [11]. HER2 expression levels were evaluated by immunohistochemistry and scored according to the percentage of cells staining positive: 0 (no staining), 1+ (1–25%), 2+ (26–50%), 3+ (51–75%), and 4+ (76–100%). HER2 staining localized primarily to the cell membrane. Overexpression was defined as greater than 2+ staining. HER2 was overexpressed in 45.3% of the patients' tumors, which was similar to the 42.6% detected from the initial biopsy specimens. Overexpression of HER2 was found to be correlated with decreased response to preoperative chemotherapy and event-free survival. At 5-years, patients whose tumors overexpressed HER2 had a 40% event-free survival compared to 78% for patients with low or undetectable levels of HER2 expression. The difference in event-free survival remained significant even when 13% of patients who presented with metastatic disease were excluded from the analysis (47% versus 79%) [12].

Zhou et al. reviewed HER2 expression from 25 patients treated at their institution from 1981 to 1996. They included in their analysis 25 primary tumor samples and 12 specimens from metastatic lung lesions. Immunohistochemistry was defined as positive if greater than 25% of tumor cells demonstrated immunoreactivity. Amplification was defined as positive if greater than 10% of the cells demonstrated more than two signals or if more than three cells showed a large number of signals by FISH probe for the *HER2* gene. They found focal to diffuse cytoplasmic staining in 44% of the primary tumor samples and 58% of the pulmonary metastases. HER2 expression was not found to be correlated with response to chemotherapy. However, patients whose tumors stained positive for HER2 were found to have a significantly worse metastasis-free survival. To evaluate for amplification of the *HER2* gene, FISH was performed on 12 samples. Increased signal consistent with amplification was observed in six of seven immunostain-positive samples and two of five immunostain-negative samples. In the two immunostain-negative samples which were found to have amplification of *HER2*, the immunohistochemistry revealed focal HER2 staining which did not meet the criteria for positive [13].

In 2004, Fellenberg et al. attempted to address some of these issues with immunohistochemistry by assessing HER2 expression at the level of mRNA by real-time reverse-transcription PCR (RT-PCR). To enrich the samples, they used laser microdissection to isolate osteosarcoma cells for analysis. They evaluated 17 pretreatment biopsies from a single institution using histologic response as their primary clinical endpoint. They found that HER2 mRNA could be detected in all the samples tested. HER2 expression was significantly elevated in patients who demonstrated a poor histologic response to preoperative chemotherapy. When they analyzed the samples for protein expression by immunohistochemistry, they found strong cytoplasmic staining in all the samples. There was no correlation between mRNA levels and protein expression of HER2 [14].

Ferrari et al. published a report on a cohort of 19 patients who presented with localized disease who subsequently experienced a pulmonary relapse. They examined HER2 expression by immunohistochemistry according to the percentage of cells staining positive on the membrane, 0–4+. The tumor was considered to be positive if it exhibited 2+ or greater staining. They found HER2 to be expressed in 32% of the primary tumors, and 53% of the patients had at least one nodule expressing HER2. The accordance rate, defined as the presence of the same expression pattern in the primary and metastatic samples, was 42%. Patients with HER2-positive primary tumors had a shorter recurrence-free interval of

17.2 months versus 31.8 months for patients with HER2-negative primary tumors. Likewise, patients with HER2-positive primary tumors were more likely to recur with multiple pulmonary metastases [15].

A large, single-institution, retrospective analysis of HER2 expression in osteosarcoma in 84 patients treated on two similar protocols was published by Scotlandi et al. in 2005. They examined pretreatment biopsy specimens, using two different antibodies, and for half of the specimens three different antibodies. They defined expression as having greater than 25% of the cells stain positive. They detected HER2 expression in 32% of the samples with a pattern of focal to diffuse cytoplasmic staining. Between the two antibodies tested, they found a concordance rate of 78%. For the samples tested with the third antibody, similar results were obtained with 28% of the samples positive for HER2 expression. Patients with HER2-negative tumors exhibited an event-free survival of greater than 60% compared to approximately 40% for those expressing HER2 [9]. This analysis demonstrated cytoplasmic staining for HER2 in osteosarcoma with a high rate of concordance using multiple antibodies.

HER2 Is Not Prognostic in Osteosarcoma

Nine studies have reported that HER2 expression is not prognostic in osteosarcoma. In 2001, Maitra et al., using immunohistochemistry and FISH, examined 21 diagnostic biopsy specimens from a single institution. For immunohistochemistry analysis, they defined as positive only cell membrane staining, excluding cytoplasmic and nuclear staining, and graded according to a four-tier grading scheme: negative, low, medium, and high. They did not find HER2 overexpression by immunohistochemistry in any of the samples. Likewise, they did not detect any amplification of the *HER2* gene by FISH [16].

Kilpatrick et al., in the same year, reported on a retrospective analysis from two centers between 1985 and 2000. They examined HER2 expression by immunohistochemistry comparing two different antibodies as well as decalcified versus non-decalcified specimens. Staining was scored from 0 to 3+. Positive was defined as 2+ or 3+: weak to moderate staining in more than 10% of cells or moderate to strong staining in more than 10% of cells. None of the osteosarcoma specimens demonstrated staining for HER2 on the cell membrane. Focal cytoplasmic staining in more than 10% of the cells was found in 83% and 98% of the samples, using the different antibodies. There was poor agreement between the antibodies in the extent of cytoplasmic staining. Neither antibody demonstrated a correlation with response to preoperative chemotherapy, metastasis, or survival [17].

Thomas et al. performed a retrospective analysis of osteosarcomas in a single institution from 33 patients. They graded the immunohistochemical staining according to a five-tier system: negative, cytoplasmic, low-positive membranous, medium-positive membranous, and high-positive membranous. None of the samples demonstrated staining for HER2 on the cell membrane. Forty-seven percent of the specimens demonstrated diffuse cytoplasmic staining. None of the samples had HER2 mRNA amplifiable by RT-PCR [18].

Anninga et al. evaluated 15 pretreatment biopsy specimens as well as 12 specimens including postchemotherapy resections or pulmonary, distant bone, or local relapse specimens. They evaluated the samples by quantitative real-time RT-PCR (qPCR) and by immunohistochemistry. Tumor samples were scored 0–3+ according to the level of membrane staining. Cytoplasmic staining was not considered positive. Of the 27 evaluable specimens, only one sample (from a pre-treatment biopsy) displayed membranous staining, which was scored as moderate. Focal cytoplasmic staining was detected in two other samples. None of the samples had overexpression of HER2 mRNA when compared to a HER2 overexpressing cell line. In the one sample with HER2 membranous staining, FISH did not reveal *HER2* amplification [19].

A collaborative project involving four institutions evaluated HER2 expression in 22 samples from 20 patients. Immunohistochemistry was graded from 0 to 3+ according to level (>10% of

cells) and intensity (mild, moderate, strong) of membranous staining. Scores of 0 and 1+ were considered to be negative. Four of the samples (18%) showed focal positivity for HER2 (1+ grading). None of the samples revealed amplification of HER2 by fluorescence in situ hybridization. When the authors interpreted 1+ staining as positive, univariate analysis did not reveal a statistically significant difference in survival in the two groups [20].

Somers et al. reviewed 34 samples from 18 patients in a single institution. They graded the immunostaining from 0 to 3+ according to the intensity of membrane staining. Cytoplasmic staining was graded as 0. They found that four osteosarcoma specimens from two patients displayed HER2 immunostaining. Two revealed cytoplasmic staining (0), and two cytoplasmic and membranous staining (1+). None of the samples were evaluated as having overexpression of HER2 by immunohistochemistry. None of the samples demonstrated *HER2* gene amplification by FISH. In 39% of the tumors, aneuploidy (having multiple signals to the FISH probe) was detected in less than 10% of the cells. They also noted that four samples exhibited three nuclear signals in greater than 50% of the cells, which they state is suggestive for trisomy 17. None of the tumors with increased signal by FISH probe displayed expression for HER2 [21]. Since there was no concordance between the increased chromogenic signal and immunohistochemistry, the authors concluded that the increased signal should not be interpreted as amplification of the gene.

HER2 gene amplification was evaluated by Willmore-Payne et al. using FISH as well as multiplex and monoplex PCR. They also performed immunohistochemistry on the samples, grading from 0 to 3+. Cytoplasmic staining was graded as 0. In the initial 21 cases evaluated by multiplex PCR and FISH, there was no evidence of *HER2*gene amplification. Of these cases, 11 demonstrated cytoplasmic staining for HER2 by immunohistochemistry, which were all graded as 0. No samples demonstrated membranous staining. Given the negative findings, they obtained an additional 35 paraffin blocks from 26 patients from another institution to perform monoplex PCR and FISH. Again, they were not able to detect any *HER2* gene amplification. In these 26 patients, they detected 2 samples with cytoplasmic staining for HER2 by immunohistochemistry, and 1 sample with 1+ membranous staining [22].

Bakhshi et al. evaluated HER2 expression by immunohistochemistry in 63 patients. They delineated the pattern of staining as cytoplasmic versus membranous. They graded the samples according to the percentage of cells stained: 0, 0–10%; 1+, 11–30%; 2+, 31–50%; 3+, 51–100%. They observed HER2 staining (1+ and greater) in 47.6% of samples. All of the samples demonstrated cytoplasmic staining, and four samples demonstrated both cytoplasmic and membranous staining. Positive staining for HER2 was not correlated with metastatic disease at presentation [23].

The Children's Oncology Group analyzed samples from a clinical trial of trastuzumab in osteosarcoma. They evaluated 191 samples from 149 patients for whom there were confirmed histologic diagnosis of osteosarcoma, adequate staining, and survival information. HER2 overexpression was evaluated by immunohistochemistry and graded according to the percentage of cells staining positive: negative (no staining), 1+ (0–25%), 2+ (26–50%), 3+ (51–75%), and 4 (>75%). Positive for HER2 overexpression was defined by a grade of 3+ or 4+. According to these criteria, the investigators found that HER2 was overexpressed in 13.4% of the samples evaluated. HER2 overexpression did not correlate with survival [24].

HER2 Is a Positive Prognostic Indicator in Osteosarcoma

Adding to the controversy over the relevance of HER2 in osteosarcoma, Akatsuka et al. published a report of 81 patients with localized disease from 2 centers. They evaluated initial biopsy specimens for HER2 expression by immunohistochemistry. The samples were graded from 0 to 3+ based on the percentage of

cells staining positive: 0, negative; 1+, 1–30%; 2+, 31–75%; and 3+, 76–100%. The section with the highest degree of staining was used as representative, and overexpression was defined as tumors with 2+ or 3+ staining. They found that 63% of the tumors had overexpression of HER2. HER2 expression did not correlate with response to chemotherapy. Overexpression of HER2 was significantly correlated with event-free survival. At 5 years, the event-free survival of patients with overexpression of HER2 was 72% compared to 46% for patients without HER2 overexpression [25]. In a separate report, these authors also demonstrate that the rate of HER2 expression is lower in metachronous pulmonary metastases as compared to initial biopsy specimens [26].

Summary of HER2 Expression Studies

A summary of the results is provided in Table 5.1. A meta-analysis published in 2010 evaluated the association of HER2 overexpression with prognosis in osteosarcoma. Of the 28 evaluable reports, 23 were excluded. In the remaining five reports, the authors had difficulty with standardization of the cohorts as the reports as described above used different modalities to evaluate HER2 overexpression, different antibodies, and different criteria for the evaluation of immunohistochemistry staining. The authors conclude that HER2 positivity revealed a trend for a 1.26-fold higher risk of death, which was not statistically significant [27]. Another major confounder of the meta-analysis was the lack of standardization of the populations and the treatments across the studies.

In conclusion, interpreting HER2 expression in osteosarcoma is complicated by differences in the definition of positivity in the different studies. In most of the studies, HER2 was found to be expressed to some degree in 13%–98% of patient samples by immunohistochemistry. The HER2 gene in the majority of the studies is not amplified.

HER2 Targeted Therapies in Osteosarcoma

HER2 Directed Monoclonal Antibodies

Trastuzumab is a humanized, monoclonal antibody targeting HER2 that is FDA approved for HER2 overexpressing breast cancer as well as gastric cancer based on pivotal studies that showed improvement in outcomes for these patients [28, 29] . Other HER2-directed antibodies include pertuzumab and lapatinib. Due to prior preclinical studies showing HER2 expression in osteosarcoma along with its potential poor prognostic significance, trastuzumab was studied in a Phase 2 trial of patients with newly diagnosed metastatic osteosarcoma in combination with cytotoxic chemotherapy. Among 96 evaluable patients on study, 41 had tumors expressing HER2. All patients received the same chemotherapy backbone, and HER2-positive patients received trastuzumab concurrently with chemotherapy for 34 weeks. No difference in event-free (32% in each) or overall survival (59% in HER2 positive vs. 50% in HER2 negative) was seen between the two groups suggesting that the addition of trastuzumab to cytotoxic chemotherapy in HER2-positive patients did not provide additional clinical benefit [30]. However, trastuzumab has not been evaluated in a randomized trial in HER2-positive patients. Despite the failure of trastuzumab to improve outcomes in patients with osteosarcoma, HER2 remains as an antigen of interest, and other approaches to use this protein as a therapeutic target are being evaluated.

HER2-Specific Chimeric Antigen Receptor (CAR)-Modified T Cells

Chimeric antigen receptor-modified T cell (CAR T cell) is a form of adoptive cellular therapy that has been tremendously successful in some hematological malignancies leading to complete remission rates of greater than 80% [31]. A

Table 5.1 Studies evaluating HER2 as a potential prognostic biomarker in osteosarcoma

Study (Year)	Sample size (*n*)	HER2 assay	HER2% positive	Outcome
Studies reporting poorer survival with increased HER2 expression				
Onda (1996) [10]	26	Immunoblotting IHC Southern	Membranous: 42% 0	3-year survival HER2–84% HER2+ 14%
Gorlick (1999) [12]	53	IHC	Membranous: 42.6%	5-year EFS HER2–78% HER2+ 40%
Zhou (2003) [13]	25 primary 12 metastases 7 IHC pos 5 IHC neg	IHC FISH	Cytoplasmic: 44% Cytoplasmic: 58% 85.7% 40	HER2+ associated with worse metastasis-free survival
Fellenberg (2004) [14]	10 good response 7 poor response	RT-PCR IHC	0% 85% Cytoplasmic: 100%	Histologic response: mRNA levels 94% predictive of histologic response
Ferrari (2004) [15]	17	IHC	Primary: 32% Metastases: 53%	Recurrence-free interval: Her2–31.8 months Her2+ 17.2 months
Scotlandi (2005) [9]	84	IHC	28–32%	HER2+ associated with worse EFS
Abdou (2016) [40]	57	IHC	Cytoplasmic: 56% Membranous: 16%	HER2+ membranous staining associated with worse metastasis-free survival and EFS
Studies that did not report a correlation between HER2 expression and survival				
Maitra (2001) [16]	21	IHC FISH	0% 0%	Not reported
Kilpatrick (2001) [17]	41	IHC	Membranous: 0% Cytoplasmic: 83–98%	No association with survival outcomes
Thomas (2002) [18]	66	IHC RT-PCR	Membranous: 0% Cytoplasmic: 47% 0%	Not reported
Anninga (2004) [19]	27 27 1	RT-PCR IHC FISH	0% Membranous: 3.7% Cytoplasmic: 7.4% 0%	Not reported
Tsai (2004) [20]	22 22	IHC FISH	Focal: 18% 0%	No association with short-term survival outcomes
Somers (2005) [21]	34 34	IHC microarray CISH microarray	Membranous and cytoplasmic: 5.8% Cytoplasmic: 5.8% 0%	Not reported
Willmore-Payne (2006) [22]	47 46	FISH PCR IHC	0% 0% Membranous: 0% Cytoplasmic: 4.3%	Not reported
Bakhshi (2009) [23]	63	IHC	Cytoplasmic: 41.2% Membranous and cytoplasmic: 6.3	No difference in HER2 expression in patients with metastatic disease or high-grade disease

(continued)

Table 5.1 (continued)

Study (Year)	Sample size (*n*)	HER2 assay	HER2% positive	Outcome
Ma (2012) [41]	63	IHC	60%	HER2+ associated with the presence of metastatic disease. EFS not reported
Studies reporting improved survival with increased HER2 expression				
Akatsuka (2002) [25]	81	IHC	63%	5-year EFS HER2–46% HER2+ 72%

IHC immunohistochemistry, *RT-PCR* reverse transcription polymerase chain reaction, *FISH* fluorescent in situ hybridization, *CISH* chromogenic in situ hybridization

CD19 CAR (tisagenlecleucel) has recently been FDA approved for B-cell acute lymphoblastic leukemia. CARs directed toward antigens expressed in solid malignancies are also being developed and studied but face certain unique challenges. These include identification of an antigen that is ubiquitously expressed highly in a tumor but not in normal tissues, and immune suppressive microenvironment of many solid tumors including osteosarcoma and longevity/persistence of CAR T cells in the host, specifically in the tumors, that would be required for sufficient activity. Researchers have continued to try to improvise CAR T-cell development to overcome some of these challenges by adding co-stimulatory molecules to first-generation CARs and other combinatorial approaches. In the case of osteosarcoma, although HER2 is not expressed ubiquitously or at very high levels, HER2 CAR T cells have been studied both in preclinical and in clinical settings as it was believed that these challenges of HER2 expression could be overcome by this adoptive therapy. Indeed, in osteosarcoma cell lines, treatment with HER2 CAR T cells induced immune responses by generation of IFN-ϒ and IL-2 with killing of target cells in HER2-specific manner. In vivo, HER2 CAR T cells led to tumor regression in tumors produced by a low HER2 expressing cell line LM7 [32]. Further, coculture with HER2 CAR T cells decreased the ability of osteosarcoma cells to form sarcospheres. This was also seen in osteosarcoma cells harvested from mouse tumors that were previously treated with HER2 CAR T cells. These data suggested that HER2 CAR T cells targeted tumor-initiating cells and could potentially be of benefit in preventing metastatic spread of the disease [33].

The first attempt to treat a patient with HER2 CAR T cells was eventful leading to fatal respiratory failure in a patient with colorectal cancer within a few minutes of infusion of cells [34]. The HER2 CAR vector was a third-generation CAR containing a single-chain variable fragment (scfv) derived from trastuzumab fused to CD8 hinge and transmembrane domains followed by CD28, 4-1BB, and CDzeta signaling domains. The cells were infused following a lympho-depleting conditioning regimen. Patient developed significant respiratory distress and pulmonary infiltrates within 15 minutes of infusion and eventually succumbed. The investigators believed that this patient had a severe cytokine storm in the lung due to reactivity with low levels of HER2 expression in lung parenchyma. Since then, three clinical trials have been completed using different HER2 CAR T constructs including one in HER2 expressing sarcomas [35–37]. In this study, of the 19 enrolled patients, 16 had metastatic or recurrent osteosarcoma. This HER2 CAR T cell used a different antibody clone called FRP5 which had lower HER2 affinity than trastuzumab in a second-generation CAR design. No dose-limiting toxicities were observed in this study after cells were infused without any prior lympho-depleting therapy. HER2 CAR T cells persisted for at least 6 weeks in seven of the nine evaluable patients who received greater than 10^6 cells/m^2. Of the 16 osteosarcoma patients, 2 were not evaluable, 10 had progressive disease, 3 patients had stable disease for ≥12 weeks and subsequently underwent

tumor removal and remain in remission, and 1 patient had partial response for 9 months after second infusion. The median overall survival for all 19 patients was 10.3 months (range 5.1–29.1 months). This study concluded that HER2 CAR T-cell therapy was feasible in patients with sarcoma, cells can persist for 6 weeks or longer without significant toxicities, and there was a preliminary signal of efficacy thus providing a rationale for future studies of HER2 CAR T cells with other immunomodulatory approaches in osteosarcoma [37].

Trastuzumab Deruxtecan (DS-8201)

An alternative approach to target surface proteins on cancer cells is via antibody–drug conjugates (ADCs). ADCs comprise of an antibody to a surface protein of interest such as HER2, a linker and a payload cytotoxic agent. The goal of an ADC is to be able to deliver large doses of the cytotoxic agent specifically to the malignant cells that express the antigen without exposure to normal tissues, which would not be tolerable if administered systemically. DS-8201 is one such ADC where the humanized monoclonal HER2 antibody is linked to a topoisomerase 1 inhibitor payload called DXd via a self-immolative enzymatically cleaved linker. No specific preclinical data currently exist with DS-8201 in osteosarcoma, but in other preclinical studies with adult cancer cell lines, DS-8201 showed activity against both low and high HER2-expressing cell lines [38]. A phase 1 trial of DS-8201 was recently completed in adults with advanced breast and gastric tumors. No maximum tolerated dose was reached. The most common grade 3 events were lymphopenia, neutropenia, and anemia. Three serious adverse events were reported which included febrile neutropenia, cholangitis, and intestinal perforation. Of the 23 evaluable patients, 6 had low HER2-expressing tumors. Forty-three percent (10 of 23) of the patients had an objective response, and 91% (21/ 23) achieved disease control [39]. A phase 2 clinical trial is currently under development in adolescents with recurrent HER2-positive osteosarcoma.

HER2 is one of the many antigens that are expressed on cell surface in osteosarcoma. Others include but are not limited to disialoganglioside 2 (GD2) and B7-H3 (CD276). ADC provides a unique approach to target any or many of these. However, the success of these ADCs will depend on the specificity of the target, a linker that can easily deliver and detach the drug at its cellular target as well as the potency of the cytotoxic agent being used.

Targeting Surface Proteins

Numerous approaches are being taken to target HER2 as already described but trials targeting other surface receptors have also been conducted. As one example, the Children's Oncology Group completed a phase 2 trial in recurrent osteosarcoma of an antibody–drug conjugate, glembatumumab vedotin, which targets the surface protein GPNMB. This leads one to consider how one should think about these targeting approaches. In targeting surface proteins, there have been several approaches utilized as depicted in Fig. 5.1. A simplified way of thinking about these approaches is considering the potency of the therapeutic agents with antibody–drug conjugates having less ability to kill protein-expressing cells as compared to the CAR T cells. This indeed may be the basis of the difference in activity observed with trastuzumab versus CAR T cells. Indeed the drug conjugates achieve some of their therapeutic index by requiring proliferation for cellular cytotoxicity by the drug component of the molecule, which is typically a micro-tubule inhibitor, topoisomerase inhibitor, or DNA damaging agent. This allows some minimization of toxicity on host cells that express the protein target. As an overly broad generalization, antibody–drug conjugates have had limited toxicity, and as such the ideal surface protein target would be expressed in all or nearly all osteosarcoma samples. Certainly, if it is expressed in a subset of patients, it would be critical for the protein expression to be on the patients who do not have disease eradication with standard treatment. If the surface protein expression is highly restricted to

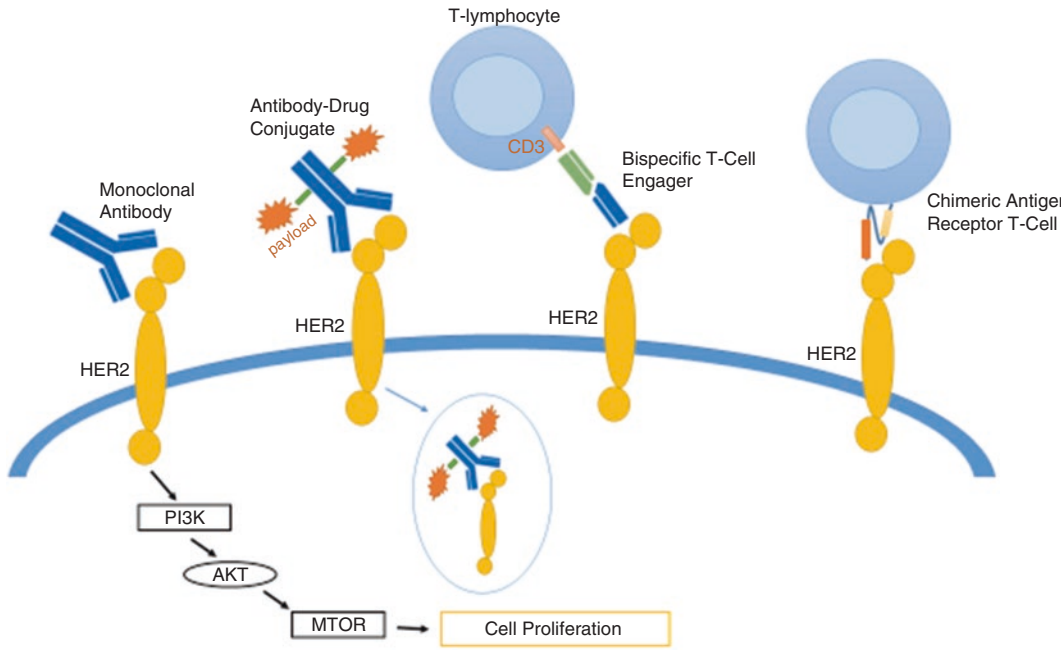

Fig. 5.1 Approaches for therapeutically targeting surface HER2 protein

osteosarcoma and not essential normal tissues, as has been the case for cancer–testis antigens in the context of other malignancies, a cellular therapy approach may be more efficacious. Regardless of the targeting approach, a key consideration in the success or failure of these approaches, as illustrated by resistance to CD19 CAR T cells, is the ability of the cancer cells to survive despite down-regulation of the surface protein. Unfortunately, none of these studies have been undertaken for HER2 or other surface protein targets in osteosarcoma. Perhaps, CRISPR screening and dependency maps, which have been created to a limited extent for osteosarcoma, may help in defining what targets may be relevant.

Conclusion

HER2 is expressed in a subset of osteosarcoma samples and continues to be explored as a potential therapeutic target. Our knowledge of both the surfaceome of osteosarcoma and how to target these proteins continues to expand at a rapid pace. We remain hopeful that these approaches will overcome the stalled progress in improving the outcomes of patients with osteosarcoma.

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Aerosolized Chemotherapy for Osteosarcoma

6

Nancy Gordon, Kumar Felix, and Najat C. Daw

Abstract

Inhalation therapy remains a suitable approach to treat lung diseases including cancer. This approach has been used to deliver various therapies including chemotherapy. The rationale for using the inhalation route vs. the systemic route has been the fewer side effects encountered when drugs are administered via inhalation. Furthermore, this approach overcomes one of the major limitations of systemic chemotherapy that results from inability of the drug to reach high concentrations in the lungs. Local delivery overcomes this limitation and spares exposure of vital organs to the drug, resulting in a more effective delivery system.

Pulmonary metastasis of osteosarcoma (OS) remains a major cause of death and is very difficult to treat. Using various OS mouse models, we demonstrated that aerosol chemotherapy causes regression of pulmonary metastases and improves survival of mice with OS. In these studies, we used gemcitabine, a nucleoside analog that is effective against various solid tumors. An initial phase I study done in Europe in patients with primary lung cancer demonstrated aerosol gemcitabine therapy to be feasible and safe. In this chapter, we describe different chemotherapeutic agents delivered by inhalation to treat lung diseases with an emphasis on an ongoing study of aerosolized gemcitabine for patients with solid tumors and lung metastases developed at the MD Anderson Cancer Center that uses a convenient approach to track patient lung health with the ultimate goal of implementing this therapy at home.

Keywords

Osteosarcoma · Inhalation therapy · Aerosol · Gemcitabine · Lung metastases

Introduction

Approximately 20% of osteosarcoma (OS) patients present with metastatic disease at the time of diagnosis, and the most common metastatic site is the lung. Pulmonary metastasis is the main cause of death in these patients [1, 2]. Inhalation therapy has been used for many years as a therapeutic approach for many lung diseases, particularly asthma. It was not until recently that this approach was used for the treatment of other

N. Gordon (✉) · K. Felix
Department of Pediatrics Research, MD Anderson Cancer Center, Houston, TX, USA
e-mail: ngordon@mdanderson.org

N. C. Daw
Department of Pediatrics – Patient Care, MD Anderson Cancer Center,
Houston, TX, USA

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diseases such as chronic obstructive pulmonary disease (COPD) and various chronic pulmonary infections such as Pneumocystis carinii pneumonia, respiratory syncytial virus infection, and cystic fibrosis [3–5]. Clinical oncologists adopted this alternative approach only three decades ago to treat tumors that affect the lungs in order to increase tumor exposure to the therapeutic agent and therefore enhance its antitumor effect [6]. There are limitations on the use of systemic chemotherapy as drugs are unable to reach high concentrations in the lung, therefore, resulting in poor clinical outcome [6, 7]. Local delivery overcomes this limitation as it offers direct delivery of high drug concentrations to the affected organ, spares exposure of vital organs, and provides a more convenient delivery system as it allows for self-administration [8]. Aerosol therapy has many potential applications. It can be used as a single therapy for lung tumors, as adjuvant therapy in conjunction with systemic chemotherapy, or for chemoprevention.

The lungs are unique organs as they have double exposure, internally through the pulmonary blood flow and externally by exposure to air flow. Circulating tumor cells are usually shed from the primary tumor, and the pulmonary bed is continuously exposed to these cells. Lung metastases can result from a variety of tumors such as breast cancer, colon cancer, prostate cancer; osteosarcoma (OS); soft tissue sarcoma; Kaposi's sarcoma; melanoma; and others. It constitutes a major cause of death. Inhalation therapy offers a unique approach to target these processes as surgical treatment depends on the number and location of lesions in the lung in addition to the patient's general status.

Advantages and Potential Limitations of Aerosolized Chemotherapy

Studies have demonstrated that drug concentration in lung tumors is low after systemic administration and this may be a reason for treatment failure [6]. Direct delivery of drug to the affected organ offers various advantages over systemic delivery. These include the local delivery of drugs to the lungs and airways with lower doses and potentially fewer side effects, the use of a noninvasive delivery system that avoids the first-pass metabolism of the drug in the liver and faster systemic absorption by the large surface area of the alveoli with the local administration of soluble drugs [8]. Furthermore, inhalation treatment offers comfort to patients as it can be potentially self-administered providing a convenient and simple approach to the treatment of primary or metastatic cancer in the lung.

In order for the aerosolized chemotherapy to be effective, it must be delivered at a sufficient concentration. The aerosol particle size is one of the most important determinants of the aerosol dose and distribution in the lungs. Aerosol particles with a mass median aerodynamic diameter (MMAD) of 5–10 um are deposited in large airways, and 1–5 um in the small airways and alveoli. The inhaled drug may reach the tumor either by direct penetration or through the blood supply [8]. A number of drugs used for systemic administration have been used as inhalation therapy to regionally treat primary or metastatic cancer in the lung [9–12]. Even though aerosolized chemotherapy was reported in 1968, oncologic use of inhalation therapy has been limited likely because of concerns about pulmonary toxicity to the patient as well as safety of administration to the individual administering the therapy and to the environment. Indeed, several chemotherapy agents such as irinotecan, gemcitabine, paclitaxel, and docetaxel can cause severe pulmonary reactions when the appropriate dose is not used [13]. Therefore, the safety and toxicities, particularly pulmonary toxicity, should be properly evaluated for any new drug considered for aerosol administration.

Preclinical Studies

Several proofs of concept studies have been performed to assess the pharmacokinetic advantages of aerosolized chemotherapy and determine the

safety and antitumor efficacy of this approach [14–18]. Using a canine model, pulmonary deposition of 14C-labeled doxorubicin administered by aerosol was compared to the intravenous administration using the same dose. Higher radioactivity was detected in the lungs when chemotherapy was delivered by aerosol, and systemic levels of the drug were very low [6]. Similarly, studies by Koshkina et al. demonstrated higher liposomal paclitaxel concentrations (measured by liquid chromatography) in the lung extracts from mice given drug by inhalation compared to intravenously. In addition, drug clearance from the lungs was slower after inhaled liposomal paclitaxel allowing for better local effect [19].

The efficacy of aerosolized chemotherapy has been tested using experimental mouse models of lung metastasis. Animals placed on a sealed plastic box were exposed to aerosolized chemotherapy delivered by a jet nebulizer. The estimated dose of aerosolized chemotherapy deposited in the lungs was calculated by defining the drug concentration in a specific aerosol volume, the volume of aerosol inspired by the animal in 1 minute, the deposition index, and the duration of treatment. Efficacy of aerosolized liposomal 9-nitocamptothecin or aerosolized gemcitabine was demonstrated using this approach [17, 18, 20]. In addition, we demonstrated the efficacy of aerosolized gemcitabine using three OS mouse models: the LM7 human xenograft mouse model, the murine DLM8 subcutaneous model, and the murine K7M3 orthotopic mouse model [14, 20]. Aerosol gemcitabine significantly inhibited the growth of primary tumors and established lung metastases in addition to preventing metastatic spread without toxicity to normal tissues. By contrast, intraperitoneal administration of a similar dose of gemcitabine inhibited the primary tumor growth but failed to affect the growth of lung metastases or prevent metastasis to the lungs [20].

Aerosol gemcitabine given to dogs with OS lung metastases proved to be well tolerated and safe and showed antitumor activity. Aerosol gemcitabine was administered twice weekly on a Monday/Wednesday or Tuesday/Thursday schedule; a total dose of 50 mg per week proved to be safe [21]. Additional studies in a nonhuman primate lung carcinoma model demonstrated aerosol gemcitabine to be feasible and safe. The safety of nine weekly inhalations of gemcitabine at a target dose of 1 mg/kg body weight in three baboons was confirmed [15].

Clinical Studies

The above preclinical studies led to the evaluation of aerosolized chemotherapy in the clinic. This treatment approach was originally tested in patients with primary lung cancer using aerosolized 5-fluorouracil. This study showed that the drug was directly incorporated and metabolized in the respiratory tract with no trace of the drug found in the serum and higher drug concentration found in the lung tumors compared to the myocardium, pancreas, and spleen. Relatively high levels were also found in the esophagus and the stomach, likely due to swallowing at the time of therapy [22]. Further, clinical evaluation of aerosolized liposomal 9-nitro-20(S)-camptothecin (L9-NC) was tested in patients with cancer that originated in or metastasized to the lung. Partial remission was demonstrated in two patients with uterine cancer and three other patients with primary lung cancer had stable disease. A potential systemic effect of aerosolized L9NC was also demonstrated in a patient who had a partial remission of a liver metastasis [23]. Based on these results, further clinical studies were developed [9, 24, 25].

The toxicity profile of aerosolized doxorubicin and liposomal encapsulated cisplatin was investigated in patients with metastatic tumors to the lungs. A maximum tolerated dose was established, and the usual systemic toxicities of these therapies were not observed [9, 25]. The safety and efficacy of inhaled lipid cisplatin was investigated in 19 patients with relapsed/progressive OS metastatic only to the lung. Inhaled lipid cisplatin was administered via nebulizer every 2 weeks. There was no hematologic toxic-

ity, ototoxicity, or nephrotoxicity, the typical toxicities seen with intravenous cisplatin. The majority of the toxicities observed were pulmonary, such as cough and dyspnea, and were transient and reversible. Pulmonary function tests showed no significant or permanent abnormality. Serum cisplatin levels after inhaled lipid cisplatin administration were significantly lower than those after intravenous cisplatin administration. Cisplatin deposition within the tumors was comparable to that in the surrounding lung tissue. Sustained benefit was limited to 3 of 8 patients who had lesions ≤2 cm, and those who underwent metastasectomy [25]. Lastly, a phase I trial of aerosolized gemcitabine in adults with non-small cell lung cancer was conducted in Europe [10]. Eleven patients were treated using a dose escalation of gemcitabine given once a week for 9 weeks. The starting dose was determined on the basis of preclinical experiments in rodents and nonhuman primates [15, 16], and doses ranged from 1 mg/kg to 4 mg/kg. On average, the total dose delivered to the patient's lung was 42 ± 16% of the dose placed in the nebulizer. There was no hematologic toxicity, nephrotoxicity, or neurotoxicity. At the 4 mg/kg dose, one patient experienced a grade 4 pulmonary toxicity (bronchospasm), which was the dose-limiting toxicity. Grade 2 and 3 toxic effects included fatigue, vomiting, dyspnea, and cough. The overall response was minor response in one patient, stable disease in four patients, and progressive disease in four patients. This study demonstrated high concentrations of the drug deposited in the lungs and low concentration in the blood, which translated to low systemic toxicity. The study did not determine the maximum tolerated dose, as there was no dose expansion at the dose level where the dose-limiting toxicity was encountered or at the dose level below that.

Pulmonary metastasis remains the major reason for treatment failure in patients with metastatic or recurrent OS with no added benefit from the multiple approaches taken within the past 30 years [1]. Based on the previously described preclinical and clinical studies, investigators at the MD Anderson Cancer Center (MDACC) have designed a phase I/II trial of aerosol gemcitabine for treatment of patients with solid tumors and lung metastases. Gemcitabine was selected for evaluation because it is effective against solid tumors and its formulation does not contain any chemical compound incompatible with aerosol delivery, it is soluble in saline, and it has not shown any local irritant effects [26]. The trial evaluates the safety and feasibility of aerosol gemcitabine administered twice weekly to patients 12–50 years of age. In addition to identifying the maximum tolerated dose and recommended phase II dose, pharmacokinetic studies are performed to evaluate for spillover of drug into the circulation. After the maximum tolerated dose is determined, the drug will be evaluated in an expansion cohort of OS patients. In addition, the study includes secondary and exploratory objectives to preliminary assess (1) the antitumor activity of aerosol gemcitabine and histologic response in tumor specimens, (2) the local effect of aerosol gemcitabine on immune infiltration in the lungs, and (3) the effect of treatment on autophagy, apoptosis, expression of heat shock protein 27 (HSP27), evidence of DNA strand breaks (γ H2AX) as a measure of drug penetration into the lungs, and expression of human equilibrative nucleoside transporter-1 (hENT1) as a measure of possible gemcitabine resistance [27]. The rationale for examining autophagy and HSP27 is based on preclinical data showing that aerosol gemcitabine induces autophagy and inhibits autophagy to either enhance or decrease sensitivity to chemotherapy [28] and a potential role of HSP27 to define whether chemotherapy-induced autophagy will lead to survival or death [29]. Gemcitabine is a nucleoside analog that once inside the cell gains activity by becoming triphosphated. The end result is DNA strand breaks which justifies the evaluation of γ H2AX. Lastly, since studies in pancreatic cancer demonstrated evidence of hENT1 expression when tumors were resistant to gemcitabine [27], expression of hENT1 will be examined in tumors from patients treated

with aerosolized gemcitabine to assess for potential therapeutic resistance.

Drug Administration

In the MDACC inhaled gemcitabine trial, sodium chloride is used to dilute study drug in the nebulizer bowl to achieve the desired volume of nebulization. A chemotherapy dispensing pin is used to draw up/measure the unit dose volume required for one treatment. This volume is added to the nebulizer bowl and mixed with the volume of sodium chloride necessary to achieve the desired volume of nebulization. A minimum volume of 3 ml is considered sufficient for optimal nebulization efficiency. A breath-actuated nebulizer (AeroEclipse® II Breath-Actuated Nebulizer) driven by a standard portable air compressor is used for aerosol treatments (Fig. 6.1).

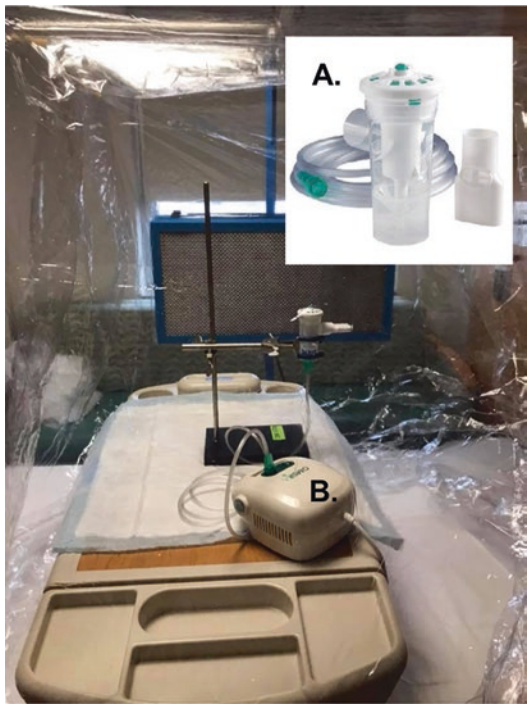

Fig. 6.1 Aerosol equipment. AeroEclipse® II Breath Actuated Nebulizer (**a**) connected to portable compressor (**b**)

This nebulizer delivers aerosolized drug on inhalation only, allowing for breaks in treatment without drug loss and minimizing environmental contamination. Time of nebulization depends on the minute tidal volume of the person inhaling and the volume of solution for nebulization, which is determined by the dose.

Safety Considerations

Aerosol therapy has safety implications to the patient, care provider, and environment. In the MDACC inhaled gemcitabine trial, baseline pulmonary function tests including forced expiratory volume in 1 second (FEV1) and forced vital capacity (FVC) are assessed before initiation of therapy. Symptoms, oximetry, and pulmonary function are monitored with every treatment by remote spirometry using the GoSpiro by Monitored Therapeutics (Dublin, OH). Patients are taught how to perform remote spirometry and record their pulse and oxygen saturation from a pulse oximeter. A minimum oxygen saturation of 93% is required to receive a treatment, and a significant decline in lung function (>10% change in FEV1) requires patient evaluation. The spirometer uploads and transmits raw numbers and flow-volume curves via Bluetooth to an Android tablet (GoHome) provided to patients and seamlessly transmits this information to a web portal and data is collected in a HIPAA-compliant web-based database (REDCap). The GoSpiro home spirometer meets the American Thoracic Society/European Respiratory Review criteria for accuracy and reproducibility.

Until the safety of aerosol therapy is established, treatments are delivered in the hospital in a negative pressure room utilizing a HEPA air filter room and a respiratory canopy tent. Patients wear disposable chemotherapy-resistant gowns and hairnets. Healthcare workers use standard personal protective equipment such as gloves, disposable chemotherapy-resistant gowns, and eye protection. The healthcare provider is required to wear an N95 respirator to enter the tent in case of an emergency. Once treatment is

completed, the area is wiped out to remove any residual aerosol drug.

Chemotherapeutic agents are known to be hazardous agents. Fear of environmental adverse events remains an issue for the healthcare personnel. Input from individuals specialized in Environmental Health and Safety and Employee Health is critical. Particle studies can help evaluate for the presence of drug on various surfaces after a patient treatment and to determine the precautions necessary to ensure the safety of drug delivery to the patient and care providers. Outpatient implementation of inhaled chemotherapy to treat tumors that affect the lung would require additional work to alleviate concerns of the healthcare personnel and to establish safety of this approach to the patient and the care provider.

Summary and Potential Implications

Aerosol delivery of agents offers a novel approach to local treatment of lung metastases, a common pattern of treatment failure in OS. Preclinical studies using aerosolized delivery of chemotherapy demonstrated the effectiveness of this approach in various tumors including OS. The feasibility and safety of aerosol gemcitabine was assessed in a phase I study of adults with lung cancer and is being studied in an ongoing phase I study in patients aged 12–50 years with solid tumors and lung metastases at MDACC. Inhaled lipid encapsulated cisplatin was well tolerated in heavily pretreated OS patients and did not appear to have the typical toxicities associated with intravenous cisplatin. Safety measures for the administration of aerosol chemotherapy include the use of personal protective equipment protection gear and a breath-actuated nebulizer for drug delivery. Particle studies to assess for the presence of the drug after treatment can aid in establishing safety to the environment. If aerosolized delivery of chemotherapy proves to be feasible and safe, it can potentially offer a convenient and more effective way to treat metastatic OS to the lungs while minimizing systemic toxicity. Further work is needed to ensure the safety of this treatment approach to the patient, the care provider and the environment, and to demonstrate its efficacy in patients.

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7 The Histone Deacetylase Inhibitor Entinostat/Syndax 275 in Osteosarcoma

Simin Kiany, Douglas Harrison, and Nancy Gordon

Abstract

The prognosis for metastatic osteosarcoma (OS) is poor and has not changed in several decades. Therapeutic paradigms that target and exploit novel molecular pathways are desperately needed. Recent preclinical data suggests that modulation of the Fas/FasL pathway may offer benefit in the treatment of refractory osteosarcoma. Fas and FasL are complimentary receptor-ligand proteins. Fas is expressed in multiple tissues, whereas FasL is restricted to privilege organs, such as the lung. Fas expression has been shown to inversely correlate with the metastatic potential of OS cells; tumor cells which express high levels of Fas have decreased metastatic potential and the ones that reach the lung undergo cell death upon interaction with constitutive FasL in the lung. Agents such as gemcitabine and the HDAC inhibitor, entinostat/Syndax 275, have been shown to upregulate Fas expression on OS cells, potentially leading to decreased OS pulmonary metastasis and improved outcome. Clinical trials are in development to evaluate this combination as a potential treatment option for patients with refractory OS.

Keywords

Osteosarcoma · Fas/FasL · Histone deacetylase inhibitors · Gemcitabine

Introduction

Metastatic osteosarcoma (OS) carries a poor prognosis and options for successful treatment and eventual cure are few. Despite dramatic progress in the 1970s and 1980s in the treatment of non-metastatic OS, the outcomes have not changed in several decades. The exact molecular mechanisms underlying drug resistance and development of metastatic disease remain unknown. Furthermore, the contribution of the organ microenvironment remains unexplored. Novel therapeutic approaches for OS lung metastasis and refractory/recurrent disease are desperately needed [1–9].

Similar to other cancer types, targeted therapy and immunotherapy are potential treatment alternatives which have yet to be fully evaluated in OS. Immunomodulatory agents have long been considered for OS as a way to enhance the immune response [2, 3]. In fact, several studies suggest that OS may be amenable to treatment

S. Kiany · N. Gordon (✉)
Department of Pediatrics Research, MD Anderson Cancer Center, Houston, TX, USA
e-mail: ngordon@mdanderson.org

D. Harrison
Department of Pediatrics – Patient Care, MD Anderson Cancer Center, Houston, TX, USA

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with immune-based therapies including immune checkpoint inhibitors [4–9]. Furthermore, there are several ongoing clinical trials which focus on the use of targeted therapies for recurrent and refractory OS. These include denosumab (anti-NFκB ligand), glembatumumab vedotin (anti-glycoprotein NMB), dinutuximab (anti-GD2), sirolimus (mTOR inhibitor), and VEGFR inhibitors (apatinib, lenvatinib, cabozantinib). In the present chapter, we provide the rationale for an alternative combination therapy using a specific histone deacetylase (HDAC) inhibitpr, entinostat/Syndax 275 in combination with the nucleoside analog, gemcitabine for the treatment of OS.

Fas and the Fas Signaling Pathway

Fas (CD95) is a cell surface death receptor that belongs to the tumor necrosis factor receptor (TNFR) superfamily. Interaction of Fas with its cognate ligand, FasL (CD95L), induces apoptosis in Fas-expressing cells. Fas is expressed on several different cell types including tumor cells, whereas FasL expression is restricted to immune cells (activated T and NK cells) and privilege organs, such as the lung [10]. The Fas/FasL signaling pathway is involved in immune homeostasis and immune and tumor surveillance.

As with all death receptors, Fas has a conserved death domain (DD) in its cytoplasmic tail that is crucial for the initiation of Fas-induced apoptosis. Fas and FasL ligation results in oligomerization and aggregation of the Fas receptor, which then leads to death-inducing signaling complex (DISC) assembly at the cellular membrane. DISC consists of Fas receptor, Fas associated with a death domain (FADD) adaptor molecule, procaspase-8, procaspase-10, and the cellular FLICE-like inhibitory protein (c-FLIP). DISC formation results in procaspase-8 activation, which later leads to cleavage of various intracellular proteins and ultimately apoptosis.

Fas Expression and Its Role in OS Lung Metastasis Formation

Fas-induced apoptosis is involved in tumor cell death and regulation of tumor development. Multiple studies have demonstrated that the absence of the Fas signaling pathway in primary tumors is associated with poor prognosis [11–14]. Tumor cells downregulate their Fas expression to escape from FasL-mediated apoptosis induced by activated immune cells [11, 14, 15]. Altered Fas expression can also affect a tumor's metastatic potential [16, 17].

OS most commonly metastasizes to the lungs. Metastases to the lungs are often resistant to salvage chemotherapy [18]. Our laboratory has previously demonstrated an inverse correlation between the metastatic potential of human OS cells with Fas expression [19]. The LM7 cell, a subline of the SAOS human OS cell line obtained by recycling the cells seven times through the lungs of nude mice, expresses low levels of Fas [20], whereas the SAOS cells express high levels of Fas. SAOS cells cannot induce pulmonary metastasis when injected intravenously (i.v.), whereas LM7 cells form metastasis in the lung when injected i.v [21]. Similarly, K7 mouse OS cells, which express high levels of Fas, are not metastatic whereas K7M3 cells, derived from K7 after recycling the cells through the lungs, express low levels of Fas and form lung metastases when injected i.v. In addition, K7M3 cells form primary tumors in the bone if injected into the tibia and metastasize to the lung spontaneously. The primary bone tumor that develops in the tibia homogeneously expresses Fas, while lung metastases have low to no Fas expression [22]. Because FasL is constitutively expressed in the lung, we hypothesized that when OS cells express a functional Fas receptor, they will undergo cell death due to Fas/FasL-mediated apoptosis as they approach the lung microenvironment. On the other hand, Fas^- OS cells will survive and form lung metastasis. We also showed that LM7 cells transfected with the full-length Fas gene

expressed a higher level of Fas and formed significantly fewer and smaller pulmonary nodules compared to control-transfected LM7 cells [21]. Conversely, blocking the Fas signaling pathway in K7M3 and K7 mouse OS cells by transfection with Fas-associated death domain (FADD) dominant-negative (FDN) plasmid resulted in lower sensitivity to FasL-mediated apoptosis in vitro and enhanced metastatic potential to the lungs. Lung nodules from mice injected with the FADD_DN-transfected cells contained both Fas-positive and Fas-negative cells [15, 22]. These results support our hypothesis that Fas expression influences OS cells metastatic potential. A functional and intact Fas/FasL signaling pathway is key to the development of OS lung metastases. We further confirm these findings by injecting wild-type K7M3 and K7 cells into an FasL-deficient gld mice and found an increase in the number of lung tumors with both Fas-positive and Fas-negative cells [15, 22] suggesting that in the absence of FasL in the pulmonary epithelium, Fas^+ tumor cells can survive and grow in the lungs. Subsequent analysis of patient samples supported our pre-clinical findings. Immunohistochemistry staining for Fas expression of 38 OS lung metastatic patient samples revealed 60% of the samples to be Fas negative, 32% to be weakly positive, and 3.2% (only one sample) to be strongly positive. Fas-positive expression was only detected in patients who had received chemotherapy prior to lung metastasis resection suggesting that treatment may contribute to Fas upregulation in OS tumors. Indeed, we further demonstrated that gemcitabine [23], interleukin -12 [24], entinostat/syndax275 [25], and 9-Nitrocamptothecin [26] upregulated Fas expression on OS cells which then resulted in the regression of established lung metastases.

Taken together, our findings address the importance of the Fas/FasL signaling pathway in the metastatic potential of OS and suggest that therapies able to upregulate Fas expression may add benefit in the treatment of OS lung metastases.

Gemcitabine and Its Effect on Osteosarcoma

Gemcitabine (2′,2′-difluorodeoxycytidine, dFdC) is a chemotherapeutic agent that has been approved for the treatment of various solid tumors including non-small-cell lung carcinoma, pancreatic, breast, and ovarian cancers. Gemcitabine is a deoxycytidine analog and its antitumor activity is the result of its ability to inhibit DNA replication and ultimately lead to cell death [27]. It has been tested in multiple pre-clinical and clinical settings [28–33], including OS [34–45]. Gemcitabine in combination with docetaxel remains a standard well-tolerated salvage chemotherapy regimen in the treatment of multiple sarcomas. However, it has only shown modest efficacy in relapsed/refractory OS [46–50]. Ofer Merimsky and colleagues reported gemcitabine treatment prolongs disease stabilization in 70% of patients with bone sarcomas resistant to doxorubicin [35]. A phase II clinical trial of the combination of gemcitabine and sirolimus demonstrated promising results in patients with relapsed and progressing OS [43]. Other gemcitabine combinations have not been as successful, however. Specifically, the addition of gemcitabine to carboplatin, for example, did not show benefit as compared to carboplatin alone in dogs with OS [37].

Based on our preliminary findings in the laboratory that the Fas-FasL pathway is implicated in the metastatic potential of OS, we hypothesized that agents that upregulate Fas expression could provide therapeutic benefit as the presence of FasL in the lung microenvironment will lead to cell death. Indeed, we demonstrated [23–26] in vitro that gemcitabine upregulated Fas expression in various OS cell lines and enhanced cell sensitivity to FasL in the lung. Inhibition of the Fas/FasL signaling pathway abolished the gemcitabine therapeutic effect, suggesting that an intact Fas pathway is important to the therapeutic efficacy of gemcitabine [22]. Other groups have similarly reported that gemcitabine induced growth inhibition, cell cycle arrest, and apoptosis

in canine OS cell lines [36, 38]. Consistent with our findings, in vitro culture with relatively low concentrations of gemcitabine significantly increased functional Fas receptor expression in lung, colon, breast, and pancreatic tumor cell lines [51, 52].

Using two OS mouse models (K7M3 and LM7), we demonstrated aerosol gemcitabine to have therapeutic effect. Gemcitabine therapy resulted in significant increase in Fas expression, enhanced apoptosis, and subsequent regression of lung metastases. Aerosol gemcitabine further inhibited the growth of a subcutaneous OS primary tumor [22, 53]. We also confirmed in vivo the importance of the Fas/FasL pathway in the therapeutic efficacy of gemcitabine as aerosol gemcitabine therapy given to gld mice whose FasL function is impaired, resulted in increased Fas expression in OS lung metastasis but no therapeutic effect [22]. Similarly, dogs with OS lung metastasis treated with aerosol gemcitabine demonstrated increased Fas expression, apoptosis, and percentage of tumor necrosis [54]. Takashi Ando and colleagues have also demonstrated that systemic administration of gemcitabine results in a decrease in primary tumor growth, increased cell apoptosis, and decreased pulmonary metastasis in an OS mouse model [38]. Taken together, these results provide a rationale for the use of gemcitabine in combination with other agents shown to upregulate Fas expression to further enhance gemcitabine therapeutic effect against OS.

Histone Deacetylase (HDAC) Inhibitors

Epigenetic modifications, such as DNA methylation and acetylation, induce chromatin remodeling and altered gene expression. Defects in epigenetic regulation may result in loss or gain of gene function and lead to onset and progression of human diseases including cancer [55].

Histone acetyltransferases (HATs) and histone deacetylases (HDACs) are responsible for histone modifications. HAT stimulates gene transcription through the transferring of acetyl moieties to histone's N-terminal lysine residues, which results in a less compact chromatin state. The opposing activity of the HDAC enzymes contributes to transcriptional repression by removing the acetyl moieties, creating a more compact chromatin leading to less gene expression. 18 HDACs have been identified in humans and are classified into four groups. Class I contains HDAC 1, 2, 3, and 8; Class II contains HDAC 4, 5, 6, 7, 9, and 10; Class III contains sirtuins and Class IV contains HDAC 11. Studies suggest that aberrant function of HAT and HDAC is often linked to tumorigenesis and poor prognosis in cancer [56]. Therefore, targeting these two enzymatic activities may provide therapeutic means to treat several malignancies associated with faulty epigenetic modifications [57, 58].

Several HDAC inhibitors have been shown to have anti-cancer effects. HDAC inhibitors regulate gene transcription by limiting the accessibility of transcription factors and RNA polymerase activities at the promoter level. HDAC inhibitors belong to four structural classes: (I) hydroxamic acids (hydroxamates); (II) benzamides; (III) short-chain fatty (aliphatic) acids; (IV) cyclic tetrapeptides; and (V) sirtuin inhibitors. In recent years, several HDAC inhibitors, with various target specificities and pharmacokinetics, have been under evaluation in clinical and preclinical studies. Thus far, four have received FDA approval for cancer treatment: vorinostat (SAHA), Belinostat (PXD-101), panobinostat (LBH589), and Istodax (romidepsin) [59, 60].

HDAC inhibitors have demonstrated a broad range of effects on tumor cells including cell death, growth arrest, and cell cycle suppression. In the clinical setting, tumor debulking and differentiation, prevention of angiogenesis, and enhancement of host immune response have been attributed to HDAC inhibitors [58]. Studies demonstrated that HDAC inhibitors are selectively more cytotoxic to cancer cells than normal cells, suggesting a potential therapeutic benefit of these drugs for the treatment of cancer [61, 62]. It has been shown that class I HDACs (1, 2, 3 and 8) play a key role in the pathogenesis of OS [63, 64]. Entinostat/syndax-275, a member of the benzamide group, is a narrow-spectrum HDAC inhibitor and affect HDAC class I with limited

effect on HDAC 8 [65]. Entinostat/syndax-275 is in several phase I/II clinical trials for the treatment of solid and hematologic malignancies.

Entinostat/Syndax-275 and Its Effect on Osteosarcoma

It is well known that HDAC inhibitors can inhibit human and canine OS cell growth by promoting apoptosis, mostly through Fas-mediated or caspase-dependent pathways. For example, treatment with valproic acid prior to incubation with doxorubicin resulted in less cell growth and more apoptosis both in canine and human OS cells. In addition, valproic acid and doxorubicin combination therapy in a canine OS subcutaneous xenograft model led to significantly less tumor growth compared to either alone [66]. Further combination of two epigenetic modifying drugs, the DNA methylation inhibitor, Zebularine, and the HDAC inhibitor suberoylanilide hydroxamic acid (SAHA) showed significant human and canine OS cell growth inhibition. Inhibition was more effective in cell lines with a more aggressive gene expression profile [67]. Similarly, co-treatment with a DNA methyltransferase inhibitor, 5-Aza-dC, and HDAC inhibitor trichostatin A effectively reduced cell proliferation of the multidrug resistance OS cell line HosDXR150, whereas single treatment had only a minor effect on cell viability [27]. Lastly, SAHA in combination with cisplatin decreased cell proliferation and enhanced OS cell apoptosis via caspase activation [68, 69].

HDAC Effect on the Fas/FasL Apoptotic Pathway

HDAC inhibitors can sensitize tumor cells to Fas-mediated apoptosis using different mechanisms. For example, apicidin and depsipeptide (FR901228) increased apoptosis in acute promyelocytic leukemia cells and uveal melanoma by inducing upregulation of Fas/FasL expression [70–72]. Another study demonstrated the HDAC inhibitor PCI-24781 to induce apoptosis in acute leukemia cells through activation of caspase-8 and FADD [73]. In OS cells, FR901228 inhibited cell growth both in vitro and in xenograft mouse models. FR901228 upregulated FasL mRNA and cell surface expression, activated caspase-8 and -3 and ultimately induced Fas-mediated apoptosis [74].

We also have demonstrated that therapeutically achievable doses of entinostat/syndax-275 while having limited cytotoxic effect on OS cell growth in vitro activate the Fas pathway and enhance Fas mRNA and protein expression. Combination treatment entinostat/syndax-275 and FasL significantly increased OS cells' sensitivity to FasL as demonstrated by enhanced caspase cleavage/activity and reduced clonogenic growth. Blocking the Fas pathway reversed this effect [25, 75]. Intranasal administration of entinostat/syndax-275 at a dose of 0.13 mg/kg (which is approximately 200-fold less than the therapeutically effective oral dose described before) in mice with established OS lung metastasis resulted in reduced metastatic tumor growth [13]. In addition, oral administration of entinostat/syndax-275 in mice with OS pulmonary metastasis resulted in tumor growth inhibition and increased survival rate. Histopathological examination showed a higher level of apoptosis and lower level of cellular FLICE inhibitory protein (c-FLIP) expression in the lung tissues of treated mice. No evidence of drug toxicity was observed in the treated group of mice [75].

Despite sufficient evidence to demonstrate that entinostat/syndax-275 activates the Fas pathway in OS, studies in our lab demonstrated that this HDAC inhibitor did not increase the expression of Fas on the cell surface. Instead, entinostat/syndax-275 treatment led to redistribution of Fas to membrane lipid rafts and downregulation of cellular c-FLIPmRNA and protein expression. c-FLIP knockdown in OS cells resulted in the redistribution of Fas to lipid rafts and enhanced sensitivity to FasL-induced cell death [75, 76]. Our findings were consistent with other studies demonstrating that the HDAC inhibitor FR901228 downregulated c-FLIP in both chronic lymphocytic leukemia cells and Fas-resistant OS cells and enhanced their sensitivity to Fas-mediated apoptosis [77, 78]. Entinostat/syndax-275 has also been shown to downregulate

c-FLIP in chronic lymphocytic leukemia (CLL) cells and induce caspase-dependent apoptosis [79]. Similarly, 7 days of treatment with valproic acid sensitized OS cells to Fas-mediated cell death without enhancing Fas expression on the cell surface [80].

c-FLIP is a key regulator of Fas-mediated apoptosis. c-FLIP, a catalytically inactive caspase-8/-10 homolog, interferes with activation of procaspase-8 at the death-inducing signaling complex (DISC) level and prevents Fas-induced apoptosis [81]. Many studies showed that c-FLIP was overexpressed in various cancer cells and its expression is liked with tumorigenesis and poor survival [75, 82–84], highlighting a potential mechanism by which cancer cells resist to death receptor-induced apoptosis. The expression of c-FLIP has also been correlated with resistance to several chemotherapy drugs [70, 85]. We also evaluated c-FLIP expression in patient primary and pulmonary OS samples using immunohistochemistry. C-FLIP expression was significantly higher in pulmonary nodules than in primary tumors. Similar results were observed in our human xenograft models [76]. Taken together, these findings suggest that the overexpression of c-FLIP as an inhibitor of the Fas-signaling pathway may contribute to the survival and growth of OS cells in a FasL+ lung microenvironment. Therefore, the downregulation of c-FLIP in entinostat/syndax-275-induced Fas signaling may be therapeutically beneficial for the treatment of OS lung metastasis.

Gemcitabine and Entinostat/Syndax-275 as Potential Salvage Regimen for Osteosarcoma Lung Metastasis

The above preclinical data suggests that the use of therapeutic agents able to upregulate Fas expression, increase Fas localization to lipid rafts, or decrease cFLIP expression may offer benefit in the treatment of OS. The combination of gemcitabine and entinostat/syndax-275 – both of which have been shown to enhance Fas expression in OS cells – has not been studied in pediatric patients with refractory or recurrent pulmonary OS. Therefore exploitation of the Fas/FasL pathway as a potential therapeutic option for patients with refractory OS seems appropriate. A phase I/II clinical trial of the combination is under development at MD Anderson Cancer Center. This clinical trial will evaluate feasibility and safety of the combination therapy entinostat/syndax-275 and gemcitabine and determine whether there is potential utility for patients with refractory/relapsed OS. To this end, the primary objective of the study is to determine the maximum tolerated dose (MTD) of entinostat/syndax-275 when it is given in combination with gemcitabine in pediatric patients with recurrent sarcoma and recommend a phase 2 dose of the combination therapy. Secondary objectives include: 1) To determine the disease control rate at 4 months for pediatric patients with recurrent unresectable pulmonary OS when treated with gemcitabine in combination with entinostat/syndax-275 and 2) To estimate the disease-free survival for the subset of pediatric patients with recurrent pulmonary OS that has been fully resected after treatment with gemcitabine in combination with entinostat/syndax-275. It is expected that this trial will serve as a potential therapeutic alternative for patients with refractory OS. Several approaches have been taken to treat OS. However, none have shown significant benefit as there has been no impact in survival. It is of paramount importance that therapies that move into clinical trials have a scientific rationale. Here we present enough preclinical evidence to support combination therapy gemcitabine and entinostat/syndax275 for refractory OS. Therefore, results from this study holds promise as an alternative to treat patients with OS.

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8 Relapsed Osteosarcoma Trial Concepts to Match the Complexity of the Disease

Damon R. Reed, Pooja Hingorani, and Peter M. Anderson

Abstract

Osteosarcoma relapses not only herald a very poor prognosis but also opportunities to treat this genetically diverse complex cancer in new ways. This review will attempt to show that the field is a rapidly evolving one in which not only cytotoxic agents but also local control strategies and the immune system can be harnessed to improve the prognosis of relapsed patients. The molecular heterogeneity and the difficulty of effectively treating most common patterns of relapse with surgery and/or radiation (lung and/or bone metastases) have been responsible for a wide variety of approaches to learning whether agents are active against osteosarcoma. This chapter will highlight past, current, and potential future approaches to provide more effective systemic therapy for the problem of recurrent metastases of osteosarcoma. These include single-agent trials with a wide variety of agents, radiopharmaceuticals, and immune therapies. Finally, how such efforts are integrated into more effective local control strategies is also discussed.

Keywords

Adjuvant chemotherapy · Tyrosine kinase inhibitors · Radiopharmaceuticals · Bone-specific therapies · Cryoablation · Stereotactic body radiotherapy (SBRT) · Abscopal response · Immune therapy · Antibodies · Immune modulators · Cell therapy · CAR-T-cells

D. R. Reed (✉)
Cancer Biology and Evolution Program, Adolescent and Young Adult Oncology Program, Department of Interdisciplinary Cancer Management and Sarcoma Department, H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL, USA
e-mail: damon.reed@moffitt.org

P. Hingorani
Department of Pediatrics, U.T. MD Anderson Cancer Center, Houston, TX, USA
e-mail: phingorani@mdanderson.org

P. M. Anderson
Cleveland Clinic, Pediatric and Taussig Cancer Institutes, Department Pediatric Hematology/Oncology and BMT, Cleveland, OH, USA

Case Comprehensive Cancer Center, Cleveland, OH, USA
e-mail: andersp@ccf.org

Relapsed Osteosarcoma

Because of the significant resources required to conduct a study and the hundreds of patients needed to answer a question in the newly diagnosed osteosarcoma patient population, most clinical trials are conducted to find an efficacy signal in relapsed patients [1]. Relapsed osteosar-

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coma remains challenging to treat, and patients with relapsed disease have poor overall survival of less than 20% at 5 years. The main predictors of survival after osteosarcoma recurrence include the time to first recurrence, disease burden, and ability to achieve complete surgical remission (CR) after recurrence [2, 3]. Solitary pulmonary nodule and greater than 24 months to the first recurrence are favorable prognostic factors. The Cooperative Osteosarcoma Study Group (COSS) data on patients with first osteosarcoma relapse and those with second and subsequent relapses suggest that the median time to the first relapse is 18 months from the time of original diagnosis. Other studies have suggested this time interval to be 15 months from the original diagnosis. The median time to second relapse from the first relapse is around 8 months, and all subsequent relapses are 6 months. Five-year overall survival rates for patients with the first relapse who are able to obtain a second surgical remission were reported at 39% as compared to 32% for patients who are able to achieve a third surgical remission in the COSS data. Data from the Rizzoli Institute reported 5-year event-free actuarial survival of 38% after first metastasectomy and 32% after second metastasectomy suggesting that patients who achieve a complete resection after second relapse have the same probability of surviving as compared to patients who achieve a complete resection after first relapse [4]. While rare survivors of unresectable disease were reported in this series together, these data point to the fact that the most important factor for survival after pulmonary relapse is the ability to achieve a complete surgical resection.

Past Relapsed Trials and a Proposed Efficacy Bars

An analysis of several prior Children's Oncology Group (COG) phase 2 trials that included patients with recurrent OS showed that patients with unresectable or measurable disease had a 4-month event-free survival (EFS) of 12% (CI 6–19%), while patients with complete resected disease had a 12-month PFS of 20% (CI 10–34%) [5]. This data helped to determine a baseline for outcomes for the design of future trials in patients with relapsed OS as objective response is uncommon in this disease and is, therefore, not a good measure of efficacy of a novel agent. Thus, recent trial designs through COG have focused on two distinct populations of relapsed patients: those with resectable disease and those with unresectable disease. This strategy is also being used on a more global scale with investigators recognizing that RECIST response is not an adequate marker for response in OS.

Using the above historical controls of EFS as a comparator, COG has conducted four clinical trials in the recurrent OS since 2012 (Table 8.1). Two of these trials, AOST1322 and AOST1521, were conducted only in patients with measurable disease, while AOST1421 was conducted only in patients with completely resected pulmonary disease. AOST1321 was unique in having both the above cohorts, which were analyzed separately. While all four agents failed to meet the set efficacy bars for consideration to be studied in a larger Phase 3 trial, several important lessons were learned. These study designs required small numbers of patients (19–39) to evaluate the first efficacy signal. Accrual rate was significantly greater than anticipated based on historical data for these national osteosarcoma-specific trials highlighting an unmet need for relapsed patients [6]. As a result, resources required to conduct these studies were limited and ideal in a resource-constraint environment. In addition, the majority of these trials had novel correlative biology objectives, which will potentially help identify new biomarkers in OS.

Another class of agents that has been studied extensively in OS by investigators outside of COG includes multi-tyrosine kinase inhibitors (TKIs) such as sorafenib, regorafenib, cabozantinib, lenvatinib, and apatinib. While all of these TKIs have a varying profile of targets, most of them met their individual study's efficacy bar of improving progression-free survival (PFS) in OS patients (Table 8.1). Seemingly inhibition of angiogenesis pathways seems to play some role in the observed activity with all members inhibiting VEGF having some activity and saracatinib

Table 8.1 Recently completed trials

Drug trial number/name mechanism/ target	Primary endpoint	Progression-free survival (PFS)	Objective response rate (ORR)
Measurable disease			
Eribulin [8] NCT02097238/AOST1322 Microtubule inhibitor	4-month PFS in >/= 5/19 patients AND >/= 2/19 RECIST response	mPFS 38 days; 0% 4-month PFS	0%
Glembatumumab [9] NCT02487979/AOST1521 Antibody drug conjugate against glycoprotein non-metastatic B protein	4-month PFS in >/= 5/19 patients AND >/= 2/19 RECIST response	4-month PFS 3/19 patients	1/19 patients PR
Denosumab NCT02470091/AOST1321 RANK ligand antibody	4-month PFS in >/= 5/19 patients	4-month PFS 1/15 patients	0%
Sorafenib [10] NCT00889057 VEGFR, PDGFR, Raf	4-month PFS	4mo PFS 46%	ORR 8%
Lenvatinib [11] NCT02432274 VEGFR (1-3), FGFR (1-4), PDGFRα, KIT, RET	4-month PFS	4-month PFS 33% mPFS 3.4 mth	ORR 8%
Regorafenib [12] NCT02389244/REGOBONE VEGFR, TIE2, KIT, RET, Raf, BRAF, PDGFR, FGFR	PFS	mPFS 16.4 weeks; 12 week PFS 62% 24 week PFS 35%	ORR 8% (2 PR)
Regorafenib [13] NCT02048371/SARC024	PFS	mPFS 3.6 months	ORR 14%
Cabozantinib [14] NCT02243605 VEGFR-2, MET, AXL	6-month PFS; 6-month ORR	mPFS 6.2 months;	ORR 12%;
Apatinib [15] NCT02711007 VEGFR2	4-month PFS; ORR at 3 months	mPFS 4.5 months	ORR 43%
Completely resected disease			
Denosumab NCT02470091/AOST1321 RANK ligand antibody	>/= 2/19 RECIST response 12-month DCS of >/= 15/39 patients	Results pending	
Dinutuximab + GM-CSF NCT02484443/AOST1421 Anti-GD2 antibody	12-month DCS of >/= 15/39 patients	Results pending	
Saracatinib SARC12 NCT00752206	12-month DCS	Results pending	

notably not having an activity (personal communication), albeit studied in the resected population [7]. Taken together, these data are intriguing and worthy of further study in a definitive Phase 3 trial in OS. However, it remains a challenge to know which if any of the targets for TKIs are the most important to inhibit biologically, and this remains to be further determined.

Having discussed recently completed trials in the relapsed population, we turn to currently open clinical trials as well as discussions of optimizing clinical trial participation through effective communication to patients and families with recurrent osteosarcoma along with maximizing quality of life through supportive care.

Current Landscape of Clinical Trials in OS

Table 8.2 lists many varieties of clinical trials currently open for osteosarcoma. The majority of these are early phase trials (Phase 1 or 2) with OS cohorts included in them and have varying eligibility criteria as well as efficacy endpoints. While data from these trials will be immensely helpful, a more concerted and unifying approach is needed internationally to design OS-specific trials to truly have an impact on improving survival.

Clinical Trial Participation

While participation in an available clinical trial is the preferred strategy in most instances with relapsed or progressive disease, several factors need to be taken into consideration before enrolling a patient on to a clinical trial as participation in a trial requires significant commitment of time and resources both from the patient/family and the treating institution. Some features worthy of discussion before making an informed decision to participate in a clinical trial include ensuring that participants understand that participation in

Table 8.2 Open clinical trials for relapsed osteosarcoma

Name/agent(s)	Mechanism of action/other information	NCT Identification#
Energy Therapies		
153-Sm-DOTA + RT	Bone-seeking beta-emitter +radiotherapy	NCT03612466
CLR131 (131-iodine)	tumor selective 131-phospholipid ether	NCT03478462
SBRT for oligo-metastases	Stereotactic body radiotherapy	NCT02880319
MRI-guided HIFUS	heat with high-intensity focused ultrasound	NCT02076906
Cytotoxics and/or targeted agents or combinations		
Simvastatin +Topo + CPM	Statin + topoisomerase inhibitor + alkalytor	NCT02390843
Copanlisib	PI3K inhibitor	NCT03458728
Losartan + sunitinib	Angiotensin receptor blocker+ TKI (antiVEGF)	NCT03900793
Nab-paclitaxel + Gemcitabine	More dose dense than gemcitabine+ docetaxel	NCT02945800
Hydroxychloroquine +G/D	inhibit autophagy to reduce G/Dresistance	NCT03598595
Pazopanib + Topotecan	VEGF inhib+ topoisomerase inh	NCT02357810
MM0398+Cyclophosphamide	liposomal irinotecan + alkalytor	NCT02013336
Pediatric MATCH	COG APEC1621SC	NCT03155620
Cabazanitib	TKI (like pazopanib) COG ADVL1622	NCT02867592
Decitabine + gemcitabine	hypomethylation of DNA + gemcitabine	NCT02959164
Antibodies or immune stimulating agents		
Natalizumab	Macrophage-tumor interaction/ICAM	NCT03811886
Avelumab	Anti-PD1 (checkpoint inhibitor)	NCT03006848
Pepinemab (VX15/2503)	AntiSema4D COGADVL1614	NCT03320330
Nivolumab + Nab-rapamycin	Anti-PD1 + mTOR inhibition	NCT03190174
Mifamurtide +EI or M-API	Macrophage activator + standard chemo	NCT03643133
Nivolimab +/− azacytidine	Anti-PD1 +/− histone hypomethylation	NCT03628209
Anti-GD2 x Anti-CD3	Bispecific MAB (increase tumor=T-cell)	NCT03860207
Nivolumab +/− ipilimumab	Dual checkpoint inh. (COGADVL1412)	NCT02304458
Cellular therapies		
EGFR806 CAR-T	Cellular immune therapy with markers	NCT03618381
GD2 CAR-VSV-CTL	Cellular therapy against GD2	NCT01953900
T-cell+anti-CD3+GD2	Bi-specific MAB on T-cells+ IL-2 + GM-CSF	NCT02163093
Donor NK + Haplo BMT	Flu+CPM+3Gy TBI, then HSCT, d+7NK	NCT01200891
Biology Studies		
BOOST	Osteosarcoma Registry and Biobank	NCT03225872

trials is voluntary. They should prepare for success (the trial works to reduce disease) or failure (some or all metastases do not respond) by reviewing the main goals of the trial, i.e., safety, dose finding, or efficacy. Another way to make certain that a decision is informed is to have indications, risks, and alternatives reviewed by another physician or second opinion, especially when local sarcoma expertise is lacking. Sometimes virtual visits can provide a reasonably efficient and effective means of providing a second expert opinion for the patient in terms of prognosis and all potential options applicable to a specific case when the local caregiver may not be fully aware of all trial options [16].

If possible and if in the patient's best interest, some local control measures can be done before clinical trial participation in order to have the best chance of an adequate period of observation on clinical trial therapy to determine the efficacy of the treatment being investigated. This may involve unilateral thoracotomy with the removal of metastases on the contralateral side if the trial is not effective especially if oligometastatic disease and years of interval from the last therapy. Another strategy is to biopsy and cryoablate painful lesions or bone (non-measurable) lesions before trial participation. A third strategy is to use stereotactic body radiation therapy (SBRT) for oligometastatic disease and, if the clinical scenario is such that all cannot be treated, leave 1–3 "indicator" lesions to facilitate clinical trial participation.

For the unfortunate situations involving too numerous to count (TNTC) osteosarcoma lung and/or bone metastases, it is important to involve palliative care specialists and have advance directives in place in case of performance and clinical deterioration before starting any additional therapy or a clinical trial with little hope of being successful in the long run. What is best for a particular patient may involve discussion of lifestyle priorities, various options near their home, prior therapy (what worked and did not and for how long), what is needed to stay healthy, and required clinical trial observations. Resources such as lifextraordinary.org website can help families in a study share their story, organize their own care team, and obtain additional financial resources through crowdfunding. This can be critical to reducing anxiety, sustaining prolonged effort, and avoiding "battle fatigue."

Next Steps: Trial Designs and Efforts Toward Improving Outcomes

As discussed, available clinical trials would be prioritized over off-label therapies in almost every relapsed osteosarcoma setting. A recent review in bone sarcomas found general clinical practice across several centers to be rather uniform and identified clear areas of unmet need [17]. To maximize enrollment and to facilitate correlative studies, ideally trials should be designed to match common clinical scenarios. While the objective of trials is to improve survival, this has not been convincingly achieved with recent front line trials [18–20]. Because the biology and underlying vulnerabilities of osteosarcoma have yet to be characterized, trials should facilitate correlative biology and at a minimum attempt to collect relapse tumor specimens to better understand the biology of osteosarcoma. A short interval of neoadjuvant therapy toward a potential resection can be considered in trial design to both evaluate the effect of therapy in terms of clinical response and to enhance an understanding of the effect of therapy on the tumor through correlative science on the resected specimens. Any resected osteosarcoma samples, especially when primary tissue also exists, should be handled in a way that maximizes the potential biologic utility of samples once the diagnostic material has served its purpose for optimal clinical care. This includes not subjecting materials to acid decalcification and when possible freezing tissue is close to the time of resection as possible. Figure 8.1a outlines commonly explored clinical trial scenarios and ongoing biology work in osteosarcoma. Using recently published trial data in OS, we can estimate accrual to be about 50 patients per year in the completely resected population and about 80 patients per year in the unresectable group [6].

Given the above, what are the current roadblocks and best ideas to overcome them in the osteosarcoma field regarding clinical trials?

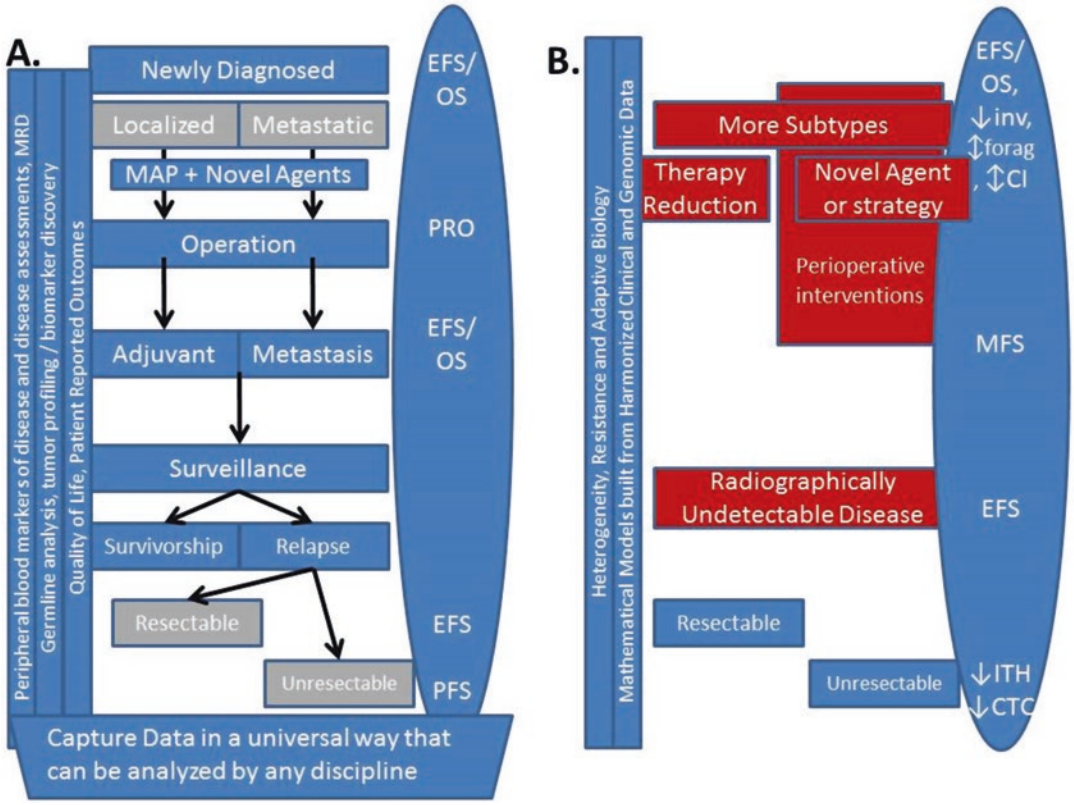

Fig. 8.1 Osteosarcoma clinical scenarios and correlate work in current and potential future trials. (**a**) Current clinical scenarios for trials are the gray boxes in newly diagnosed and relapsed populations typically investigating the addition of an agent to MAP or testing an agent in an unselected relapse population against a historical endpoint. (**b**) New potential directions for trials deign in red along with novel endpoints. Vertical boxes in both A and B highlight ongoing biology efforts highlighted from working groups and being collected on active trials. Ongoing liquid biopsy work may provide an opportunity to better define a complete response, and ongoing aggregation of clinical and biologic information may allow for subtyping of osteosarcoma beyond localized and metastatic. With improved detection and measuring of the MRD state, both novel scenarios and endpoints can be envisioned in future osteosarcoma work. (Abbreviations: ITH intra-tumor heterogeneity, inv invasiveness, forag foraging, CI chromosome instability, MFS metastasis-free survival, CTC circulating tumor cells, PRO patient-reported outcomes)

Several groups have assembled to tackle this question. This has included bringing together members of the basic science, pathology, veterinary, clinical, translational, murine modelers, radiation oncologists, surgeons, and advocates through various venues. Some of these working groups have reported their findings and conclusions. A combined QuadW, Curesearch Foundation, and COG sponsored meeting concluded that paucity of relapsed tumor biology, lack of prognostic markers, and lack of predictive model systems were the key translational knowledge gaps. The group furthermore proposed circulating tumor DNA studies, determining germline genetic abnormalities in osteosarcoma patients and creating patient-derived xenograft models using metastatic and relapsed tumor specimens as the ways to close these gaps [21]. An ongoing European sarcoma networking meeting reported the importance of AYA enrollments in 2011, summarized the 2015 workshop, and conducted a timely meeting in May 2019. The 2015 report highlighted the promising fields of genomics,

drug resistance and pharmacogenomics, translational efforts, and immunotherapy [22]. With the myriad of stakeholders present at this meeting, there was a better understanding of how basic science insights could impact future trials and how trials can best improve tissue sample access for scientific discovery as an example of how trials could be more innovatively designed. In addition, there is increasing recognition between the North American and European investigators that there is an urgent need for data harmonization across all groups to be better able to collaborate on and compare clinical trial outcomes across studies which is a big limitation currently.

While the optimism exudes from these meetings with hopes for a near-term discovery to be translated into positive clinical trials, continuing to better understand the underlying biology of the disease is ultimately needed to design and conduct more effective trials. Toward this effort, several recent publications have emerged on investigating copy number change as predictive to response of targeted agents [23], enhancer regions pliancy contributing to metastatic disease [24], TP53 mutation type being important in metastatic potential [25], and single-cell sequencing that can capture genetic changes, even that from chemotherapy, over time in osteosarcoma [26]. While groups have published sequencing results in osteosarcoma, the largest effort, TARGET, remains in the analytic stage with data available to researchers but lacking a comprehensive manuscript [27]. In addition, the Children's Oncology Group's Osteosarcoma Biology Group, an international group of over 50 researchers that share unpublished data through monthly webinars, devised provocative questions that could help focus research toward questions that would be transformative if answered. These questions included disease ontology including inherited predisposition and osteosarcoma initiation events that lead to the tremendous structural variations that characterize the disease. The underlying biology of established tumors through epigenetic states of osteosarcoma, mechanisms of metastasis, and immune evasion was also highlighted. Finally, characterizing the best predictive models of the disease and optimizing clinical trial designs were highlighted in the final seven provocative questions [28]. Furthermore, there is a general hope that subtyping of osteosarcoma, either through genetic characteristics or phenotypic characteristics, may be helpful in future trials.

Clinical trial design is another important consideration in OS to ensure the efficacy endpoints are relevant to this disease. Due to limited patient numbers with relapsed or progressive disease, only the most compelling novel therapeutic agents can be studied at any given time. Therefore, it is important to consider how to best answer the objective within the context of specific clinical trial design. Importantly, while the importance of metastasis biology has been emphasized for years in osteosarcoma, it remains an aspiration to design a trial with metastasis prevention as an endpoint. This is due to this endpoint being difficult to measure in an unselected osteosarcoma population. The preclinical criteria emphasized to prioritize agents through a past working group included the target being identified in micrometastases, activity in murine tail vein metastasis models, thresholds of metastasis-free survival in canines when given as monotherapy (8-month delay) or with chemotherapy (24 months) and a defined human dose and schedule in addition to activity in comparative oncology models like canines [29].

As specific agents and pathways are discussed at length elsewhere in this book, we focus on conceptual future trial considerations. In addition to potential novel clinical scenarios to conduct trials outlined in Fig. 8.1b, we discuss how advances in technology and understanding may impact future osteosarcoma trials. In Table 8.3, we capture some current thoughts and potential future directions depending on the answers to questions like these: Is the MAP backbone permanent? When should chemotherapy be timed around surgery? How to test agents that only target early metastasis? What will advances in MRD mean for trials? Should immune therapy be incorporated? How to test ideas preclinically and how much dependence on results in models? Which models?

Table 8.3 Possible future directions and impacts on osteosarcoma trials

	Current state	Path forward	Impact on trials
Standard of care	Off-label use common, trial enrollment preferred	Off-label use captured, trials and real-world data inform next trial	Decentralization of ideas for trials. Increased ability of individuals and advocates to test ideas.
Data	Silos of data, much unusable in EHR	International collaboration to harmonize important data elements in a trial as well as outcome measures	Decentralization of background data for trials Allows seamless collaboration on future clinical trials in both patient accruals and outcome comparisons
MAP	Rigidly applied in with little regard to toxicity and risk(s)	Timing, number of cycles may vary between patients depending on the response, agents matched to other therapies or MAP +additional therapies. Some patient with surgery only	More variety in approaches and need to collect information to compare.
Surgery timing	Week 11	Varies with standardized handling and collection of samples	Correlates and biology studies can impact design
Trial designs	Clear bars for efficacy and working toward phase 3 to improve cure rates in the newly diagnosed population	Adopt more nimble trial designs that require fewer patients and resources and allow for changes during a trial based on real-time data; think beyond safety and efficacy, engage basic scientists early in trial design to incorporate relevant biological correlates; include quality of life measures	Allows for more efficient processes such as rapid start, fewer interruptions, addition, or deletion of different trial arms as needed ultimately leading to more data with less resources; learn from even negative trials
Tumor biology	Imperfect understanding of the initiation and targetable drivers of osteosarcoma	Identifying the high impact gaps in tumor biology knowledge; collaborate to share ideas and resources between scientists early in the process	Foster rapid discovery of novel biomarkers and targets with clinical relevance and avoid duplicative efforts
Metastasis biology	They are already there, MAP	Osteosarcoma is dynamic and therapy around the time of surgery may be particularly effective	Interventions and endpoints to detect activity for agents that are not cytotoxic
MRD Threshold	CT scan, 3–5 mm in lung. Higher thresholds by MRI, plain films or bone scan	ctDNA, miRNA, or other peripheral fluid-based technology with improved sensitivity and specificity	More decision points and more possible time points for intervention. Complicates intervention, conduct, and power
Immune therapy	Aspirational	Has a role in selected patients	How to combine immune approaches with surgery, radiation, and chemotherapy rationally?
Models	Available and investigated	A standard suite of well-characterized and freely available models known to predict clinical trial outcomes	Preclinical and comparative studies designed in the context of the planned trial. Correlative biology conducted preclinically focuses on the trial design and interpretation of both positive and negative results.
Stakeholders	Active voice and provide resources and direction. Multiple groups working in parallel with early collaborative efforts.	Break down academic, industry, and nonprofit silos to work as a large team together for the development of new agents for clinical use; foster public-private partnership; involvement of patient advocacy early to in the process of drug development	Rapid bench to bedside translation if academia and industry work together from an early stage; focused drug development for pediatric cancers; better drug availability

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9 Using Liquid Biopsy in the Treatment of Patient with OS

David S. Shulman and Brian D. Crompton

Abstract

Liquid biopsies encompass a number of new technologies designed to derive tumor data through the minimally invasive sampling of an accessible body fluid. These technologies remain early in their clinical development, and applications for patients with osteosarcoma are actively under investigation. In this chapter, we outline the current state of liquid biopsy technologies as they apply to cancer generally and osteosarcoma specifically, focusing on assays that detect and profile circulating tumor DNA (ctDNA), microRNAs (miRNA), and circulating tumor cells (CTCs). At present, ctDNA assays are the most mature, with multiple assays demonstrating the feasibility of detecting and quantifying ctDNA from blood samples of patients with osteosarcoma. Initial studies show that ctDNA can be detected in the majority of patients with osteosarcoma and that the detection and level of ctDNA correlates with a worse prognosis. Profiling of ctDNA can also identify specific somatic events that may have prognostic relevance, such as 8q gain in osteosarcoma. miRNAs are stable RNAs that regulate gene expression and are known to be dysregulated in cancer, and patterns of miRNA expression have been evaluated in multiple studies of patients with osteosarcoma. While studies have identified differential expression of many miRNAs in osteosarcomas compared to healthy controls, a consensus set of prognostic miRNAs has yet to be definitively validated. Recent studies have also demonstrated the feasibility of capturing CTCs in patients with osteosarcoma. The development of assays that quantify and profile CTCs for use as prognostic biomarkers or tools for biologic discovery is still in development. However, CTC technology holds incredible promise given the potential to perform multi-omic approaches in single cancer cells to understand osteosarcoma heterogeneity and tumor evolution. The next step required to move liquid biopsy technologies closer to helping patients will be wide-scale collection of patient samples from large prospective studies.

Keywords

Liquid biopsy · Circulating tumor DNA · MicroRNA · Circulating tumor cells · Prognosis · Diagnosis · Biomarker · Surveillance · Tumor evolution · Sarcoma · Osteosarcoma

D. S. Shulman · B. D. Crompton (✉)
Pediatric Hematology/Oncology, Dana-Farber/Boston Children's Cancer and Blood Disorders Center, Boston, MA, USA
e-mail: BrianD_Crompton@DFCI.HARVARD.EDU

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The Emerging Field of Liquid Biopsy in Cancer

"Liquid biopsies" hold incredible promise to transform the way we treat patients with osteosarcoma. Liquid biopsy describes an array of assays designed to extract tumor information from body fluid, including peripheral blood, cerebral spinal fluid, urine, or effusions, that may be more easily accessible than tissue from a surgical biopsy. These technologies provide a noninvasive opportunity to study cancer biology and derive clinically useful information at numerous times during a patient's treatment for cancer. Specific advancements include a more comprehensive understanding of disease biology, a means of risk stratification, a method to measure treatment response, a tool for early identification of relapses, and a means to identify mechanisms of treatment resistance. While numerous studies now show that tumor material can be detected in the blood of patients with cancer, tumor-derived nucleotides, tumor cells, and cell fragments remain a very small fraction of the components of the blood, even in cancer patients with a high burden of disease. Therefore, the major challenge to adapting liquid biopsy technologies to each cancer type is the identification of disease hallmarks that distinguish cancer material from the patient's normal blood components. As this field of cancer biology rapidly grows, studies describing approaches for the identification of circulating tumor material in patients with osteosarcoma are just beginning to emerge. In this chapter, we aim to provide an overview of the current state of liquid biopsy across oncology, within pediatric oncology, and the nascent work that has been done to develop liquid biopsy assays for patients with osteosarcoma. Finally, we will discuss future directions for these assays and how they may ultimately improve outcomes for patients with osteosarcoma.

The first descriptions of freely circulating DNA in the peripheral blood came about in 1948 [1]. The first description of circulating DNA in patients with cancer occurred in the 1970s and 1980s [2, 3]. Since that time, assays to detect and characterize circulating tumor (ctDNA) have improved enormously through advancements in PCR and next-generation sequencing, which have become the most prevalent means of performing liquid biopsies [4–6]. Although there is a growing literature evaluating liquid biopsy in cancer, relatively few studies have demonstrated clinical utility or validity of these assays [5]. To date, two ctDNA assays have gained FDA approval for use in adult cancers [7, 8]. Nevertheless, an increasing number of studies have demonstrated early evidence for the use of ctDNA for disease diagnosis, prognostication, measurement of residual disease, identification of genomic alterations for targeted therapy, and exploration of disease biology.

More recently, numerous alternative methods of ascertaining information about a tumor through liquid biopsy have been developed including analysis of circulating RNA (primarily microRNA (miRNA)), circulating tumor cells (CTCs), extracellular vesicles (EVs), exosomes, tumor educated platelets (TEPs), proteins, and metabolites [9]. These areas of exploration all harbor an opportunity to advance the care we provide for patients with osteosarcoma in different ways. For the purposes of this chapter, we will focus on liquid biopsy strategies which have been applied in some way to osteosarcoma, which include the detection and profiling of ctDNA, CTCs, and miRNA.

Liquid Biopsy Technologies and Their Adaptation to Osteosarcoma

While ctDNA has been evaluated in many diseases as a type of liquid biopsy, conventional methods of analysis relied upon detection of recurrent hotspot mutations in genes such as *KRAS* and *EGFR*, which are common in carcinomas, but rare in sarcomas [7, 10–13]. Instead, many sarcomas harbor genomes with characteristic translocations or copy-number changes. Sarcomas require approaches to ctDNA detection and quantification that are tailored to the recurrent genomic aberrations found in these diseases as well as the particular clinical context in which

the assay will be used. Similarly, approaches to detection of CTCs require that such assays leverage characteristic features of the sarcoma tumor cell. Evaluation of miRNAs may be performed using similar techniques to those described in other cancers, but profiling results must be analyzed to identify the specific miRNAs secreted by osteosarcoma tumors. Here we describe ways in which knowledge of osteosarcoma biology may be leveraged to harness ctDNA, CTC, and miRNA assays as a means of liquid biopsy for patients with osteosarcoma.

ctDNA

The detection of ctDNA relies on the identification of somatic variants that distinguish tumor DNA from germline DNA. Several studies have shown that pediatric solid tumors harbor few recurrent single-nucleotide variants, diminishing the value of hotspot focused ctDNA assays for these diseases [14–16]. In osteosarcoma, one group demonstrated that next-generation targeted sequencing of a panel of genes designed to detect a combination of recurrent single-nucleotide variants (SNVs) and focal structural variants was able to identify ctDNA in six of eight cases of osteosarcoma [17]. However, genomic studies of the most common pediatric solid tumors would suggest that a reasonably sized panel of genes targeting only SNVs would be able to detect ctDNA in only a subset of patients [18–28].

Pediatric solid tumors are typically characterized by structural variants, including recurrent translocations and frequent copy-number alterations [14]. For tumors characterized by recurrent copy-number changes, such as osteosarcoma, whole-genome or whole-exome sequencing can be utilized to detect and quantify ctDNA [6]. Recent studies have utilized ultralow-pass whole-genome sequencing (ULP-WGS), with genome coverage as low as 0.1-1x, to identify ctDNA in diseases with genomes characterized by widespread structural events by employing computer algorithms such as the iChorCNA to use segmental and chromosomal alterations to estimate the ctDNA content of a sample [29]. This approach lies in contrast to next-generation sequencing strategies utilized for detection of ctDNA in translocation positive sarcomas, where intronic regions that typically host recurrent rearrangements are enriched for deep sequencing [30].

In osteosarcoma, landscape sequencing studies have shown that these tumors host few recurrent SNVs, have one of the most complex genomes in cancer, and frequently contain aneuploidy and chromothripsis [20, 27]. Unlike other types of pediatric solid tumors, copy-number and translocation events appear to be nearly stochastic, increasing the challenge of bringing a low-cost sequencing technology to the identification of ctDNA in the blood. In recent work, ULP-WGS was used to effectively detect ctDNA in patients with localized, metastatic, and recurrent osteosarcoma [30]. While this approach has limitations in terms of sensitivity for ctDNA, this technique is well adapted to the osteosarcoma genome. As ctDNA assays become more adaptive, it may be possible that unique CNAs and rearrangements harbored within each individual's tumor may provide an opportunity to develop patient-specific ctDNA assays. However, such an approach has yet to be described for patients with osteosarcoma.

Another unique hallmark of the cancer genome is the methylation pattern of DNA. Numerous studies have demonstrated that different cancer types harbor unique methylation patterns that can distinguish each cancer from normal tissues and other cancer types. Recent studies have demonstrated the feasibility of utilizing methylation profiling to categorize small round blue cell tumors, such as Ewing sarcoma, osteosarcoma, desmoplastic small round cell sarcoma, and synovial sarcoma [31]. Similar methylation profiling has been applied to sequencing methylomes in cell-free DNA demonstrating a similar ability to detect ctDNA and differentiate cancer types based on methylation profiles [32, 33]. While this has been accomplished for osteosarcoma using tissue sequencing, it has not been performed using ctDNA [31]. This may ultimately prove to be a sensitive means of early detection in patients at risk of sarcomas, improving diagnosis in situations in which diagnostic

tissue is not attainable and improving disease surveillance.

Circulating Tumor Cells

Circulating tumor cells (CTCs) are intact tumor cells found in the bloodstream as single cells or clusters and have been postulated to exist since it was first understood that tumors could metastasize to other locations in the body. The term CTC generally refers to tumor cells derived from solid tumors that do not otherwise circulate in the blood, as opposed to malignancies of the blood. CTCs may be viable or apoptotic at the time of analysis, with viable CTCs likely representing tumor cells with the potential to form metastases [34]. CTCs are typically isolated through positive detection using markers on the surface of the tumors cells or physical cell characteristics such as size, electrical charge, density or deformability or through negative detection by removing noncancerous cells from a blood sample. Traditionally, analysis of CTCs focused simply on detection and enumeration of CTCs; however, advances in single-cell analysis have opened the door to a wide range of studies, including single-cell sequencing, epigenome analysis, and protein profiling [35, 36]. More recent studies have demonstrated the utility of a combination approach using CTC enrichment and RNA sequencing [37].

CTCs can now be reliably detected in patients with carcinomas using endothelial surface markers, primarily EpCAM [38]. Similar attempts to identify sarcoma cells have utilized surface markers, such as CD99 in Ewing sarcoma using flow cytometry [39, 40]. Vimentin has been shown to be a more ubiquitously expressed surface marker on sarcoma cells; [41] however, both vimentin and CD99 lack specificity with baseline expression of both markers on other circulating non-tumor cells. GD2 is another potential surface marker for isolating osteosarcoma CTCs [42, 43]; however, further work must be done to define solid tumor- and osteosarcoma-specific surface markers for CTC isolation. More recent attempts have utilized size selection for detection and isolation of CTCs in sarcomas and successfully isolated CTCs from patients with osteosarcoma [44].

miRNA

MicroRNAs are small (approximately 22 nucleotides in length) double-stranded RNAs that are thought to regulate gene transcription at the cellular level [45]. MicroRNAs were first characterized in the 2000s and, due to their relative stability, can be found ubiquitously in a variety of bodily fluids [46, 47]. These small double-stranded RNA sequences are produced in normal cells, and their expression is thought to be dysregulated in the cancer cell with the potential to act as oncogenic regulators of gene expression. miRNAs can be analyzed using RNA sequencing (RNA-Seq), quantitative PCR (qPCR), or microarrays and are being evaluated for a range of applications including early detection of cancer, diagnosis of cancer, and prognostication. While there is now a large literature evaluating these RNA profiles, little is known about the role in cellular regulation and the packaging of these molecules in the cytoplasm and extracellular space. It is believed that they are typically transported in extracellular vesicles, apoptotic bodies, high-density lipoprotein structures, and complexes with Argonaute proteins [47, 48]. As a biomarker, miRNAs are typically analyzed as a miRNA profile, consisting of a number of specific miRNAs, and the relative frequencies of each miRNA are analyzed as profiles relative to normal controls. These profiles may be developed through unbiased genome-wide profiling of miRNAs or by preselecting miRNAs for evaluation. These profiles may be used for early detection, diagnosis, or prognostication. Given that miRNAs have a role in regulating transcription, they may also eventually inform our understanding of disease biology.

The most extensive clinical studies of miRNA have evaluated miRNA profiles in patients at high risk of lung cancer. Two large studies have demonstrated that miRNA profiling may be able to augment low-dose CT screening in identifying

high-risk patients in need of a biopsy [49, 50]. miRNAs have been characterized in patients with osteosarcoma [51], and a number of studies have attempted to identify miRNA profiles associated with high-risk disease; however at this time, these studies have shown contradictory results, and larger studies with test and validation cohorts are needed.

Liquid Biopsy Applications in Cancer and Osteosarcoma

Early Detection and Diagnosis

The ability to detect genetic hallmarks of cancer in liquid biopsies has engendered optimism that these technologies may be used to augment traditional biopsies for cancer diagnosis. Such diagnostic liquid biopsies may be particularly beneficial in instances where viable tissue is difficult to obtain or the quality or quantity of a biopsy is not sufficient to arrive at a definitive diagnosis. For patients at an elevated risk of developing a malignancy, liquid biopsies may be a way to augment cancer screening regimens designed to detect cancer early, when tumors are expected to be more amenable to treatment.

Multiple lines of evidence suggest that ctDNA may be detectable at the time of diagnosis and prior to diagnosis in patients with osteosarcoma. While no published studies have detected prediagnostic ctDNA in patients who are later proven to have osteosarcoma, a previous study of a cohort of 72 patients with localized osteosarcoma with available banked plasma demonstrated that ctDNA was detectable using an ultralow passage whole-genome sequencing assay in 57% of newly diagnosed patients without prior knowledge of the tumor genome [52]. New means of collecting and isolating ctDNA and enhanced analytic algorithms are expected to increase the sensitivity of such assays for detection of osteosarcoma ctDNA.

While the majority of cases of osteosarcoma are thought to be sporadic, cancer predisposition syndromes, including Li–Fraumeni syndrome, and environmental exposures, such as prior treatment with radiation or chemotherapy, are known to increase the risk of developing osteosarcoma [53]. Although there are no published studies of liquid biopsies detecting occult osteosarcoma, there now exist multiple case reports of ctDNA being detected in women with no known existing tumor undergoing cell-free DNA prenatal testing, who were subsequently found to have cancer [54, 55]. One recent study has shown that using patient-specific NGS panels, ctDNA can be detected in patients with osteosarcoma and no radiologic detectable disease, speaking to the potential sensitivity of this assay in osteosarcoma [17]. These studies suggest that ctDNA assays may be adapted for early detection in cancer patients with an increased risk of developing malignancies, including osteosarcoma. Efforts are underway to improve the sensitivity of ctDNA assays which would be expected to improve the utility of these tests for early-cancer detection.

While the initial studies of liquid biopsies in osteosarcoma have focused on identifying patterns of DNA mutations, new studies in cancer now demonstrate that somatic methylation changes can be utilized to discriminate tumor DNA from germline DNA. This approach has the added benefit of being able to predict the type of tumor present in the patient when ctDNA can be detected by methylation patterns [32, 33]. CTCs may also provide diagnostic information, but studies to demonstrate the feasibility of such an approach remain aspirational. Multiple studies have also suggested that miRNA may be useful in discriminating the presence of osteosarcoma in patients compared to healthy controls [56, 57]; however, no specific miRNAs have shown promise across multiple studies, and it remains to be seen whether these biomarkers will be useful for diagnosing osteosarcoma.

Improving Risk Stratification of Newly Diagnosed Patients

Risk stratification of patients at the time of diagnosis remains an ongoing challenge in the clinical care of patients with osteosarcoma. The only existing strong prognostic factors for poor

outcomes for patients with high-grade disease remain the presence of metastatic disease and having an axial primary tumor [58, 59]. Further, for some patients, the presence of metastatic disease may be ambiguous if there are small pulmonary nodules of unclear significance. Conceptually, liquid biopsy may correlate with disease burden, or be associated with the presence of micrometastatic disease, and may provide an excellent biomarker for identification of high-risk patients, especially in instances where the presence of metastatic disease is not clear. Prognostication using liquid biopsy can be achieved through multiple approaches, including quantification of ctDNA or CTCs, or identification of high-risk genomic features such as Myc overexpression, or high-risk miRNA profiles.

Multiple studies have now demonstrated correlations between ctDNA quantification and stage and tumor size, although primarily in adult carcinomas [60–62]. Not surprisingly, early studies subsequently showed that ctDNA detection was associated with poor outcome. In one early study of patients with colorectal cancer, patients with detectable ctDNA had a 2-year overall survival of 48% compared to 100% for those without detectable ctDNA [63]. While there has not yet been a study attempting to correlate tumor size with ctDNA levels in osteosarcoma, we have demonstrated that binary ctDNA detection and increasing ctDNA levels are associated with event-free survival and overall survival in patients with localized osteosarcoma [52]. Although no ctDNA studies have shown that genomic features identified in ctDNA were associated with poor outcome, multiple genomic features in osteosarcoma identified in tumor tissue have been demonstrated to correlate with a poor outcome [26]. 8q gain was readily detectable in 74% of patients with detectable ctDNA in patients with localized osteosarcoma.

MicroRNAs have also been evaluated as potential prognostic markers in patients with osteosarcoma. A number of miRNAs have been evaluated, including miR-21, miR-106a miR-199a-3p, miR-143, miR-221, and miR-34b, however with varying results, sometimes upregulated and sometimes downregulated [64–70]. While these miRNAs seem to be detectable in the peripheral blood of patients with osteosarcoma, further work is needed to elucidate which miRNAs are consistently dysregulated and hold the potential for useful diagnostic and prognostic biomarkers.

Monitoring Response to Therapy and Detecting Relapse

Utilization of liquid biopsy for disease monitoring and surveillance holds particular promise in osteosarcoma given the challenges of using traditional imaging to gauge response to treatment [71] and the significant radiation exposure from CT scans during surveillance [72]. Further, markers of minimal residual disease (MRD), which have profoundly impacted the treatment of hematologic malignancies, are lacking in solid tumors.

All three analytes mentioned in this chapter hold the potential to improve disease monitoring and surveillance in osteosarcoma. To use ctDNA for disease monitoring, assays capable of quantifying ctDNA must be utilized. The iChor algorithm which discriminates ctDNA from genomic cfDNA by identifying copy-number variations is validated down to 3% ctDNA [29]. While it is not known whether this exceeds the threshold of radiologic detection for patients with osteosarcoma, it is likely that more sensitive assays will be required for ctDNA to be useful for disease monitoring and MRD detection. Nevertheless, case reports have demonstrated that ctDNA levels change following the treatment of osteosarcoma [30, 73]. To increase sensitivity of these assays, a number of strategies could be employed, including using patient-specific panels of copy-number changes or SNVs either using NGS or PCR-based assays [74]. Conversely, machine-learning techniques that differentiate tumor DNA from germline DNA are gaining increasing use and would likely prove useful for patients with osteosarcoma given the degree of copy-number changes seen in the osteosarcoma genome.

Similarly, CTCs and miRNA may prove useful for monitoring patients with osteosarcoma. Both CTC levels and miRNA levels are known to

change over time in patients with osteosarcoma [44, 67, 68]. However, the threshold for detection of these analytes even with the current technology is not well understood.

Understanding Tumor Heterogeneity and Evolution

Osteosarcoma harbors an extremely complex genome with yet unanswered questions about driving genomic alterations that may be further elucidated through deep sequencing of serial liquid biopsy samples [23, 25, 26, 75, 76]. Understanding spatial heterogeneity in osteosarcoma, like many solid tumors, has been hampered by sampling error of conventional biopsies, especially in patients with metastatic disease. Temporal heterogeneity, or how the osteosarcoma genome changes over time, has also remained elusive given that serial tumor biopsies are not routinely performed in adolescents and young adults. Sequencing of ctDNA samples will allow for exploration of temporal and spatial tumor heterogeneity. For example, these approaches have allowed for ctDNA-based identification of genomic copy-number changes specific to metastatic disease that were not present in primary tumor samples in patients with metastatic breast cancer [77]. Similarly, CTCs may prove to be an additional key analyte to explore tumor heterogeneity using a variety of single-cell genomic approaches.

While studies of osteosarcoma tumor biology using liquid biopsy are lacking, multiple studies of neuroblastoma have begun to demonstrate the promise of liquid biopsy for elucidating tumor genomic heterogeneity. Two studies utilizing a combination of whole-exome sequencing and targeted panel sequencing of plasma samples from patients with neuroblastoma demonstrated that (1) ctDNA provides an avenue to identify somatic mutations or copy-number changes associated with metastatic disease potential that may be missed when sequencing the primary tumor and (2) that sub-clonal events seen early in the disease course may become clonal events following treatment [78, 79]. Such an approach provides compelling evidence that deep sequencing of serial ctDNA samples from patients with osteosarcoma may deepen our understanding of spatial and temporal tumor heterogeneity and facilitate identification of driving events and markers of resistance to chemotherapy. As an increasing number of samples from patients with osteosarcoma are collected for ctDNA analysis, these questions are prime for exploration in the coming years.

The Path to Clinical Implementation

To date, liquid biopsy has not entered the clinical care of patients with osteosarcoma. Yet, as we have attempted to outline, liquid biopsy holds great promise for improving the care we provide to patients with osteosarcoma. We believe these assays may inform care across the clinical spectrum including early detection, diagnosis, risk stratification, on-therapy monitoring, detection of relapse, and detection of markers of resistance and sensitivity to therapy. A path to clinical implementation for liquid biopsy assays was outlined in a 2017 joint statement from the American Society of Clinical Oncology and College of American Pathologists [5]. Based on these recommendations, assays must demonstrate (1) analytic validity, or the ability to detect a targeted variant with accuracy, reproducibility, and reliability; (2) clinical validity, meaning the ability of the assay to divide a clinical group into multiple cohorts with significantly different outcomes; and finally (3) clinical utility, which means that knowledge gained from the assay can be used to significantly improve clinical care and outcomes. To date, only a handful of assays have gained regulatory approval in Europe and the United states for selection of patients for targeted therapies [7, 11, 12, 80–82]. No assays have gained regulatory approval for use in children. However, there are assays that are being utilized by clinicians for patient care, even without regulatory approval for a specific pediatric indications, most notably, the Guardant360 assay. At this time, the analytic validity, clinical validity, and clinical

utility of miRNA and CTC assays remain under investigation.

While there are a number of assays that have been developed for analysis of ctDNA, CTCs, and miRNAs in patients with osteosarcoma, for these to move toward the clinic, large prospective studies are needed that are sufficiently powered to demonstrate clinical validity. These studies could take the form of biology studies, in which patients receiving standard of care provide blood samples at prespecified time during their care. Furthermore, the inclusion of liquid biopsy studies on new clinical trials enrolling patients with osteosarcoma should be considered whenever possible. Given the rarity of osteosarcoma, these studies must necessarily be multicenter and require close collaboration. These studies can serve the basis for demonstrating clinical validity and then inform future therapeutic trials designed to improve outcomes.

Summary

In this chapter, we've attempted to outline the current state of liquid biopsy in oncology, pediatric oncology, and what has been done to bring liquid biopsy to patients with osteosarcoma. While ctDNA was described decades ago, and miRNAs and CTCs have been well established in other diseases, the study of liquid biopsy in osteosarcoma remains relatively new. Nevertheless, given early successes of liquid biopsy in diseases such as non-small cell lung cancer, as well as preliminary studies in osteosarcoma, we believe that these technologies may ultimately improve the care we provide to patients with osteosarcoma.

The evidence to date suggests that ctDNA, CTCs, and miRNAs are all ripe for analysis in patients with both localized and metastatic osteosarcoma at diagnosis and throughout treatment. The most mature clinical studies demonstrate that ctDNA may be a prognostic biomarker for patients with localized osteosarcoma. This is now being evaluated in a large multicenter study. Further studies of CTCs and miRNA in larger clinical studies will be key to determine how best these assays can inform clinical care.

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Part III

Novel Immunotherapeutic Approaches

10 Genetically Modified T-Cell Therapy for Osteosarcoma: Into the Roaring 2020s

Christopher DeRenzo and Stephen Gottschalk

Abstract

T-cell immunotherapy may offer an approach to improve outcomes for patients with osteosarcoma who fail current therapies. In addition, it has the potential to reduce treatment-related complications for all patients. Generating tumor-specific T cells with conventional antigen-presenting cells ex vivo is time-consuming and often results in T-cell products with a low frequency of tumor-specific T cells. Furthermore, the generated T cells remain sensitive to the immunosuppressive tumor microenvironment. Genetic modification of T cells is one strategy to overcome these limitations. For example, T cells can be genetically modified to render them antigen specific, resistant to inhibitory factors, or increase their ability to home to tumor sites. Most genetic modification strategies have only been evaluated in preclinical models; however, early clinical phase trials are in progress. In this chapter, we will review the current status of gene-modified T-cell therapy with special focus on osteosarcoma, highlighting potential antigenic targets, preclinical and clinical studies, and strategies to improve current T-cell therapy approaches.

Keywords

Pediatric cancer · Osteosarcoma · Cancer immunotherapy · T-cell therapy · Gene therapy · Chimeric antigen receptor · Tumor antigens

Introduction

Adoptive T-cell therapy refers to the isolation of allogeneic or autologous T cells, followed by ex vivo manipulation, and subsequent infusion into patients for therapeutic gain [162]. Channeling the cytotoxic killing and specific targeting ability of T cells through adoptive transfer has the potential to improve outcomes for patients with osteosarcoma. An early example of adoptive T-cell therapy for osteosarcoma was reported by Sutherland and colleagues [178]. A 14-year-old girl who had the same human leukocyte antigen (HLA) type as her mother received unmanipulated maternal lymphocytes. Lymphocytes isolated from the patient post-infusion killed osteosarcoma cells in vitro, but the patient had only a minimal clinical response prior to disease progression. Since Sutherland's report, significant advances in immunotherapeutic techniques have taken place.

C. DeRenzo (✉) · S. Gottschalk
St. Jude Children's Research Hospital, Department of Bone Marrow Transplantation and Cellular Therapy, Memphis, TN, USA
e-mail: Chris.DeRenzo@stjude.org; Stephen.Gottschalk@stjude.org

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Cell therapy with conventional T cells has shown promise in several clinical settings [16, 82, 162]. Examples include donor lymphocyte infusions (DLI) after stem cell transplantation to treat CML relapse [93], infusion of Epstein-Barr virus (EBV)-specific T lymphocytes to treat EBV-related lymphomas and nasopharyngeal carcinoma [10–12, 34, 110, 174], infusion of tumor-infiltrating lymphocytes (TILs) to treat melanoma [45, 124, 162], and the infusion of virus-specific T cells to prevent and treat viral-associated disease in immunocompromised patients [65, 87, 98, 101, 185].

Since the ex vivo generation of T cells specific for tumor-associated antigens (TAAs) is often cumbersome, investigators have developed genetic modification strategies to render T cells TAA specific [19, 44, 83, 167, 193]. For example, infusion of T cells genetically modified with chimeric antigen receptors (CAR) specific for CD19 (CD19-CAR) has shown remarkable success resulting in FDA approval of two CD19-CAR T-cell products [54, 58, 119, 120, 139, 144, 145]. While CAR T-cell therapy shows promise for some patients with solid tumors [2, 6, 62, 63, 111, 137, 150, 195, 207], responses have been considerably less impressive compared to CD19-CARs. Besides rendering T cells tumor-specific, genetic modifications enable the generation of T cells with enhanced effector functions (Table 10.1). While these approaches have been mainly evaluated in preclinical models, some are already being actively explored in the clinic. In this chapter, we will review the current status of gene-modified T-cell therapy for patients with osteosarcoma, highlighting potential antigenic targets, preclinical and clinical studies, and strategies to improve T-cell therapeutic approaches.

Table 10.1 Genetic modifications for T-cell therapy for osteosarcoma

Goal	Introduced gene class	Example
Antigen-specificity	Receptors	αβ TCR, CAR, BiTE
T-cell expansion	Costim molecules	CD40L, CD80, 41BBL
	Domains of costim molecules	CD27, CD28, 41BB, OX40, ICOS, MyD88/CD40, DAP12
	Cytokines	IL12, IL15, IL18
Resistance to inhibitory tumor environment	Costim molecules	CD40L, CD80, 41BBL
	Domains of costim molecules	CD27, CD28, 41BB, OX40
	Cytokines	IL7, IL12, IL15, IL18
	Dominant negative receptors	DN TGFβ receptor
	Chimeric cytokine receptors	IL4/IL2, IL4/IL7, TGFβ/41BBL
	Constitutive active cytokine receptors	C7R
	shRNAs, TALENs, CRISPR/Cas9	FAS, PD-1, CTLA-4, TIM-3
	Constitutive activated kinases	AKT
Improve T-cell homing to tumor sites	Chemokine receptors	CCR2b, CCR4, CXCR1, CXCR2
Safety	Inducible suicide genes	HSV-tk; caspase 9
	Cell surface markers	CD20, tEGFR

BiTE Bispecific T-cell engager, *DN* dominant negative, *HSV-tk* Herpes simplex virus thymidine kinase, *IL* interleukin, *TGFβ* transforming growth factor β, *tEGFR* truncated epidermal growth factor receptor

T-Cell Therapy Targets for Osteosarcoma

Developing successful antigen-specific T-cell therapy depends on the availability of specific TAA. Once a TAA is identified, TAA-specific T cells can be either generated using conventional antigen-presenting cells or by gene transfer to recognize and induce killing of TAA-positive osteosarcoma.

TAA are potential candidates for immunotherapy, including T-cell therapy, if they are (1)

expressed at higher than normal levels on tumor cells compared to nonmalignant host cells; (2) are normally only expressed during fetal development or at immunoprivileged sites, such as the testes; (3) contain novel peptide sequences created by gene mutation; (4) are viral antigens; (5) are antigens produced by epigenetic changes, or (6) are antigens on non-transformed cells in the tumor microenvironment [21, 158, 188]. Unaltered tissue-differentiation antigens on tumors can also be targets for T-cell immunotherapy, but only if the associated tissues are not essential for life and/or their products can be replaced [188]. For example, CD19-CAR T-cell therapy induces regression of CD19-positive malignancies but also leads to long-term depletion of normal, CD19+ B cells, which can be remedied by the infusion of intravenous immunoglobulin (IVIG) [18, 58, 67, 85, 92, 144, 169].

For osteosarcoma, numerous TAA have been described that are summarized in Table 10.2. These include activated leukocyte cell adhesion molecule (ALCAM, CD166) [196], B7-H3 [115, 125], epidermal growth factor receptor (EGFR) [136], ephrin type-A receptor 2 (EphA2) [146], fibroblast activation protein (FAP) [204], G melanoma antigen (GAGE) family members [78], GD2 (a disialoganglioside; not a protein tumor associated antigen) [41, 108, 203], GD3 [41], human epidermal growth factor receptor 2 (HER2) [2, 57], interleukin 11 receptor alpha (IL11Rα) [72], insulin-like growth factor 1 receptor (IGF-1R) [73, 113], melanoma-associated antigen (MAGE) [176], melanoma cell adhesion molecule (MCAM, also called MUC18) [123], NKG2D ligands (MICA, MICB, ULBP1, 2, 3) [47], New York esophageal squamous cell carcinoma 1 (NY-ESO-1) [78, 105], papillomavirus binding factor [184], tumor endothelial marker 1 (TEM1, also called endosialin or CD248) [166], and receptor tyrosine kinase-like orphan receptor 1 (ROR1) [73]. Other TAA for osteosarcoma-targeted T-cell therapy are being elucidated and should help inform future clinical trials.

Table 10.2 Tumor-associated antigens expressed in osteosarcoma

Target antigen	Cell surface expression	Preclinical in vivo studies[a]	T-cell clinical studies[b]
ALCAM (CD166)	+	+	–
B7-H3	+	+	–
EGFR	+	–	+
EphA2	+	–	–
FAP	+	–	–
GAGE 1,2,8	–	–	–
GD2	+	+	+
GD3	+	–	–
HER2	+	+	+
IL-11Rα	+	+	–
IFG-1R	+	+	–
MAGE A1-6,10, 12; C2	–	–	–
MCAM (MUC18)	+	–	–
NKG2D ligands	+	+	–
NY-ESO-1	–	–	–
Papillomavirus binding factor	–	–	–
ROR1	+	+	–
TEM1	+	–	–

[a]using T cells vs. osteosarcoma; [b]including patients with osteosarcoma

ALCAM activated leukocyte cell adhesion molecule, *CLUAP1* clusterin-associated protein 1, *EGFR* epidermal growth factor receptor, *EphA2* ephrin type-A receptor 2, *FAP* fibroblast activation protein, *GAGE* G melanoma antigen, *GD2* disialoganglioside, *HER2* human epidermal growth factor receptor 2, *IL11Rα* interleukin 11 receptor α, *IFG-1R* insulin-like growth factor 1 receptor, *MAGE* melanoma-associated antigen, *MCAM* melanoma cell adhesion molecule, *NY-ESO-1* New York esophageal squamous cell carcinoma 1, *ROR1* receptor tyrosine kinase-like orphan receptor 1, *TEM1* tumor endothelial marker 1

Genetic Approaches to Render T Cells Specific for Osteosarcoma

Since the ex vivo generation of conventional antigen-specific T cells is often cumbersome and unreliable, investigators have developed genetic approaches to rapidly generate antigen-specific T cells. These include the forced expression of α/β

T-cell receptors (TCRs), CARs, and bispecific T-cell engagers (BiTEs) [13, 29, 77, 191, 192]. Here we will focus our discussion on α/β TCRs and CARs.

α/β TCR Modified T Cells

Conventional TCRs are composed of α and β chains that form heterodimers. TCRs recognize peptides, which are derived from proteins and are presented on major histocompatibility complex (MHC) molecules on the cell surface. Isolating TCRs for adoptive T-cell therapy requires the generation of TAA-specific T-cell clones and subsequent isolation and cloning of the TCR α and β chains [187]. In general, a large number of T-cell clones need to be screened, and isolated TCRs often are of low affinity requiring additional affinity maturation. Following isolation, genes encoding the α and β chains are cloned into retroviral or lentiviral vectors and then used to transduce T cells [158]. Since T cells express endogenous α/β TCRs, mispairing between endogenous α/β and transgenic α/β TCR chains is a common problem. Several approaches have been developed to overcome this limitation, including the introduction of disulfide bonds or use of murine sequences to favor dimerization of transgenic α/β TCR chains [33, 55]. Silencing the expression of endogenous α/β TCRs by shRNAs, zinc-finger nucleases, or CRISPR/CAS9 gene editing are other options [103, 134, 148, 165, 182].

α/β TCRs have been isolated for several TAA including CEA, GP100, MAGEA3, MART1, and NY-ESO-1 [74, 81, 127, 129, 140, 157, 159, 180]. So far the safety and efficacy of α/β TCR T-cell therapy has been evaluated mainly for patients with melanoma, but studies have also been conducted for patients with sarcoma, colon cancer, and multiple myeloma. One of the first studies in humans with transgenic α/β TCR T cells was conducted by Morgan et al. and demonstrated that the infusion of autologous polyclonal T cells expressing MART1-specific α/β TCRs was safe and induced objective tumor responses in 2 out of 15 lymphodepleted patients with melanoma [127]. To increase the response rates, the same group infused T cells expressing high affinity MART1- and gp100-specific α/β TCRs. While response rates increased, several patients developed toxicities, including skin rash, uveitis, and/or hearing loss, which were not associated with antitumor responses [81]. Recognition of normal tissues expressing low levels of CEA has also been reported for the adoptive transfer of CEA-specific α/β TCR T cells [140]. In contrast, infusion of NY-ESO-1-specific α/β TCR T cells was well tolerated with objective responses for 11/18 patients with synovial sarcoma and 11/20 patients with melanoma [160]. In addition, clinical studies indicate that NY-ESO-1-specific α/β TCR T cells induce clinical responses in patients with multiple myeloma without off-target effects [157]. As mentioned above, affinity maturation is frequently used to increase the activity of α/β TCRs. However, this can lead to recognition of related antigens resulting in severe adverse events. For example, infusion of MAGE A3-specific α/β TCR T cells caused fatal neurotoxicity due to recognition of MAGE A12 as well as fatal cardiac toxicities due to recognition of titin [121, 129].

Thus clinical studies so far have not only demonstrated the potency of adoptively transferred α/β TCR-modified T cells but also their clinical limitations. Nevertheless, active exploration of α/β TCR-modified T-cell therapy is warranted for patients with osteosarcoma.

CAR-Modified T Cells

Antigen-specific T cells can also be generated by the transfer of genes encoding CARs [46, 114, 167]. CARs consist of an ectodomain that confers antigen specificity, a hinge, a transmembrane domain, and an endodomain that contains signaling domains derived from the T-cell receptor CD3-ζ chain and costimulatory molecules such as CD28 or 41BB. Depending on the number of costimulatory domains, CARs are referred to as first generation (no), second generation (one), or

third generation (two) CARs. CARs targeting multiple pediatric malignancies have been developed [1, 2, 23, 51, 54, 56, 58, 62, 69, 72, 115, 116, 120, 131, 137, 150, 164, 168]. CAR ectodomains are most commonly generated by joining the heavy and light chain variable regions of a monoclonal antibody (MAb), expressed as a single-chain Fv (scFv) molecule. CARs recognize unprocessed antigen on tumor cell surfaces and do not require peptide presentation on MHC molecules.

CAR T-cell therapy has several advantages compared to α/β T-cell therapy. Because CARs do not require antigen presentation on MHC molecules, generation of CAR T cells for patients does not require HLA matching. This property also renders CAR T cells resistant to tumor escape mechanisms, such as downregulation of HLA molecules and defects in the MHC class I processing pathway. A second advantage is that MAbs already exist for numerous surface antigens, obviating the need of cumbersome α/β TCR isolation. Additionally, CAR T cells recognize carbohydrate and glycolipid antigens, in addition to protein antigens [41, 114, 167]. Furthermore, CARs confer T-cell specificity in a single molecule unlike artificial α/β TCRs, which require the expression of two molecules that are prone to heterodimerization with the endogenously expressed α/β TCR chains. A potential drawback of CARs is that, in general, only cell surface molecules are recognized. However, the isolation of scFvs that recognize HLA-molecule/peptide complexes has allowed the generation of CARs that recognize peptides derived from intracellular proteins [112, 122, 153, 170, 201].

Multiple osteosarcoma TAAs have been evaluated for gene-modified T-cell targeting in preclinical animal models and/or clinical trials, including those specific for HER2, GD-2, B7-H3, IL11Rα, ALCAM (CD166), IGF-1R, NKG2D ligands (MICA, MICB, ULBP1/2/3), and ROR1 (Table 10.2). Of these approaches, HER2-CAR T cells have been comprehensively evaluated preclinically and in early phase clinical trials. While HER2 is not gene amplified in osteosarcoma, 60–70% of osteosarcoma are HER2+, and HER2-positivity is associated with poor outcomes [57, 130]. Preclinically, T cells expressing a second-generation CAR, derived from the monoclonal antibody FRP5, with a CD28.ζ-endodomain showed promising antitumor activity in both local and lung metastatic osteosarcoma models [1]. In addition, HER2-CAR T cells had potent antitumor activity against osteosarcoma sarcospheres, which are enriched in osteosarcoma-initiating cells [155]. However, safety concerns were raised in regards to targeting HER2 with CAR T cells in humans. One patient, who received high dose chemotherapy followed by the infusion of 1×10^{10} high affinity third-generation HER2-CAR T cells plus IL2, developed respiratory failure within 12 hours of T-cell infusion and died [128]. Subsequently, up to $1 \times 10^{8}/m^{2}$ T cells expressing a second-generation HER2-CAR were given to pediatric and adolescent patients with sarcoma. While the infusions were safe, infused T cells did not expand significantly, and antitumor activity was limited [2]. Of 17 patients treated, 4 had stable disease for up to 14 months. Three patients had tumor removed after treatment. HER2-CAR T cells were present in two of these three tumors, and one tumor had ≥90% necrosis on pathologic examination. Given the safety data generated from this study, lymphodepletion was subsequently added to enhance HER2-CAR T-cell expansion and persistence. An early report demonstrated CAR T-cell expansion in 9 of 11 patients treated with lymphodepleting chemotherapy followed by HER2-CAR T cells [132]. Eight patients developed low-grade cytokine release syndrome (CRS) that resolved with supportive care, and thus far, treatment was felt to be safe. One patient had a complete response to treatment, three had stable disease, and five had progressive disease [132]. In addition, one patient, who was in complete remission with very aggressive, recurrent disease prior to lymphodepletion and HER2-CAR T-cell infusion, remains in complete remission with a follow-up of >3 years. Given these promising results, HER2-CAR T cells could provide additional benefits to patients earlier in treatment, for example, as con-

solidative therapy for patients with HER2+ tumors that are metastatic at diagnosis.

GD2 is another osteosarcoma TAA that has been extensively evaluated, mainly for patients with neuroblastoma. Pule et al. expressed a first-generation GD2-specific CAR on Epstein-Barr virus (EBV)-specific T cells and treated 11 children with advanced neuroblastoma [111, 150]. Three patients had complete responses (sustained in 2), while an additional two with bulky tumors showed substantial tumor necrosis. Heczey et al. used a combinatorial approach with anti-PD-1 antibody, lymphodepleting chemotherapy, and third-generation GD2-CAR T cells with CD28 and OX40 costim domains for patients with relapsed/refractory high-risk neuroblastoma [62]. Results demonstrated the therapy was safe, albeit with limited clinical response. For patients with osteosarcoma, a clinical trial using varicella zoster virus (VZV)-specific GD2-CAR T cells in combination with VZV vaccine and lymphodepleting chemotherapy is underway (NCT01953900). Results from this and other studies should provide insight into the risks and benefits of using GD2-specific T cells for treating patients with osteosarcoma. If multiple CAR T-cell therapies are deemed safe, we envision future trials combining CARs targeting multiple osteosarcoma TAAs to limit antigen escape.

B7-H3, also called CD276, is another promising TAA found on a high percent of osteosarcoma samples [125]. B7-H3 functions to inhibit T-cell activation [97, 104] and is associated with osteosarcoma invasiveness and increased metastatic potential [194]. Majzner and colleagues reported that second-generation B7-H3-CAR T cells with a 41BB costim domain have anti-osteosarcoma activity in both local and lung metastatic models [115]. Clinically, B7-H3 antibodies have been systemically infused on early phase trials, including one treating pediatric patients with osteosarcoma (NCT02982941). B7-H3-specific T cells are not currently in clinical trials. However, a bispecific B7-H3xCD3 antibody (MGD009), which activates host T cells to target B7-H3+ tumors, is being evaluated as monotherapy (NCT02628535) or in combination with PD-1 blockade (NCT03406949), demonstrating that T-cell targeting of B7-H3 on solid tumors is an active area of research. Given these data, B7-H3-CAR T-cell trials are expected soon.

In summary, CAR T cellshave shown promising antitumor activity in preclinical animal models, and initial clinical experiences are encouraging. However, several challenges remain including in vivo T-cell expansion and persistence, the inhibitory tumor microenvironment, T-cell trafficking to tumor sites, and safety. As reviewed in the next section, we and others believe that additional genetic modifications of T cells have the potential to overcome these obstacles.

Genetic Approaches to Enhance the Effector Function of Osteosarcoma-Specific T Cells

Enhancing T-Cell Expansion and Persistence In Vivo

Dramatic T-cell expansion and long-term persistence post infusion of adoptively transferred T cells has been observed in lymphodepleted patients post hematopoietic stem cell transplantation or in patients that have been lymphodepleted with chemotherapy and/or radiation prior to T-cell transfer [45, 54, 58, 65]. Since T-cell expansion post antigen recognition requires costimulation, investigators have most commonly included CAR endodomains derived from costimulatory molecules CD28 or 4-1BB, discussed in a recent review [189]. The optimal costimulatory domain to include in new CAR T cell constructs is largely unknown because direct comparisons are rarely performed in humans. Numerous preclinical studies have documented the benefit of added costimulation [17, 20, 149, 173]; however, only two studies in humans have done “head-to-head” comparisons to date [156, 169]. Savoldo et al. compared first-generation CD19-CARs with a ζ-domain to second-generation CD19-CARs with a CD28.ζ-domain

[169]. While CD28 costimulation enhanced expansion of adoptively transferred CAR.CD28.ζ T cells compared to CAR.ζ T cells, the effect was limited. Ramos and colleagues reported outcomes for patients with non-Hodgkin's lymphoma, who received simultaneous infusion of second-generation CD19-CD28.ζ- and third-generation CD19-CD28.41BB.ζ-CAR T cells [156]. In this study, third-generation CD19-CARs had improved expansion and longer persistence compared to second-generation CARs. These findings were most pronounced for patients with low disease burden and low circulating CD19+ B cells, indicating that third-generation CARs may have superior effector function for patients with low CD19 antigen load. While these studies provide insight into commonly used costimulatory domains for CD19+ malignancies, comparison of costimulatory domains on a broad scale for patients with osteosarcoma is not currently feasible, and preclinical evaluation remains critical to guide the choice of costimulatory domain(s) for genetically modified T cells in clinical trials.

While CD28 and 41BB are the most commonly used costimulatory domains, development of noncanonical costimulatory domains or strategies to provide costimulation with a second molecule expressed in CAR T cells are actively being explored. A recent study demonstrated that mesothelin-specific CD4- and CD8-CAR T cells require different costimulatory signals for optimal persistence against solid tumors in vivo [60]. Intriguingly, CD4-CARs persisted best with ICOS costimulation and CD8-CARs best with 41BB. Given that persistence of both CD4- and CD8-CAR T cells are likely important for long-term antitumor activity, these findings could prove critical insight for developing the next generation of genetically modified T-cell therapies. While intriguing and important, applicability of these findings for designing new CARs is challenging. Optimal costimulation cannot be predicted without preclinical empiric evaluation, making widespread use of this strategy unlikely with current technologies. Conceivably, new techniques for predicting optimal CAR T-cell costimulation could be developed in the years to come. This would relieve a large burden imposed by current methods of carefully evaluating multiple costimulatory domains for each new CAR product developed.

Providing costimulation via an inducible system is another technique for enhancing CAR T-cell expansion and persistence against solid tumors. Two separate groups developed CAR T cells with an inducible MyD88 and CD40 costimulatory domain (iMC). For this method, MyD88 and CD40 are part of a single construct that contains dimerization domains, which can be activated by rimiducid, also known as chemical inducer of dimerization or CID. At baseline, iMC domains are inactive and signal only when treated with CID [49, 118]. Importantly, Mata and colleagues incorporated iMC costim into first-generation HER2-CAR (HER2iMC-CAR) T cells and compared effector function to second-generation HER2-CARs against osteosarcoma in vitro and in vivo. Notably, the second-generation CAR used here was the same CAR used in clinical trials discussed above (HER2.CD28-CAR). In the presence of CID, HER2iMC-CAR T cells had significantly enhanced: (i) proliferation, (ii) cytokine production, and (iii) anti-osteosarcoma activity compared to second-generation HER2-CAR T cells. This "remote control" system is now being evaluated in PSCA-specific CAR T cells for adults with solid tumors (NCT02744287). Given that iMC or other inducible constructs could be incorporated into nearly any genetically modified T-cell product, results from this study and others should inform decisions on using it for patients with osteosarcoma.

Costimulatory ligands also show promise for enhancing CAR T-cell expansion and persistence. When activated, T cells upregulate costimulatory receptors such as 41BB. In this regard, investigators showed that second-generation CAR T cells modified to constitutively express 41BB ligand (41BBL) on the cell surface demonstrate significantly enhanced function compared to T cells containing a standard third-generation CAR, with CD28 and 41BB incorporated into the endodomain [208]. This benefit extends beyond

41BBL, as other tumor necrosis factor superfamily ligands, such as CD40 ligand showed a similar benefit [38]. Importantly, CD19.CD28-CARs with 41BBL as a second costimulatory molecule have been used to treat patients on a phase I clinical trial (NCT03085173). An early report from this study describes a positive safety profile [138]. Twenty-five adult patients with lymphomas were treated. Sixteen patients experienced low-grade CRS (grade 1 or 2), and none had severe CRS. Eight patients had neurotoxicity with two cases reported as grade 3. Twenty-one patients were evaluable for response at the time of the report, and 12 achieved a complete response [138]. With promising results coming out of this trial, similar methods are likely to be adopted for other CAR T-cell targets.

Other options to enhance expansion and persistence in vivo include transgenic expression of cytokines (discussed below) and vaccination post-infusion to boost T-cell expansion. Lastly, most studies have been conducted with unselected T cells. Some studies indicate that it might be advantageous to express CARs in T cells that are specific for viruses, so that infused cells could be boosted by vaccination (e.g., influenza) [35] or by viruses, which are present latently in humans (e.g., EBV) [150]. In addition, expressing CARs in T-cell subsets, such as central memory T cells, has the potential to enhance T-cell persistence [7, 179].

Genetic Modifications to Overcome Tumor-Mediated Immunosuppression

Malignant cells including osteosarcoma and their supporting stroma develop an intricate environment to suppress the immune system [8, 50, 53, 66, 152, 186]. They (1) secrete immunosuppressive cytokines such as transforming growth factorβ (TGFβ) or IL10, (2) attract immunosuppressive cells such regulatory T cells (Tregs) or myeloid-derived suppressor cells (MDSCs), (3) inhibit dendritic cell maturation, (4) express molecules on the cell surface that suppress immune cells including FAS ligand (FAS-L) and PD-L1, and (5) create a metabolic environment (e.g., high lactate, low tryptophan) that is immunosuppressive.

Three broad approaches have been developed to overcome tumor immune suppression: (1) increasing CAR T-cell activation, for example, by enhanced costimulation (discussed above) or by local production of transgenic cytokines, (2) engineering CAR T cells to be resistant to immune evasion strategies used by the tumor, and (3) targeting cellular components of the tumor stroma. Any one may affect more than one mechanism of tumor immunosuppression [39, 99].

CAR T cells can be engineered to produce immunostimulatory cytokines by transgenic expression of cytokines such as IL-15 [70, 75, 94, 151], which improves CAR T-cell expansion and persistence in vivo. In addition, it renders T cells resistant to the inhibitory effects of Tregs by activation of the phosphoinositide 3-kinase (PI3K) pathway [143]. Results from a clinical trial using GD2-CAR invariant natural killer T cells modified to secrete IL-15 for patients with neuroblastoma (NCT03294954) should provide important insight into adapting this strategy for patients with osteosarcoma. Alternatively, transgenic expression of IL-12 in CAR T cells acts directly to enhance T-cell activity [24, 26, 28, 205]. In addition, IL-12 reverses the immunosuppressive tumor environment by triggering apoptosis of inhibitory tumor-infiltrating macrophages, dendritic cells, and MDSCs through a FAS-dependent pathway [88], resulting in enhanced antitumor activity of adoptively transferred T cells in several preclinical animal models. While there are safety concerns in regard to constitutive IL-12 expression [206], CAR T cells secreting IL-12 are actively being explored via compartmental injection to treat patients with advanced stage solid tumors (NCT02498912). Additionally, CAR T cells modified to secrete IL-18 show promise in preclinical solid tumor models [3, 27, 71]. Another approach to provide cytokine signaling to gene modified T cells without the presence of cytokine is through a constitutively active IL-7 receptor [171].

Conversely, instead of themselves being engineered to produce cytokines, CAR T cells can be engineered to be resistant to cytokines such as IL-4 and TGFβ that inhibit their cytolytic function. TGFβ is widely used by tumors as an immune evasion strategy [202], since it promotes tumor growth, limits effector T-cell function, and activates Tregs. These detrimental effects of TGFβ can be negated by modifying T cells to express a dominant-negative TGFβ receptor type II (DNR), which lacks most of the cytoplasmic kinase domain [9, 12, 48]. DNR expression interferes with TGFβ-signaling and restores T-cell effector function in the presence of TGFβ, and long-term results describing benefits of this strategy for patients with EBV-positive lymphomas were recently published [12].

Engineering T cells to actively benefit from inhibitory signals generated by the tumor environment is also possible, by converting inhibitory signals into stimulatory signals [4, 100, 107, 126, 197, 199]. For example, linking the extracellular domain of the TGFβ RII to the endodomain of toll-like receptor (TLR) 4 results in a chimeric receptor that not only renders T cells resistant to TGFβ but also induces T-cell activation and expansion [197]. Chimeric IL-4 receptors are another example of these "switch receptors." Many tumors secrete IL-4 to create a TH2-polarized environment. Multiple reports have shown that expression of chimeric IL-4 switch receptors, consisting of the ectodomain of the IL-4 receptor and the endodomain of the IL-7Rα or the IL-2Rβ chain, enable T cells to proliferate in the presence of IL-4 and retain effector function including TH1-polarization [4, 102, 126, 199].

Silencing genes that render T cells susceptible to inhibitory signals in the tumor microenvironment may also improve T-cell function. For example, many tumor cells express FAS ligand, and silencing FAS in T cells prevents FAS-induced apoptosis [43]. Besides silencing genes, expression of a constitutively active form of serine/threonine AKT (caAKT), which is a major component of the phosphatidylinositol 3-kinase (PI3K) pathway in T cells, has also been shown to improve T-cell function [177]. caAKT-expressing T cells sustained higher levels of NF-κB and had elevated levels of antiapoptotic genes such as Bcl2, resulting in resistance to Tregs and TGFβ.

Lastly, most solid tumors have a stromal compartment that supports tumor growth directly through paracrine secretion of cytokines, growth factors, and provision of nutrients, and contributes to tumor-induced immune suppression [32, 61]. For example, we have shown in preclinical studies that T cells expressing CARs specific for fibroblast activation protein (FAP) expressed on cancer-associated fibroblasts (CAFs) have potent antitumor effects [84]. In addition, combining tumor-specific CAR T cells with FAP-specific CAR T cells enhanced antitumor activity. While some concerns have been raised in regard to targeting FAP [161, 183], our findings indicate that targeting FAP on CAFs has the potential to improve antitumor effects of adoptively transferred CAR T cells. Targeting the tumor vasculature with CARs to enhance T-cell therapy for solid tumors has also been explored [25, 133]. Targeting the tumor vasculature with vasculature endothelial growth factor receptor 2 (VEGFR2)-specific CAR T cells combined with providing tumor-specific T cells synergized in inducing tumor regression in several syngeneic, preclinical tumor models [25]. In addition, transgenic expression of VEGFR2-specific CARs and IL-12 in T cells was sufficient to eradicate tumors, indicating that combining countermeasures might potentiate effects [24].

While many of the discussed genetic modification strategies have not been explored in osteosarcoma models, these strategies could be readily integrated in current T-cell therapy approaches for osteosarcoma.

Genetic Modification of T Cells to Improve Homing to Tumor Sites

T-cell homing to solid tumor sites might be limited. For example, Kershaw et al. evaluated the safety and efficacy of first-generation folate receptor (FR)-α CAR T cells in patients with ovarian cancer [90]. Infused T cells persisted less

than 3 weeks in all but one patient and did not specifically home to tumor sites as judged by ^{111}indium scintigraphy. No antitumor activity was observed. Since then, several investigators have shown in preclinical models that the expression of chemokine receptors in CAR T cells that recognize chemokines secreted by solid tumors can enhance T-cell homing. For example, transgenic expression of chemokine receptors CCR2b or CXCR2 in T cells enhances trafficking to CCL2- or CXCL1-secreting solid tumors including melanoma and neuroblastoma [36, 89]. Another recent report demonstrates that CAR T cells modified to express CXCR1 or CXCR2 have enhanced homing to brain tumors via recognition of IL-8. Interestingly, tumors only secreted IL-8 after local radiation therapy, making this combinatorial strategy an intriguing method for enhanced CAR T-cell homing [79]. While these specific genetic modification techniques have not been implemented in clinical trials using CAR T cells, one study evaluating if CXCR2 gene modification can improve homing and antitumor activity of tumor-infiltrating lymphocytes is underway (NCT01740557).

Improving Safety of T-Cell Therapy

Toxicities can be divided into four categories: (1) toxicities due to genetic modification**,** which have not been observed with genetically modified T cells in humans so far [5, 14, 117], (2) "on target organ" toxicities (e.g., depletion of normal B cells post CD19-CAR T cells) [85], (3) "on target, off organ" toxicities (e.g., liver toxicity of carbonic anhydrase IX CAR T cells to target renal cell carcinoma) [95], and (4) systemic inflammatory syndromes [58, 85, 144].

Genetic safety switches have been developed to selectively destroy genetically modified T cells once adverse events occur. The most widely used suicide gene strategy for T-cell therapy is to introduce the herpes simplex virus thymidine kinase (HSV-tk) gene into T cells. HSV-tk phosphorylates acyclovir, valacyclovir, and ganciclovir to toxic nucleosides [31]. T cells transduced with HSV-tk are robustly killed in the presence of these medications and clinical studies demonstrate effectiveness of the strategy. A drawback to utilizing HSV-tk as a safety switch for T-cell therapy is the immunogenicity of HSV-tk, and that some patients require acyclovir, valacyclovir, or ganciclovir to treat herpetic diseases. Therefore, genetic safety switches using non-immunogenic human components have been developed, such as inducible caspase 9 (iC9) [40, 175]. As opposed to using CID to activate costimulatory domains, the drug can also be used to activate caspase-induced cell death. Once exposed to CID, T cells genetically modified with iC9 rapidly undergo apoptosis. Furthermore, repeated doses of CID can remove remaining populations of genetically modified cells expressing low levels of iC9 [209], demonstrating that administration of CID is safe and functional in clinical settings. Another approach includes the transgenic expression of CD20 or truncated EGFR (tEGFR), rendering T cells sensitive to the clinically approved MAbs rituximab or cetuximab, respectively [76, 141]. Multiple clinical trials are open using CAR T cells modified to express tEGFR as a safety mechanism (NCT03085173, NCT03618381, NCT03244306, NCT03710421, NCT02153580, NCT02159495, NCT02051257, NCT02028455, NCT03070327, NCT02028455, NCT02706405, NCT01865617, NCT02146924, NCT03389230). While suicide gene switches can selectively kill infused cells, systemic inflammatory syndromes might be difficult to control with this approach since resident immune cells, which are activated by the infused T cells, most likely contribute. Studies indicate that IL6 plays a critical role in these syndromes, and the infusion of the IL6 receptor MAb (tocilizumab) alone or in combination with steroids proved to be effective [58, 85, 144].

While suicide switches are one strategy to prevent "on target, off organ" toxicities, other strategies include the generation of T cells that are only fully activated if they encounter a unique "antigen address" at the tumor site. Examples include the development of T cells expressing two CARs in which one TAA-specific CAR has an endodomain with a ζ-signaling domain and a second CAR, specific for another TAA, provides costim-

ulation [91, 96, 200]. For this type of approach, success depends on targeting two antigens that are unlikely to be found on a given normal tissue, making antigen selection critical for translating this approach to target osteosarcoma.

Combinatorial T-Cell Therapy

As for other cancer therapies, combinatorial therapies hold promise for improving T-cell therapy for cancer [190]. These can be divided into approaches that (1) kill tumor cells without affecting T cells, (2) enhancing the expression of TAA, (3) improving T-cell expansion and persistence, and (4) reversing the inhibitory tumor microenvironment. For example, the BRAF inhibitor vemurafenib has no adverse effects on T-cell function, and combining vemurafenib with adoptive transfer of T cells enhanced antitumor effects in preclinical animal models of melanoma [42, 106]. Increasing the expression of TAA in cancer cells can be achieved with epigenetic modifiers such decitabine [30, 37].

Combining T-cell therapy with blocking antibodies specific for negative regulators of T-cell responses such as the cytotoxic T-lymphocyte-associated protein (CTLA-4) and programmed cell death 1 (PD-1) is one strategy to increase their function [86, 142, 181, 198]. The role of CTLA-4 as a negative regulator of T-cell responses has been well demonstrated in CTLA-4-deficient mice and preclinical tumor models. Based on these studies, an antibody to block human CTLA-4 (ipilimumab) was developed, and a phase III randomized clinical trial showed that 23% of patients with metastatic melanoma survived more than 4 years following ipilimumab treatment, leading to FDA approval [68].

Similarly, combining T-cell therapy with MAbs that block PD-1 and/or its ligands (PD-L1 and PD-L2) is another promising approach. Clinical trials evaluating the safety and efficacy of PD-L1 antibodies reported encouraging objective clinical response rates for patients with advanced solid tumors [15, 147]. In addition, multiple reports have demonstrated benefits of blocking the PD-1/PD-L1 axis to enhance adoptive cell transfer in preclinical models [22, 80, 154].

As mentioned in section "Enhancing T-cell Expansion and Persistence In Vivo," the administration of vaccines is an attractive strategy to boost adoptively transferred T cells. Several groups have shown that vaccines augment the effectiveness of adoptive T-cell therapy in preclinical animal models [109, 135, 172]. Besides provision of antigen, providing potent costimulation and/or cytokines was critical for the observed effects. However, limited experience is available in humans except for an ongoing clinical trial in which patients are vaccinated with an autologous DC vaccine post α/β TCR T-cell transfer.

Lastly, reversing the immunosuppressive tumor microenvironment with small molecule inhibitors is another approach to enhance the antitumor activity of adoptively transferred T cells. For example, blocking STAT3 in combination with the adoptive transfer of T cells resulted in enhanced antitumor effects [52, 64]. In addition, several preclinical studies have highlighted the benefit of combining oncolytic viruses with the adoptive transfer of CAR T cells [59, 163].

Conclusions

T-cell therapy has shown promising results in early phase clinical studies especially for patients with hematological malignancies. For solid tumors including osteosarcoma, T-cell therapy has shown promise in preclinical studies but formidable challenges remain in developing safe and effective T-cell therapies for treating patients with osteosarcoma. These include target antigen selection, limited in vivo T-cell expansion and persistence, T-cell trafficking to tumor sites, and the hostile tumor microenvironment. Genetic modification of T cells and combining T-cell transfer with other therapies are promising strategies to overcome these obstacles.

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Conflict of Interest SG has patents and patent applications in the field of T-cell therapy and gene therapy for cancer and is a member of the data safety monitoring board of Immatics US, Inc.

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11 Muramyl Tripeptide-Phosphatidyl Ethanolamine Encapsulated in Liposomes (L-MTP-PE) in the Treatment of Osteosarcoma

Paul A. Meyers

Abstract

The recruitment of autologous macrophages to attack osteosarcoma represents a novel immunotherapy approach to the treatment of osteosarcoma. Muramyl tripeptide-phosphatidyl ethanolamine encapsulated in liposomes (L-MTP-PE) was derived as a compound with the ability to stimulate macrophages to destroy autologous osteosarcoma tumor cells. Preclinical studies including studies in dogs with spontaneously arising osteosarcoma showed the ability of L-MTP-PE to control microscopic metastatic disease in osteosarcoma. A pivotal clinical trial led to the approval of L-MTP-PE for the treatment of newly diagnosed osteosarcoma in over 40 countries.

Keywords

Osteosarcoma · Muramyl tripeptide · Immunotherapy · Macrophages · Adjuvant therapy

Introduction

The idea that the immune system could be activated to attack cancer is an old one. In 1891, Coley reported his experience at the Memorial Sloan Kettering Cancer Center (MSKCC). He used direct injections of bacteria into tumors to cause infection which in some cases led to regression of sarcomas [1]. In the ensuing century, a variety of immune effector cells have been tested for their anticancer properties including tumor-infiltrating lymphocytes, lymphokine-activated killer cells, and genetically modified T cells. Immune stimulating agents such as interferon have been used to treat melanoma. There was less attention paid to the macrophage as a potentially active antitumor immune effector cell. Liposomal muramyl tripeptide phosphatidyl ethanolamine (L-MTP-PE) was developed to stimulate monocytes and macrophages to become tumoricidal against autologous tumor cells and has undergone extensive testing in preclinical, phase I, phase II, and phase III trials and was ultimately approved as adjuvant therapy for the treatment of osteosarcoma.

Background

Bacille Calmette-Guerin (BCG) is a bacterium that was derived from the tuberculosis bacterium by repeated passage to obtain an isolate of

P. A. Meyers (✉)
Memorial Sloan Kettering Cancer Center, New York, NY, USA
e-mail: meyersp@mskcc.org

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attenuated virulence that could be used as a vaccine against tuberculosis. In the early decades of the twentieth century, BCG was used as an adjuvant to stimulate the immune system in patients with cancer. BCG is part of the armamentarium of modern cancer therapy. In the treatment of superficial cancer of the urinary bladder, injection of BCG into surface malignancies of the bladder leads to spontaneous regression [2].

Zwilling and Campolito showed that BCG could stimulate pulmonary macrophages to become tumoricidal in an autologous model [3]. Namba et al. showed that this tumoricidal activity resided in a component of the BCG cell wall [4]. Ellouz et al. isolated peptidoglycans from the BCG cell wall and reported that a synthetic analogue, N-acetyl-muramyl-L-alanine-D-isoglutamine, or muramyl dipeptide (MDP) preserved the activity of the intact cell wall [5]. Benacerraf et al. reported that MDP was an effective immune adjuvant [6]. Fidler and colleagues reported that packaging lymphokines in liposomes resulted in improved activation of immune effector cells [7]. They also reported that MDP encapsulated in liposomes could lead to macrophage destruction of autologous tumor cells [8]. Fidler's group reported that intravenous administration of MDP encapsulated in liposomes could prevent the development of pulmonary metastases in a murine model [9].

MDP is a small molecule and disappeared rapidly from the circulation following intravenous administration [10]. Small molecules like MDP leak rapidly from liposomes. Fidler's group modified MDP by adding a third peptide to created muramyl tripeptide (MTP). They also linked MTP to phosphatidyl ethanolamine so that the resulting liposomes incorporated the MTP into multilamellar membranes [11]. Kleinerman and Fidler used the resulting agent liposomal muramyl tripeptide phosphatidyl ethanolamine (L-MTP-PE) to demonstrate autologous tumoricidal activity in human models [12].

Clinical Trials

The first trials of L-MTP-PE in humans were carried out at the MD Anderson Cancer Center (MDACC). The first phase I trial reported mild to moderate side effects, including chills, fever, nausea, and malaise [13]. The maximum tolerated dose (MTD) was reported to be 6 mg/m^2. Radiolabeled L-MTP-PE was taken up by the reticuloendothelial system including the liver, spleen, lungs, and nasopharynx. Kleinerman studied the peripheral blood monocytes from the patients who participated in the phase I trial and reported activation of tumoricidal activity in monocytes in 24 of 28 subjects [14]. The dose of MTP which achieved the best immune stimulation was 0.5–2.0 mg/m^2, lower than the MTD of 6 mg/m^2.

When patients with osteosarcoma are initially diagnosed, most of them do not have clinically detectable metastatic disease. In the absence of systemic therapy, 80–90% of them will go on to develop metastatic disease, and the great majority of the metastases are pulmonary [15]. L-MTP-PE had been shown to induce autologous tumoricidal activity in human monocytes and macrophages . L-MTP-PE had been shown to prevent the development of pulmonary metastases following intravenous injection of tumor cells in murine models. This suggested that L-MTP-PE might be a useful adjunct in the treatment of osteosarcoma.

Most anticancer drugs are treated in models in which human tumor cell lines are grown in mice with a compromised immune system. These models, called heterotopic xenografts, are imperfect models of human disease. The cell lines have often undergone mutation so that they no longer recapitulate the human tumor. The tumors are grown in compartments that do not recapitulate the tumor microenvironment in which they arose. The lack of a competent immune system in the mice, necessary to establish the xenograft, precludes testing therapies that involve immune effector cells. Osteosarcoma arises in dogs

spontaneously and largely recapitulates human disease. Tumors arise in long bones and metastasize to the lung, and death results from pulmonary failure. Osteosarcoma in dogs represents an excellent model in which to test potential new treatments for human osteosarcoma.

MacEwen performed a prospective, randomized, double-blind, placebo-controlled trial of L-MTP-PE in dogs with osteosarcoma [16]. All the dogs underwent amputation. They were then randomly assigned either to receive L-MTP-PE or placebo. 100% of the dogs that received placebo developed metastatic disease and went on to die with a median survival of 77 days. The dogs treated with L-MTP-PE had a statistically significant improved median survival of 222 days, and 4 of 14 dogs remained alive and free of recurrence 1 year following treatment. These results supported subsequent trials in human patients including phase II trials and ultimately the phase III randomized trial.

Investigators at MDACC performed a phase II trial of L-MTP-PE in patients with osteosarcoma who developed recurrent pulmonary metastases after frontline therapy including surgery and multi-agent chemotherapy [17]. All patients had surgical removal of the pulmonary metastases. One group of patients received L-MTP-PE twice weekly for 12 weeks. A second group of patients received L-MTP-PE for 24 weeks. Progression-free survival (PFS) for the two groups was compared to a comparable group of patients treated at MDACC without L-MTP-PE (historical control). The median time to progression for the second group of patients treated for 24 weeks was 9 months, significantly longer than the median PFS of 4.5 months for historical control group. Median PFS for the second group was better than for the first group, suggesting that longer duration of therapy was beneficial. Among the patients who went on to develop pulmonary recurrence despite the administration of L-MMTP-PE, some had surgical resection of these new pulmonary nodules. Nodules resected after administration of L-MTP-PE demonstrated infiltration by monocytes and macrophages and a rim of fibrosis, supporting the conclusion that L-MTP-PE provoked an immune inflammatory response in the metastatic nodules [18].

Treatment of osteosarcoma always includes the use of systemic chemotherapy. Kleinerman investigated the interaction between chemotherapy and L-MTP-PE. She reported that doxorubicin had no effect on cytokine release or induction of tumoricidal activity in monocytes by L-MTP-PE [19, 20]. She retrieved circulating monocytes from patients before, during and after administration of chemotherapy and demonstrated no difference in the response to L-MTP-PE [21].

Investigators at MDACC and MSKCC performed a phase II study in patients with osteosarcoma which recurred after initial therapy with surgery and multi-agent chemotherapy which did not include ifosfamide [17]. Patients were treated with concurrent ifosfamide and L-MTP-PE. They reported the usual and customary toxicity with ifosfamide; there was no increased toxicity seen with concurrent administration. Administration of L-MTP-PE was associated with similar increases in circulating cytokines to that seen when L-MTP-PE was administered without concurrent ifosfamide. Some patients underwent resection of metastatic pulmonary nodules after administration of ifosfamide and L-MTP-PE. Pathologic review of the resected nodules showed tumor necrosis similar to that seen after administration of chemotherapy without L-MTP-PE; it also showed inflammatory infiltrates and surrounding fibrosis similar to that seen when L-MTP-PE was administered without concurrent chemotherapy. This study showed that chemotherapy did not interfere with L-MTP-PE activity.

Randomized Phase III Trial

L-MTP-PE had a very favorable safety profile. A phase II trial in recurrent osteosarcoma suggested that prolonged administration of L-MTP-PE was associated with decreased risk for recurrence. A prospective, randomized, double-blind study of L-MTP-PE in dogs with osteosarcoma showed a statically significant improvement in progression-

free survival and apparent cures. All of this evidence justified a phase III trial of L-MTP-PE in patients with osteosarcoma.

As the North American pediatric cooperative groups began consideration of the design of the phase III trial in osteosarcoma, there was an additional prominent question. Ifosfamide had shown activity in metastatic recurrent osteosarcoma with reports of 30–50% objective responses [22, 23]. The phase III clinical trial was designed to answer two questions:

1. The trial would be a comparison of a three-drug chemotherapy regimen with cisplatin, doxorubicin, and high-dose methotrexate to a four-drug chemotherapy regimen with cisplatin, doxorubicin, high-dose methotrexate, and ifosfamide. Would adding a fourth chemotherapy agent improve outcome?
2. Would the addition of L-MTP-PE to systemic chemotherapy improve outcome?

Osteosarcoma is a rare disease. In order to answer both questions in a reasonable period of time, we decided to use a factorial design. In factorial design, patients are randomly assigned to each intervention, but each intervention is analyzed for its effect on the entire population. All patients who received four-drug chemotherapy would be compared to all patients who received three-drug chemotherapy, ignoring whether or not they had been assigned to receive L-MTP-PE. All patients assigned to receive L-MTP-PE would be compared to all patients assigned not to receive L-MTP-PE, without considering whether they had been assigned to receive three- or four-drug chemotherapy. These marginal analyses can only be performed if there is no interaction between the two study interventions. No preclinical or clinical evidence suggested that there would be an interaction between the two study interventions, and there was no plausible biological basis to suggest an interaction [21]. The final analysis at the completion of the randomized prospective phase III trial detected no interaction [24].

The design for the chemotherapy question was an addition study. Patients assigned to treatment arm A received cisplatin, doxorubicin, and high-dose methotrexate. Patients assigned to treatment arm B received the same agents with the addition of ifosfamide. As had become widespread practice for the treatment of osteosarcoma, patients received an initial period of chemotherapy followed by definitive surgical resection of the primary tumor followed by additional adjuvant chemotherapy. Assessment of necrosis in the primary tumor after the initial period of systemic chemotherapy was performed as there is a strong correlation between the degree of necrosis in the primary tumor following initial therapy and outcome [25]. Longer periods of chemotherapy prior to definitive surgery can be associated with higher degrees of necrosis at the time of definitive surgery, so it was important to maintain an identical duration of initial chemotherapy in both arms of the study [26].

We relied on preclinical and early clinical data to decide when to introduce L-MTP-PE. All of the available evidence suggested that L-MTP-PE was more likely to provide benefit in the setting of minimal tumor burden, i.e., after definitive resection of the primary tumor and any macroscopic metastatic disease [9, 17]. Since L-MTP-PE has its maximum effect against minimal residual disease, L-MTP-PE therapy was initiated after surgical resection of the primary tumor. There were four treatment arms: A, A+, B, and B+. Patients assigned to regimen A received chemotherapy with cisplatin, doxorubicin, and high-dose methotrexate. Patients assigned to regimen B received chemotherapy with the same three drugs with the addition of ifosfamide. Patients assigned to receive L-MTP-PE were designated with the addition of a plus sign to the chemotherapy regimen; 677 patients were randomly assigned to one of the four treatment regimens at the time of study enrollment. In retrospect, this was an error in study design, because it allowed for an imbalance in the number of patients with poor necrosis after initial therapy, which is associated with worse prognosis, to one arm. This design flaw ultimately masked the treatment success of L-MTP-PE in the three-drug plus L-MTP-PE group (A+) as discussed below.

The frequency of more favorable and less favorable necrosis following initial chemother-

apy was the same when we compared patients treated with regimen A and B. Toxicities on all four arms of the study were very similar. There was no increased toxicity among the patients assigned to receive L-MTP-PE (regimens A+ and B+).

Analysis of the results of the study approximately 9 years after the last patient was enrolled (13 years after enrollment of the first patient) was reported in 2008 [24]:

1. Treatment with three chemotherapy drugs (regimen A) and four chemotherapy drugs (regimen B) achieved the same probability for both event-free and overall survival.
2. All patients assigned to receive L-MTP (with three- or four-drug chemotherapy) showed an improvement in event-free survival compared to those that received three- or four-drug chemotherapy alone. The probability for event-free survival 6 years from study entry was 67% with L-MTP-PE and 61% without. The *p* value for this difference was 0.08.
3. The same comparison showed a statistically significant improvement in overall survival. The probability for overall survival 6 years from study entry was 78% with L-MTP-PE and 70% without. The *p* value for this difference was 0.03.
4. The hazard ratio for death from osteosarcoma comparing treatment with L-MTP-PE to treatment without was 0.7.

Necrosis following initial chemotherapy in the randomized prospective trial was analyzed according to the method described by Huvos [25]. Less necrosis (Huvos grade 1 and 2 necrosis) was associated with a higher probability of recurrence and death than more necrosis (Huvos grades 3 and 4). When we analyzed the frequency of greater and lesser necrosis among the patients assigned to receive each of the four possible randomized therapies, we observed an excess of patients with less necrosis assigned to receive three-drug chemotherapy in combination with L-MTP-PE (regimen A+). Since the observation of less necrosis strongly correlates with a higher probability for recurrence, this imbalance could explain the apparent failure to observe an improved outcome for event-free survival among the patients receiving three-drug chemotherapy who were assigned to receive L-MTP-PE.

Further analysis of the imbalance in necrosis revealed that by chance most of the imbalance took place in patients older than 16 at study entry. For patients aged less than 16 at study entry, there was better balance among the study arms in the frequency of patients with greater and lesser necrosis following initial chemotherapy. This allowed us to examine the effect of the addition of L-MTP-PE to chemotherapy in 496 patients free from the confounding effect of an excess of patients with poor necrosis in one study arm. For this group of 496 children, the addition of L-MTP-PE to chemotherapy resulted in improved event-free survival. The improvement was seen with both chemotherapy regimens to the same degree. There was no interaction between the two study questions. For this group, the addition of L-MTP-PE to chemotherapy resulted in improved overall survival. The improvement was exactly the same for both chemotherapy regimens.

The hazard ration for death associated with the addition of L-MTP-PE was 0.5 ($p = 0.001$). This analysis of 496 children in a prospective randomized trial represents one of the largest experiences ever reported for osteosarcoma and demonstrates a clinically and statistically significant improvement for both event-free and overall survival when L-MTP-PE is added to chemotherapy. The benefit was independent of the chemotherapy regimen to which the patients were assigned.

Phase III Randomized Trial for Patients with Metastatic Disease at Initial Presentation

The phase III randomized trial allowed enrollment of patients with newly diagnosed osteosarcoma who presented with clinically detectable metastatic disease if the clinical assessment indicated the possibility of surgical resection of all sites of metastatic disease as well as the primary tumor. Most patients who present with metastatic

disease have metastasis limited to the lungs and resection of pulmonary nodules is feasible. The protocol specified that patients would be randomized to the same four treatment arms as the patients with localized disease. Patients would undergo resection of the primary tumor and all sites of metastatic disease prior to the initiation of L-MTP-PE. The total number of patients with metastasis who participated in the prospective randomized trial was only 91 patients which greatly decreased the ability to make statistical comparisons between the 2 interventions. We reported the results of this stratum in 2009 [27]:

1. We observed no interaction between the two study interventions, that is, addition of ifosfamide to three drug chemotherapy and addition of L-MTP-PE.
2. Both event-free and overall survival were the same for patients treated with three-drug and four-drug chemotherapy regimens.
3. Both event-free and overall survival were better for the patients who received L-MTP-PE than for those who did not. Neither of these improvements reach a conventional level of statistical significance.
4. The hazard ratio associated with the risk of death when patients who received L-MTP-PE were compared to patients who did not was 0.7, which with the same as the hazard ratio we observed for patients with localized osteosarcoma.

Compassionate Access Trial

We conducted a compassionate access clinical trial of L-MTP-PE from 2008 to 2012 [28]. Eligibility included patients who presented either with osteosarcoma with metastatic disease at initial presentation or metastatic recurrent osteosarcoma after initial therapy with surgery and multi-agent chemotherapy. Trial design called for all patients to receive L-MTP-PE, either as a single agent or in combination with chemotherapy if the treating clinician felt that chemotherapy was appropriate. We enrolled 40 patients with initially metastatic disease and 165 patients with recurrent osteosarcoma. Among the 50 patients for whom it was possible to resect all sites of clinically detectable tumor, overall survival at 2 years following study enrollment was greater than 50%. Many of these patients were treated following two or more recurrences following their initial therapy for osteosarcoma.

Regulatory Status of L-MTP-PE

The sponsor presented L-MTP-PE to the Oncology Drugs Advisory Committee of the United States Food and Drug Administration (FDA) in May, 2007. Data from the pivotal phase III randomized trial was analyzed at two time points. The first analysis with data truncated in 2003 was reported in 2005 [29]. The sponsor recognized that follow-up at the first data point was poor and worked with the Children's Oncology Group to improve ascertainment of patient status for all study participants. The second analysis, with data truncated in 2006, was reported in 2008 [24]. Although the updated data set was provided to the FDA prior to the hearing, the FDA chose to analyze and present only the earlier data set. Based on that analysis, the FDA did not grant an indication for the use of L-MTP-PE in osteosarcoma. In 2008, the sponsor presented the updated data set to the European Medicines Agency. L-MPT-PE, marketed at MEPACT (mifamurtide), was approved for treatment of osteosarcoma in patients between the ages of 2 and 30 when administered in conjunction with multiagent chemotherapy [30]. As of 2019, L-MTP-PE is licensed and approved for that indication in 45 countries.

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Natural Killer Cell Immunotherapy for Osteosarcoma

12

Brian P. Tullius, Buhvana A. Setty, and Dean A. Lee

Abstract

Natural killer (NK) cells are lymphocytes of the innate immune system that have the ability to recognize malignant cells through balanced recognition of cell-surface indicators of stress and danger. Once activated through such recognition, NK cells release cytokines and induce target cell lysis through multiple mechanisms. NK cells are increasingly recognized for their role in controlling tumor progression and metastasis and as important mediators of immunotherapeutic modalities such as cytokines, antibodies, immunomodulating drugs, and stem cell transplantation. Recent advances in manipulating NK cell number, function, and genetic modification have caused renewed interest in their potential for adoptive immunotherapies, which are actively being tested in clinical trials. Here, we summarize the evidence for NK cell recognition of osteosarcoma, discuss immune therapies that are directly or indirectly dependent on NK cell function, and describe potential approaches for manipulating NK cell number and function to enhance therapy against osteosarcoma.

Keywords

Natural killer cell · Adoptive immunotherapy · Innate immunity · Antibody-dependent cell cytotoxicity · Chimeric antigen receptor · Immunomodulating drugs

Brief Overview of NK Cell Biology

The number of natural killer (NK) cells in humans varies widely, comprising 1–32.6% (median 7.6%) of all peripheral blood lymphocytes [1]. They are identified by the lack of CD3 and the presence of CD56 and/or CD16, and make up 85% of the large granular lymphocyte population [2]. NK cells are a major component of the innate immune system whose primary function is to serve as "first responders" against virally infected and transformed cells [3]. They have direct antiviral and anticancer activity through multiple cytokine and cytotoxic effector functions, but also serve to establish a pro-inflammatory microenvironment that recruits and primes adaptive immune responses [4, 5]. Unlike adaptive T and B lymphocytes, NK cells are characterized by their ability to recognize such targets without prior sensitization. Instead, NK cells base their

B. P. Tullius · B. A. Setty · D. A. Lee (✉)
Department of Hematology, Oncology, and BMT, Nationwide Children's Hospital, Columbus, OH, USA
e-mail: Brian.Tullius@nationwidechildrens.org; Bhuvana.Setty@nationwidechildrens.org; Dean.Lee@nationwidechildrens.org

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response to targets on a balance of activating and inhibitory receptors that recognize danger and self, respectively. Activating receptors typically recognize proteins that are upregulated by cell stress or are of non-self-origin, and inhibitory receptors primarily bind human leukocyte antigens (HLA) as a form of self-recognition. NK cell effector function, including target cytotoxicity, is triggered when the balance of activating and inhibiting signals is tipped toward activation.

Activating and Inhibitory Receptors

NK cells express several families of activating receptors, including CD16 (FcR**γ**IIIa), natural cytotoxicity receptors (NCRs), NK Group 2 (NKG2) family lectin-like receptors, DNAM-1, and 2B4. In general, these activating receptors serve to recognize signs of stress or danger on target cells during immune surveillance. CD16 is the low-affinity Fc receptor which binds the Fc portion of human IgG1 and IgG3, mediating antibody-dependent cell cytotoxicity (ADCC) of antibody-labeled cells [6]. The NCRs (NKp30, NKp44, and NKp46) are activating receptors that bind virus- and stress-related proteins (such as B7-H6) [7]. The receptors of the NKG2 family are expressed as heterodimers with CD94, except for NKG2D which is expressed as homodimer [8]. NKG2D, the major activating receptor in this family, recognizes MHC class I-related chain A or B (MICA/B) and members of the UL-16 binding protein (ULBP) family, which are increased in response to cellular stress. 2B4 (a SLAM family member) recognizes other ligands of the SLAM family such as CD48, and DNAM-1 recognizes the viral receptors PVR and Nectin which are highly expressed on pediatric sarcomas [9].

The primary inhibitory receptors in NK cells are the long-tailed KIRs (which possess an immunoreceptor tyrosine-based inhibition motif (ITIM) [8]) and NKG2A, both of which bind to HLA class I molecules, preventing NK-mediated lysis of cells with normal HLA expression.

Inhibitory KIRs are specific for HLA isotypes on the basis of conserved amino acid residues at position 80. Approximately half of HLA-C alleles have the amino acid asparagine (N) at residue 80—referred to as Group C1—which confers binding to KIR2DL2 and KIR2DL3. The other half of the C alleles code for lysine (K) at residue 80 (Group C2), which confers binding to KIR2DL1. Similarly, about 40% of HLA-B alleles carry the supertypic serologic epitope HLA-Bw4 (defined primarily by threonine (T) at residue 80), which confers binding to KIR3DL1. The presence of the HLA ligand regulates the activity of these KIRs during NK cell development through a process called licensing. Thus, given both parental alleles, it is possible for the HLA type of an individual to restrict NK cell licensing to as few as one (e.g., C2/C2 homozygous and Bw4−) or as many as three (C1/C2 heterozygous and Bw4+) inhibitory KIRs.

The NK cell repertoire varies greatly between individuals. The KIR family also contains members with short cytoplasmic domains, which generally deliver an activating signal and are present or absent in many different haplotype combinations such that most individuals lack one or more KIR genes. In addition to their haplotype variability, KIR genes are highly polymorphic and are variably expressed between NK cells, and functional reactivity is educated by interaction with the host HLA haplotypes. The allelic variations in KIR have been grouped into A and B haplotypes [10], with B haplotypes having greater numbers of activating KIR genes. Individuals with the "B" haplotype are predicted to have superior NK cell-mediated antitumor effects [11].

This HLA-biased education without HLA-restricted antigen recognition (as for T cells) gave rise to the "missing-self hypothesis," which postulates that NK cells recognize and destroy autologous cells with lost or altered self-HLA class I molecules [12]. However, classical HLA class I is not always required to protect from NK cell-mediated cytotoxicity, nor is it always sufficient to prevent NK cell cytotoxicity [13].

Mechanisms of NK Cell-Mediated Killing

Upon receiving a predominance of activating signals, NK cells release granules containing perforin and granzymes directed toward the target cell. The perforins form a pore in the cell membrane, allowing entry of the granzymes to the cytoplasm to induce apoptosis by direct activation of caspase-3 [14]. NK cell activation also results in increased expression of death receptor ligands on the NK cell, such as Fas ligand (FasL) and tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) [15], which induce apoptosis via associated death receptors on target cells [16–18]. In addition to these pathways, NK cells also produce several cytokines such as IFN-**γ**, which are important in mediating the adaptive immune response against cancer [19].

Evidence for NK Cell Activity in Osteosarcoma

NK Cell Function in Patients with Osteosarcoma

The critical role of anticancer immune surveillance by NK cells is well established. NK cells also appear to play an important role in osteosarcoma (OS) prevention and treatment response. Whereas NK cells in patients with several types of cancer have been shown to have poor function, NK cells isolated from patients with OS were shown to be functionally and phenotypically unimpaired, have intact IFN signaling, and demonstrated cytolytic activity against autologous and allogeneic OS cells and other target cells [20, 21]. However, children and adolescents with osteosarcoma demonstrate a statistically significant reduction in peripheral blood NK cells at the time of diagnosis compared to healthy controls [22]. NK cells also confer a survival benefit during treatment of osteosarcoma, as the rapidity of absolute lymphocyte recovery while receiving standard frontline osteosarcoma chemotherapy regimens (MAP or MAPIE) correlates significantly with an improved event-free survival [23]. Further, IL-2 support during neoadjuvant and adjuvant chemotherapy for osteosarcoma demonstrated a significant correlation between the magnitude of NK cell expansion and enhanced survival [24].

In addition, low expression of PD-L1 (an important suppressor of immune effector function) in osteosarcoma correlates with significantly increased infiltration of NK cells into the tumor microenvironment and is associated with improved event-free survival [25]. Lastly, genomic data obtained from analysis of mRNA and miRNA from patients diagnosed with relapsed osteosarcoma show that the density of the patient's activated NK cells calculated by CIBERSORT algorithm correlates positively with a good prognosis [26]. These findings all point to the critical role NK cells have in disease-free survival of patient with osteosarcoma.

Expression by Osteosarcoma of Ligands Recognized by NK Cells

The susceptibility of tumor cells to NK cell lysis is regulated by the proportion of inhibiting and activating signals perceived upon interaction of NK cells with the target cell. It correlates negatively with expression of HLA class I antigens and positively with intercellular adhesion molecules and activating ligands on the surface of tumor cells.

Downregulation of HLA class I antigens on the cell surface can be induced by stress conditions and is correlated with increased susceptibility to NK cell killing through decreased signaling by inhibitory KIRs, a phenomenon described as "missing-self." In vitro experiments with OS cell lines of varying levels of HLA class I antigen expression show that OS cells with surface expression of HLA are less susceptible to killing by NK cells compared to cells lacking cell-surface HLA; moreover, downregulation of cell-surface HLA enhances the sensitivity of NK-resistant OS cells to NK killing. Similarly, OS target cell killing correlates with their degree of KIR-HLA incompatibility with the NK cells [27]. In vivo, OS primary and metastatic tumors

have been shown to lose or downregulate HLA class I expression, thus becoming more susceptible to NK cell killing [28].

Expression of cell adhesion molecules renders tumor cells more susceptibility to NK-mediated lysis; these molecules fortify cell-to-cell interactions and provide co-stimulatory signals that enhance the cytotoxic activity of NK cells [29, 30]. Expression of the adhesion molecules CD54 and CD58 increases the bond between target and effector cells and correlates positively with the susceptibility of OS cells to NK lysis [31–33]. In vivo, lack of CD54 expression allows the circulation of tumor cells, avoids establishing stable cytolytic conjugates, and provides means of evading NK spontaneous lysis [34]. In contrast, NK cells can enhance the inflammatory microenvironment in tumors through release of IFNγ, which upregulates these adhesion molecules and increases recognition by NK cells [9].

Several activating receptor-ligand interactions have been implicated in the interaction of NK cells with OS cells. Ligands for NKG2D and DNAM-1 activating receptors (MICA/B, ULBP, PVR, and nectin-2) are widely expressed on OS cell lines and OS tumor samples [20, 35], rendering them more sensitive to NK recognition and killing. Cytolysis of OS cells is dependent on NKG2D and DNAM-1 pathways, and blockade of both pathways is required for optimal inhibition of activated NK cells; activation through NKG2D and DNAM-1 pathways also overcomes inhibition of NK cells mediated by KIR-HLA interaction [20]. In vivo, the level of MICA expression on OS cells has been correlated with staging; expression of MICA is higher in patients with early stage disease compared to late stage, suggesting a role for MICA-NKG2D-mediated NK control of OS [35], and downregulation of MICA appears to be a common immune escape mechanism [36]. Unlike other tumor types, MICA expression on OS tumor cells is unaltered by exposure to chemotherapy [20]. NK cell recognition of OS tumor cell has also been described via the NCR receptors, although the ligands on OS cells for these receptors are unknown.

Mechanisms of Killing

True to their name, NK cells exhibit a wide range of robust direct and indirect antitumor activities. NK cells can kill tumor cells via the secretion of cytotoxic granules that contain perforin and granzyme and secretion of cytokines and other effector molecules that impact tumor survival and recruitment of adaptive immunity, ligation, and activation of death receptors (e.g., TRAIL, Fas) on tumor cells and ADCC through CD16 when combined with tumor-targeting antibodies. Moreover, their release of pro-inflammatory cytokines has a profound impact on recruitment and maturation of adaptive immune responses [19].

Several early studies demonstrated the in vitro cytotoxicity of NK cells against osteosarcoma cell lines [30, 32, 37]. The mechanism by which NK cells induce apoptosis in osteosarcoma cells may depend on both the activation status of the NK cells and the death receptor and apoptotic pathways that are intact in the osteosarcoma. The predominant pathway for activated NK-mediated lysis of some osteosarcoma cell lines is via granule-mediated release of granzyme B, such that blocking this pathway leads to complete abrogation of cytolysis [20]. However, NK cells may also induce apoptosis of osteosarcoma via granule-independent mechanisms, depending more on Fas-Fas ligand or TNF-TRAIL interactions (see Book 2, Chap. 12 “Fas Signaling as a Potential Target for the Treatment of Osteosarcoma Metastasis in the Lungs”). The importance of these pathways may be underappreciated, as they are kinetically slower and therefore less apparent in classic 4-hour in vitro cytotoxicity assays that measure loss of membrane integrity [38, 39].

As previously discussed, the cytolytic activity of NK cells is mediated by the balance of activating and inhibitory receptors. NK cells isolated, propagated, or activated by different approaches may differ as to which activating receptor(s) play the dominant role in recognition of osteosarcoma. IL-15-stimulated NK cells target osteosarcoma predominantly through DNAM1, though NKG2D remains important [20]. IL-2-stimulated

NK cells target osteosarcoma predominantly through NKG2D-NKG2D ligand interactions [40]. In vitro study of IL-15-stimulated NK cells co-cultured with an osteosarcoma cell line demonstrated decreased expression of activating receptors (NKG2D, DNAM-1, and NKp30), inhibiting direct killing [41]. In contrast, IL-21-expanded NK cells increase both NKG2D and DNAM-1 [42, 43], and TGFβ-imprinted NK cells express much higher levels of TRAIL and FasL [44]. Thus, the type of NK cell applied to immunotherapy of osteosarcoma may be an important consideration in optimizing outcomes.

Mechanisms of Immune Escape

Tumor cells may acquire diverse mechanisms to evade NK cell recognition [45]. No or low expression of adhesion molecules or ligands for activating receptors and/or increased expression of ligands for inhibitory receptors are described mechanisms adopted by tumor cells to evade NK cell surveillance. In addition, shedding of NKG2D ligands (soluble sMICA) from the membrane of tumor cells can impair NKG2D-mediated cytotoxicity by blocking the NKG2D receptors on NK cells. Furthermore, secretion of immunosuppressive cytokines and transforming growth factor-β has been associated with defective NK cell function, restricting tumor cell recognition and killing.

Both classical and nonclassical HLA class I molecules, which are ligands for inhibitory KIR and CD94/NKG2A receptors, are expressed on some OS naïve tumors and may be increased in OS cells when exposed to chemotherapy [20].

OS cell lines and tumor sample show higher expression of surface MICA compared to normal bone tissue and benign bone tumors making them theoretically more susceptible to NK cells killing. However, soluble MICA was detected in the serum of some patients with OS resulting in diminished NKG2D expression on NK cells and decreased tumor cell killing. Clinical correlation showed that in patients with OS, elevated MICA expression combined with increased soluble MICA was associated with decreased NKG2D expression on PBMC, and this combination correlated significantly with advanced and metastatic disease [35, 46]. With progression of OS, expression of MICA decreases, soluble MICA increases, and expression of NKG2D on NK cells decreases [35].

Indirect Activation of NK Cell Function

As described above, patients with osteosarcoma have important defects in NK cell function—including lower circulating peripheral NK cell numbers and decreased expression of activating receptors—and NK cell numbers are further impacted during treatment, as they are extremely sensitive to chemotherapy and radiation. As these functional and numeric NK cell deficits have been linked to poorer outcomes for patients, approaches to improve the antitumor activity of NK cells can improve clinical outcomes. These include monoclonal antibodies, cytokines, immunomodulators, and attention to chemotherapeutic regimens that enhance NK cell-mediated tumor lysis.

Monoclonal Antibodies

ADCC by NK cells requires interaction between the Fc receptor (CD16) on NK cells and the Fc region of an antibody binding to an antigen on the tumor cell surface, resulting in NK cell activation and degranulation toward the target cell.

EGFR is expressed on 90% of OS tumor samples [47]. Cetuximab, a MoAb-targeting EGFR, increases NK-dependent lysis of EGFR-expressing sarcomas. Importantly, the sensitivity to cetuximab-enhanced lysis by resting NK cells is comparable among most EGFR-expressing cell lines, including chemotherapy-resistant OS cells [48]. Although prolonged OS/NK cell co-cultures and excess of tumor cells in culture result in diminished NK cell cytotoxicity secondary to downregulation of activating receptors on NK cell surface, ADCC killing of OS by NK cells is unaltered by this suppressive mechanism [41].

NK cytotoxicity to OS cells is enhanced by Fc-FcγR interaction; epidermal growth factor receptor (EGFR)-expressing OS cells are more susceptible to NK killing in the presence anti-EGFR monoclonal antibody (MoAb) compared to EGFR-negative OS cells [48].

GD2 and GD3 are tumor-associated glycolipid antigens that are highly prevalent in osteosarcoma and are potential targets for antibody-based therapies. GD2 and GD3 are highly expressed in sarcomas of children, adolescents, and young adults [49]. Tumors that express ganglioside GD2 tend to have persistence of GD2 expression at the time of recurrence [50], including patients with osteosarcoma [49]. It has been shown that ganglioside GD2-specific antibodies can inhibit tumor cell viability without involving the immune system. Combination of GD2 with cisplatin induces apoptosis in osteosarcoma cell lines [51]. Chimeric antigen receptors (CARs) against GD2 have been used to enhance the activity of NK cells against Ewing sarcoma. The expression of CARs directed against the GD2 in activated NK cells increased the responses to GD2+ allogenic Ewing sarcoma cells and also overcame resistance of individual cell lines to NK cell lysis [52].

Cytokines

Cytokines may act directly on tumor cells as antiproliferative agents and indirectly via activation of cellular immune agents such as NK cells leading to increased lysis of tumor cells.

Interleukin (IL)-15 potentiates the cytolytic activity of NK cells by increasing NKG2D expression on cell surface and enhancing GrB release upon activation. IL-15 activation reverses impaired expression of NKG2D and DNAM-1 and impaired NK cell cytotoxicity induced by prolonged co-cultures of NK cells with OS cells, and NK cells activated with IL-15 prior to co-culture with OS cells do not downregulate activating receptors and preserve functional activity despite prolonged exposure to target cells [41]. IL-2 and IL-12 increase cytotoxicity of NK cells to NK-sensitive and NK-resistant OS cell lines by increasing the density of CD18 and CD2 receptors on the NK cell surface, enhancing the conjugate-forming capacity of NK cells to OS targets [53]. Importantly, targeted application of IL-2 to the lung by aerosolized delivery markedly improves the migration of adoptively transferred NK cells into lung metastasis, resulting in enhanced control of metastatic disease [54].

IL-12 increases expression of ICAM-1 (a ligand for CD18) on OS cell lines co-cultured with PBMCs in cell-to-cell contact [55]. In a mouse model of metastatic osteosarcoma, mice bearing pulmonary metastasis treated with IL-12 showed decreased number and size of pulmonary metastasis mediated by NK cells [56]. IFN potentiates NK-mediated lysis of OS cell lines; IFN-conjugated antibodies specifically localize tumor cells in a mouse xenograft tumor model and further increase NK cell activation and tumor cell lysis [57, 58]. IL-17 augments expression of fibronectin on OS cell lines that express the IL-17 receptor, mediating increased adhesion of NK cells to OS cells and thus enhancing NK cytotoxicity. IL-17 has no direct effect on NK cells function [37].

The common γ chain cytokines IL-15 and IL-2 have both been used successfully to activate and expand NK cells ex vivo for adoptive transfer [59]. These cytokines both activate trimeric receptors on NK cells that share two subunits in common—IL-2Rβ and IL-2Rγc—with the third subunit conferring cytokine specificity [60]. Despite this similarity, they have disparate effects on NK cell expansion that can be leveraged against osteosarcoma. K562 have been induced to express membrane-bound IL-15 to serve as a platform for NK cell expansion [61]. Use of this IL-15 platform leads to a multifold expansion of activated NK cells with increased NKG2D expression on cell surface, enhanced granzyme B release, and thus increased cytolytic activity against tumor targets. However, NK expansion to a clinically usable product is limited by senescence caused by telomere shortening. More recently, recombinant IL-15 has been used for NK cell activation and expansion through mTOR-dependent activation of STAT-5 signaling leading to improved NK cell metabolic function and

antitumor cytotoxicity [62]. Allogeneic and autologous NK cells expanded with this recombinant IL-15 have proven cytotoxicity against even chemotherapy-resistant osteosarcoma cell lines in vitro [20]. Expansion and activation of NK cells for adoptive transfer as a cancer immunotherapy has been accomplished with IL-2 stimulation as well with demonstrably increased expression of NKG2D, CD16, CD94, and NKp46 and cytolytic activity [63]. NK cells cultured as briefly as 18 hours in IL-2 have shown markedly improved cytotoxicity against both NK cell-sensitive and NK cell-resistant osteosarcoma cells in vitro with similar results seen with the use of IL-12 as the activating cytokine [53]. These cytokines were seen to increase the density of CD18 and CD2 receptors on the NK cell surface, enhancing the conjugate-forming capacity of NK cells to osteosarcoma targets. Aerosolized IL-2 has been used successfully to expand adoptively transferred NK cells in vivo in a canine model of metastatic osteosarcoma [64, 65]. This aerosolized delivery of cytokine led to better specificity in terms of expansion and activation only of the pulmonary NK cells without systemic IL-2 toxicities and was associated with improved therapeutic efficacy against pulmonary metastasis.

IL-21 is another common γ chain cytokine known to play a pivotal role in NK cell activation and maturation. Ex vivo expansion utilizing feeder cells expressing membrane-bound IL-21 can yield 30,000-fold expansion of NK cells in 21 days, with retained KIR repertoires, increased expression of CD16 and NKG2D, and superior cytokine secretion [42]. In a canine patient-derived xenograft model of osteosarcoma, adoptive transfer of membrane-bound IL-21 expanded canine NK cells led to tumor regression and suppression of metastasis. Notably, NK cell homing and antitumoral cytolytic activity against osteosarcoma were enhanced by radiotherapy [66] (see Book 2, Chap. 14 "Comparative Immunology and Immunotherapy of Canine Osteosarcoma").

IFNγ-conjugated antibodies specifically localized to tumor cells in a mouse xenograft tumor model and increased NK cell activation and tumor cell lysis [57, 58].

Although it is typically associated with NK cell suppression within the osteosarcoma tumor microenvironment, the inclusion of transforming growth factor-beta (TGFβ) during NK cell expansion and activation results in NK cells with enhanced functionality [44]. This process of TGFβ imprinting results in activated NK cells with increased cytokine secretion in response to osteosarcoma cells, improved cytolytic activity against an osteosarcoma, and resistance to the suppressive effects of TGFβ.

Cytokines can also act directly on osteosarcoma cells to make them more susceptible to lysis by NK cells. IL-12 increases expression of ICAM-1 (a ligand for CD18) on osteosarcoma cell lines, making them more susceptible to NK cell-mediated lysis [55] and improving NK cell-mediated metastatic control with decreased number and size of pulmonary metastases mediated by NK cells [67]. IL-12 may also increase expression of Fas on osteosarcoma [68], making it more susceptible to Fas-mediated lysis. IL-17 can increase NK cell-mediated lysis of osteosarcoma cells through augmented expression of fibronectin on osteosarcoma cells and subsequent increased NK cell adhesion [37].

Chemotherapy

As mentioned above, chemotherapy appears to increase expression of inhibitory ligands, but does not increase MICA [20]. Chemotherapy does increase sensitivity to ADCC by NK cells [41], and both gemcitabine [69] and cisplatin [70] may increase sensitivity of OS to direct NK cell lysis by upregulation of Fas or downregulation of anti-apoptotic proteins.

Several chemotherapeutic agents commonly used for osteosarcoma have direct effects on the osteosarcoma cells that enhance NK cell-mediated lysis. Doxorubicin, cisplatin, and etoposide have all been shown to downmodulate expression of the inhibitor of apoptosis X-IAP, sensitizing osteosarcoma cell lines to NK cell-mediated lysis via TRAIL [71]. This sensitization to TRAIL was specific to osteosarcoma cells and was not seen in normal human osteoblasts.

Cisplatin has also been shown to sensitize osteosarcoma cells to Fas/Fas ligand-mediated apoptosis via downregulation of FLICE inhibitory protein long form (FLIP-L) [70].

The taxane docetaxel and the nucleoside analog gemcitabine are commonly used in relapsed or refractory pediatric sarcoma patients, including those with osteosarcoma [72, 73]. Gemcitabine has been shown to upregulate NKG2D ligand in several other solid tumor cancer types [74–76]. While docetaxel has been shown to upregulate NKG2D expression on NK cells in vivo [77], it also inhibits NK cell cytotoxicity [78]. Irinotecan and temozolomide have similarly been used in relapsed and refractory osteosarcoma patients. Temozolomide has been shown to cause minimal reduction in NK cell cytotoxicity, but may suppress proliferation of NK cells in response to activation with IL-2.

Immunomodulators

In addition to monoclonal antibodies (mAb) and cytokines, a variety of immunomodulatory drugs have been successfully combined with NK cells to potentiate their antitumor activity and treat human malignancies [79–81].

In the setting of OS, the activity of NK cells may be weakened or enhanced by immune-modulating agents. Sodium valproate (an HDAC inhibitor) and hydralazine (a DNA methylation inhibitor) increase the expression of MICA and MICB on OS cells, but not sMICA in serum, and therefore increase the susceptibility of tumor cells to NK cell lysis [82, 83]. Moreover, hydralazine increases cell-surface expression of Fas and augments Fas-induced OS cell death, whereas valproic acid sensitizes OS cells to Fas-mediated cell death and decreases production of soluble Fas [82, 83], thus further potentiating OS sensitivity to NK cell killing. However, both HDAC inhibition [84] and DNA hypomethylation [85] can have an adverse direct effect on NK cell function, necessitating approaches that sequence drug therapy and cell therapy. A narrow-spectrum HDAC inhibitor, SNDX-275, has been shown to increase osteosarcoma killing through upregulation of Fas [86], c-FLIP [87], and MICA [85], and also augments NK cell function through upregulation of NKG2D [36] (see Book 2, Chap. 4 "Targeting the Cancer Epigenome with Histone Deacetylase Inhibitors in Osteosarcoma").

PD-1 and its ligands play a role in evasion of malignant tumor cells from the immune system. Recently, immunotherapy with anti-PD-1 inhibitors has been approved for treatment of non-small cell lung carcinoma, urothelial cell carcinoma, and Hodgkin lymphoma. In vitro studies have shown increased cytoplasmic expression of PD-1 in bone sarcomas [98]. Pembrolizumab, an anti-PD-1 antibody, has been studied as a treatment option for patients with advanced soft tissue sarcoma or bone sarcoma. Even though the primary endpoint of overall response was not met for either cohort, promising activity was seen in certain histologies and further study is underway [99].

Lenalidomide is an immunomodulatory thalidomide derivative with activity against a wide variety of cancers. Lenalidomide may enhance NK cell number and maturation through increased IL-15 levels [88]. Lenalidomide augments the activity of NK cells by enhancing ADCC of mAb against solid tumors [89], including trastuzumab and cetuximab activity against bone sarcomas [90]. Mifamurtide (MTP-PE), discussed extensively in Book 1, Chap. 11, may exert some of its anticancer effects by enhancing NK cell activity [91].

Heat treatment of OS cell lines increases their susceptibility to NK cell-mediated lysis through upregulation of heat shock protein 72 (HSP72) expression [92]. Hypoxia decreases the expression of MICA on OS cell lines in a hypoxia-inducible factor 1α (HIF-1α)-dependent manner and consequently decreases the susceptibility of tumor cells to NK cell lysis [93]. However, hypoxia does not interfere with MoAb-mediated target cell killing by ADCC [94].

NK Cell Adoptive Immunotherapy

Clinical NK Cell Sources and Trials

NK cells may be obtained in numbers sufficient for clinical use in adoptive immunotherapy by

apheresis and CD3 depletion or by ex vivo expansion. NK cells have been successfully expanded from peripheral blood, cord blood, and pluripotent or embryonic stem cells. Expansion methods have included various combinations of cytokines, cytokine fusion proteins, cytokines and OKT3, cytokines and stromal support, antibody-coated beads, and feeder cells obtained from peripheral blood or derived from EBV-lymphoblastoid cell lines or K562 (reviewed in [42]).

NK cells have been delivered by adoptive transfer to very few patients with osteosarcoma. Expanded NK cells were given as adjuvant immunotherapy after matched allogeneic transplant (C. Mackall, personal communication, ClinicalTrials.gov Identifier NCT01287104). As mentioned above, KIR-ligand incompatibility is associated with increased NK cell activity against osteosarcoma cell lines [27]. Thus, similar to the observed benefit in AML, it is likely that approaches using mismatched allogeneic donors for NK cell therapy of osteosarcoma will have a greater antitumor effect than matched or autologous NK cells. NK92 is a cell line derived from a patient with NK cell leukemia and has NK cell-like activity against tumor cell lines. Clinical grade irradiated NK92 cells have been infused in a patient with advanced osteosarcoma, though no response to treatment was observed [95]. Newer studies evaluating the use of expanded natural killer cells following cytotoxic chemotherapy are being utilized in neuroblastoma and CNS tumors, as well as other sarcomas such as Ewing sarcoma and rhabdomyosarcoma.

Future Approaches

The recent availability of clinically viable approaches for obtaining large number of NK cells now enables the clinical testing of combination therapies to enhance NK cell function and osteosarcoma sensitivity. The antigen-binding domains of all of the mAb mentioned above have been identified and genetically manipulated to generate chimeric antigen receptors (CARs) that mediate enhanced killing by T cells (see Book 1, Chap. 10). As an alternative to T cells, genetic modification to express CAR may also be applied to NK cells to further enhance their activity against osteosarcoma [96]. These CARs also have potential application for clinical development in NK cells, and CAR with NKG2D-like specificity can further improve the NK cell immunotherapy of osteosarcoma in murine models [97]. The ability to deliver large cell doses, combination with sensitizing chemotherapy, radiation, or immunomodulatory drugs, and genetic modifications will be the subjects of cutting-edge trials in the decade to come.

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Nanocapsule Delivery of IL-12

13

Justin E. Markel, Ryan A. Lacinski, and Brock A. Lindsey

Abstract

Interleukin(IL)-12 is a protein that activates T cells and macrophages to kill tumor cells. However, despite this cytokine showing strong antitumor activity in preclinical settings, translation to patients has been slowed by toxic side effects, poor distribution to peripheral tissues, and improper dosing regimens. Osteosarcoma (OS) is an aggressive primary tumor of bone that has shown particular responsiveness to recombinant (r)IL-12 in preclinical models. Poly(lactic-co-glycolic) acid (PLGA) nanospheres, an FDA-approved drug delivery vector, may be a viable delivery vector for transporting biologically active IL-12 to tissues without disturbing normal homeostasis. In this chapter, we explore the potential for using IL-12-loaded nanospheres (IL-12-NS, <1 μm in diameter) to treat cancer, describe the synthesis process, and examine a typical protein release profile while providing insight and future directions of nanoscale tumor immunotherapeutics.

Keywords

IL-12 · Nanoparticle · Nanocapsule · PLGA · Osteosarcoma · Immunotherapy

J. E. Markel · R. A. Lacinski · B. A. Lindsey (✉)
West Virginia University School of Medicine, Department of Orthopaedics, Morgantown, WV, USA
e-mail: blindsey@hsc.wvu.edu

Interleukin-12 Immunotherapy: The Past, Present and Future

Why IL-12?

Interleukin(IL)-12, a member of the IL-12 family of cytokines, has a long history of antitumor activity against a wide range of preclinical cancer models [1, 2]. Originally characterized as a potent inducer of natural killer (NK) cell cytotoxic activity, IL-12 is now recognized as a key regulator of the cell-mediated immune response and a bridge between innate and adaptive immunity [3]. Although the immunostimulatory functions of IL-12 can be induced locally with intratumoral (i.t.) injections, preclinical models suggest that more complete antitumor responses are achieved via systemic administration [4]. By influencing the differentiation of $CD4^+$ T helper (T_H) cells into T helper type 1 (T_H1) cells, IL-12 coordinates antitumor immunity through pro-inflammatory M1 activation of macrophages and stimulation of cytotoxic T (T_C) cells [5–7]. Signaling through IL-12 is also crucial for the survival and reactivation of circulating memory cells, making it an important component of long-term cancer remission [8]. Upon binding of the heterodimeric p35p40 (p70) IL-12 protein to its type 1 cytokine receptor on the surface of T cells, Janus kinase-signal transducer and activator of transcription protein (JAK-STAT) signaling pathways are propagated through the downstream effector molecule

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STAT4 [9]. In addition to its effects on the immune system, IL-12 has strong anti-angiogenic properties, making it an extremely attractive protein candidate for cancer therapies targeting metastasis and/or highly vascularized tumors [10].

Osteosarcoma (OS) is characterized by extensive disruption of the genome [11] resulting in a plethora of neoantigens that can stimulate an immune reaction if given the proper co-stimulatory signals [12], and evidence of IL-12's importance to OS immunology stems from a variety of sources: at the cytokine level, it was found that *IL-12* gene polymorphisms resulting in low serum IL-12 levels were associated with increased OS risk in a cohort of Chinese patients, with affected individuals having significantly lower levels of circulating IL-12 compared to healthy controls [13]. Moreover, IL-12 has been shown to upregulate Fas expression on OS tumor cells, an occurrence that inversely correlates with metastatic potential by increasing the likelihood of Fas-Fas ligand (Fas L)-induced apoptosis [14–16]. In a preclinical murine model, specific targeting of lung metastases through aerosol gene therapy with a polyethyleneimine-transported plasmid increased parenchymal IL-12 mRNA expression while decreasing metastatic burden [17].

At the cellular level, the relative abundance of IL-12-associated immune events in both the primary tumor microenvironment (pTMic) and systemic tumor macroenvironment (sTMac) can influence the progression of disease. Macrophages account for a large portion of the cellular content of OS primaries, and adopt anti- or pro-tumorigenic phenotypes depending on activation status, namely classical (M1) or alternative (M2), respectively. Classical activation of macrophages stimulates IL-12 production, T_H1 polarization of T_H cells, and the release of Type 1 interferons (IFNs) like IFN-γ that in turn stimulate additional inflammation and further M1 activation [18]. The drug mifurmatide, a synthetic derivative of a cell wall component of *Mycobacterium* species, is approved in Europe for the treatment of nonmetastatic OS and is an M1 activator of macrophages [19, 20]. Still, immunosuppressive influences from the tumor can override IL-12 signaling cascades and cause macrophages to adopt alternative M2 phenotypes, bestowing actions that are mainly pro-tumorigenic. These cellular changes are then reflected in both the pTMic and the sTMac, where the immune cell composition (A.K.A., the "immunophenotype," for more information see Chap. 6) can be assembled to yield insights regarding prognosis. Indeed, the presence of IL-12-secreting M1-activated macrophages (as determined by inducible nitric oxide synthase [INOS] protein expression) in the OS pTMic has been shown to correlate negatively with metastasis [21]. Figure 13.1 displays a visual summary of the major effects of IL-12 signaling.

The Past and Present (1994–2019)

Building upon a plethora of exciting preclinical data from multiple tumor types, the first Phase I clinical trial examining the clinical utility of intravenous (i.v.) recombinant human IL-12 (rhIL-12) for human cancer began in the spring of 1994 [22]. Upon enrollment in the study, all subjects were administered a preliminary rhIL-12 test dose and, if tolerated, were given a subsequent series of six high-intensity 5-day treatment cycles every 15 days. Here, objective tumor responses were observed in only 2 of the 40 subjects, with the majority of patients experiencing lymphopenia, elevated liver enzymes (occasionally dose-limiting), flu-like symptoms, and transiently increased serum IFN-γ levels that paradoxically diminished as treatment continued. Nevertheless, data from this study prompted a Phase II trial in renal cell carcinoma (RCC) that ended prematurely due to dose-limiting toxicities (DLTs); it was later realized that the test doses given to patients in the Phase I study produced an immunoprotective effect of unknown etiology that improved drug tolerability [23]; further studies investigating the clinical utility of rhIL-12 were thus motivated to construct more conservative regimens with designated rhIL-12 acclimation periods.

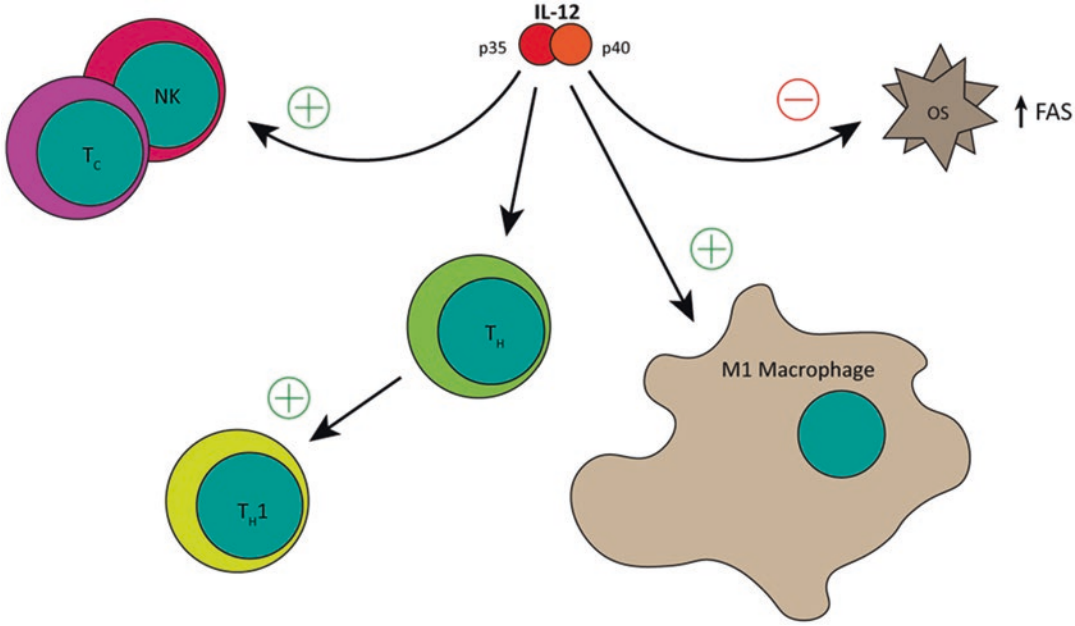

Fig. 13.1 Visual summary of the major effects of IL-12 signaling

In an attempt to mitigate systemic toxicities, most subsequent studies began to move away from i.v. rhIL-12 [24] and focus instead on subcutaneous (s.c.) and locoregional routes of delivery. One notable exception includes a 2004 Phase II clinical trial that compared the efficacy of i.v. to s.c. delivery of rhIL-12 in relapsed and refractory non-Hodgkin's lymphoma (NHL). In this study, although the objective response rates were suboptimal, patients receiving i.v. administration showed higher response rates (40%) than those dosed subcutaneously (7%); importantly, the inclusion of an rhIL-12 primer dose coupled with less protein per treatment cycle allowed for improved patient tolerability in the i.v. administration group [25]. A concurrent Phase II trial conducted for advanced cervical cancer using a similar regimen observed increased cell-mediated immune responses that did not associate with increased survival [26]. Other studies investigating i.v. therapy used dose titration to decrease toxicity and increase the longevity of IFN-γ responses (an effect that was shown to associate with clinical response) [27].

In the late 1990s, two Phase I clinical trials of s.c. rhIL-12 therapy in patients with advanced RCC and metastatic melanoma showed positive responses while markedly reducing the frequency of DLTs [28, 29]. Unfortunately, future investigations in RCC were stopped once a multicenter Phase II trial comparing rhIL-12 to IFN-α2a reported no increased efficacy [30]. Intraperitoneal (i.p.) and intravesicular (i.vs.) injections of rhIL-12-containing solutions for metastatic ovarian and bladder cancer, respectively, have also shown satisfactory patient tolerability without appreciably improving survival [31–33]. Of note, one Phase II study carrying particular importance to OS cytokine immunotherapy followed relapsed follicular NHL patients treated with s.c. rhIL-12 in combination with the anti-CD20 monoclonal antibody rituximab. Here, researchers observed decreased survival rates in the combination group versus rituximab alone [34]; these findings were later attributed to a life-threatening state of immune system over-activation known as T cell exhaustion (TCE) [35], characterized by lymphocyte anergy, systemic inflammatory response syndrome (SIRS), and ultimately multiple organ failure (MOF). As TCE is a phenomenon inherently associated with OS disease progression

[36], the exhaustive side effects of IL-12 administration must be adequately addressed to prevent further exacerbation of this deadly condition; these studies highlight both the importance of proper dosing strategies as well as the value of an adequate immune monitoring platform (see Chap. 6) when administering potentially immune-exhaustive experimental therapeutics.

Although they have presented a mixed bag of clinical findings, studies investigating locoregional IL-12 delivery in combination with other immunostimulatory cytokines, monoclonal antibodies, and radiation have also been investigated. Direct modifications to the IL-12 protein itself, such as fusion to necrosis-targeting antibodies for enhanced delivery to irradiated sites [37] and peptide truncation to decrease toxicity [38], have been published, as well as the effectiveness of IL-12 administration as an adjuvant for cancer vaccination [39]. For OS, one study showed that IL-18, a member of the IL-1 family of cytokines with similar properties to IL-12, has particular efficacy against OS when given in combination with IL-12 [40].

Another method of IL-12 delivery includes the use of genetically engineered cell types to increase i.t. IL-12 levels upon introduction into the pTMic. Fibroblasts, dendritic cells (DCs), mesenchymal stem cells, T lymphocytes (e.g., CAR T cells), and even tumor cells themselves have been engineered to release supraphysiological levels of IL-12 within the pTMic for the purposes of increasing the immunogenicity of tumor antigens [41–46]. Viruses and DNA vaccines carrying IL-12-coding genes to metastatic sites have also been studied; in 2003, Shu-Fang Jia et al. eliminated OS pulmonary metastases using aerosol therapy to direct an IL-12-coding polycationic DNA carrier directly to the lungs of mice [17]. Although this method of IL-12 delivery did not translate to the clinic, it was one of the first instances displaying the in vivo efficacy of IL-12 against metastatic OS. In 2007, a Phase I trial for pediatric cancer (including seven OS patients) was published combining cancer vaccines with genetically engineered IL-12-secreting DCs; while intranodal administration resulted in antigen-specific IFN release in vitro, no OS patients displayed noticeable improvement following vaccination [47].

Without toxic loading doses, s.c. and i.v. administration of rIL-12 does not ensure adequate distribution to peripheral tissues. To solve this issue, IL-12 can be encapsulated within biodegradable organic polymers that release their contents in a slow and controlled manner, thereby reducing side effects and prolonging antitumor activity. In 1999, Kuriakose et al. showed that tumor regression with a single i.t. dose of rIL-12-loaded polylactic (PLA) microspheres (IL-12-MS, >1 μm in diameter) was superior to multiple doses of free rIL-12 in a head and neck tumor xenograft model following the transfer of human peripheral blood lymphocytes [48]. These data were further supported a year later when Egilmez et al. discovered that tumor regression from IL-12-MS was superior not only to i.t., but also i.p. free rIL-12 in a Line-1/BALB/c murine alveolar lung adenocarcinoma model [49]. Shortly thereafter, i.t. injection of rmIL-12-loaded PLA microspheres was found to regress primary MT-901 breast cancer tumors while simultaneously inducing systemic antitumor immunity in the form of circulating, antigen-primed memory T cells that prevented tumor growth following rechallenge [50].

One possible answer to many of the above problems could be rIL-12-loaded poly(lactic-co-glycolic) acid (PLGA) nanospheres (IL-12-NS, <1 μm) for i.v. delivery. PLGA colloidal systems provide protection for and enhance the stability of the entrapped compound while providing enhanced delivery to target tissues. Indeed, while many other researchers have moved towards i.t. formulations, other literature continues to support the notion that cancers (including OS) induce systemic immune dysfunctions unrelated to the immunophenotype of the primary lesion. By using flow cytometry to assess splenic immunophenotypes, systemic immunotherapies like anti-programmed death ligand 1 (PD-L1) can reverse malignancy-induced immunosuppression [36]; therefore, the long-term goal is to use the PLGA capsule as a barrier to allow safe transit of IL-12-NS through the blood to the peripheral tissues, where they can release their protein slowly

over extended periods of time. Much of the remainder of this chapter will describe the process of synthesizing IL-12-NS for systemic administration.

The Future

Analogous to human immunodeficiency virus (HIV) anti-retroviral therapy (ART), rIL-12 will most likely represent one component of an immunotherapeutic "cocktail" tailored to the specific immunological needs of each patient. That said, there are certain themes that have arisen from three decades of IL-12 research that must be considered, of which PLGA encapsulation may help solve:

1. *Large bolus loading doses of rIL-12 are required to ensure adequate tissue distribution when delivered systemically, often resulting in DLTs that can be somewhat attenuated by dose escalation.* Upon administration of IL-12-NS, PLGA encapsulation delays the release of entrapped IL-12, thereby allowing for increased delivery to peripheral tissues.
2. *I.V. rIL-12 dosing regimens require multiple injections including dose titrations to prevent unwanted pro-inflammatory reactions (DLTs).* IL-12-NS has intrinsic dose titration properties, as encapsulated protein is released slowly and continually as the PLGA coating hydrolyzes in aqueous environments. Additionally, preliminary in vitro data show that one sample of IL-12-NS continues to elute protein up to 14 days, which may drastically reduce the number of injections needed per patient.
3. *I.V. rIL-12 cannot be targeted to specific areas of interest without direct modification of the protein itself, which may lead to decreased biological function.* Due to the presence of free carboxylic acid moieties, PLGA nanospheres can undergo a number of different surface modifications that allow for control over their in vivo biodistribution properties; some common conjugates that have been tested include monoclonal antibodies for specific cell targeting, albumins to increase the enhanced permeation and retention (EPR) effect, and chitosan for enhanced lung delivery via the formation of transient microaggregates in pulmonary capillaries [51–53].

To illustrate the concept of using IL-12-NS for the treatment of OS, consider the following scenario. OS induces systemic immune suppression in mice (Fig. 13.2A) that can be reversed by checkpoint blockade using anti-PD-L1 (Fig. 13.2B); that is, their systemic immunophenotype returns to baseline status (see Chap. 6 for more details). However, baseline status does not provide enough immune stimulation to effectively reduce tumor burden in mice with advanced disease. On the other end of the spectrum, when patients in the aforementioned Phase II study of rituximab/rhIL-12 combination therapy for relapsed and refractory NHL succumbed to DLTs, their immunophenotypes were pushed past stimulation and into the realm of overstimulation and TCE (Fig. 13.2D). However, in a background of anti-PD-L1 checkpoint blockade providing disinhibition of activated T cells, slow and sustained delivery of low dose rIL-12 (which is generally considered safe [54]) from hydrolyzing PLGA nanospheres to the sTMac may provide the systemic stimulation necessary to effectively reduce disease burden while still remaining beneath the exhaustion threshold (Fig. 13.2C); from this example, it is clear that a real-time immunophenotypes monitoring platform, like the one described in Chap. 6, would be of considerable value for this application.

Production of IL-12-Loaded PLGA Nanospheres

Double Solvent Emulsion-Evaporation Method: The Basics

Synthesizing protein-loaded PLGA nanospheres via the double solvent emulsion-evaporation (DSEE) method (AKA, the water-in-oil-in-water [$w_1/o/w_2$] method) involves three main steps: *(1) primary emulsion (w_1/o), (2) double emulsion, and (3) nanosphere isolation and purification*.

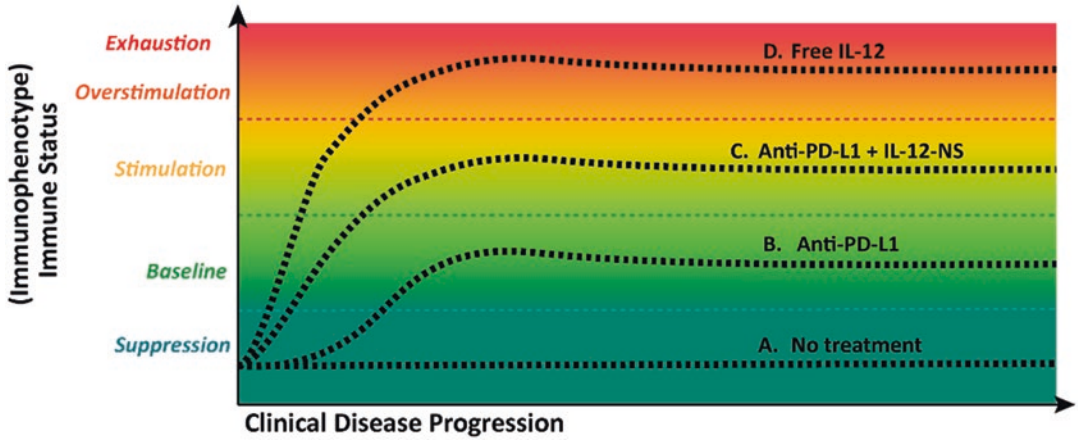

Fig. 13.2 Hypothetical application of IL-12-NS for the treatment of metastatic OS. (A) Pathologic immune suppression observed in patients with OS. (B) Systemic treatment of metastatic OS with the immune checkpoint blocker anti-PD-L1 reverses malignancy-induced immunosuppression back to baseline but does not improve survival. (C) Systemic treatment of metastatic OS with IL-12-NS in the background of anti-PD-L1 T cell disinhibition provides adequate immune stimulation to reduce disease burden without falling into the realm of exhaustion. (D) Toxic loading doses of free IL-12, which are necessary to ensure adequate distribution to peripheral tissues, push the immune system into a state of exhaustion leading to SIRS, MOF, and ultimately death

First, the drug of choice is suspended in an aqueous solution to create the internal aqueous phase (w_1). The internal aqueous phase (w_1) is then added to a solution of organic solvent (o) containing polymer and agitated briefly (usually via homogenization or sonication) to create the primary emulsion (w_1/o, Fig. 13.3a); some commonly used organic solvents include dichloromethane (DCM), acetone (AC), and ethyl acetate (EA). During this step, the emulsion can be kept in an ice bath to counteract the rise in temperature caused by agitation. Next, the primary emulsion (w_1/o) is added to the external aqueous phase (w_2) and agitated again to form the double emulsion ($w_1/o/w_2$, Fig. 13.3b). The w_2 phase contains a stabilizer (usually polyvinyl alcohol (PVA)) to prevent coalescence of the resulting emulsion droplets [55]. As the agitation/shear force increases, the droplets and hence resulting particles become smaller [56]. To solidify the droplets and form particles, the organic solvent is evaporated at the water/air interface with stirring. As the organic solvent evaporates, the PLGA precipitates, thereby encapsulating the internal aqueous phase in a spherical matrix (Fig. 13.3c). The last step involves removal of excess PLGA and non-encapsulated protein via a series of washes, flash-freezing, and lyophilization for long-term storage. By minimizing contact between the internal aqueous phase and organic solvent, this method of synthesis is suitable for the encapsulation of bioactive proteins [57].

Double Solvent Emulsion-Evaporation Method of Encapsulating rIL-12 with Homogenization

The primary emulsion is formed by adding aqueous rIL-12 to a small beaker containing PLGA in DCM and agitating on ice (Fig. 13.4a). The double emulsion is formed by combining the primary emulsion with an aqueous solution of PVA/NaCl and agitating for 8 minutes on ice (Fig. 13.4b); the addition of salt to the external aqueous phase increases protein encapsulation while decreasing the surface area of the resulting product [58]. Both agitation steps in the homogenization method are done with a standard tissue homogenizer tip set at medium speed (~175,000 RPM). The double emulsion is then stirred overnight at room temperature (RT)

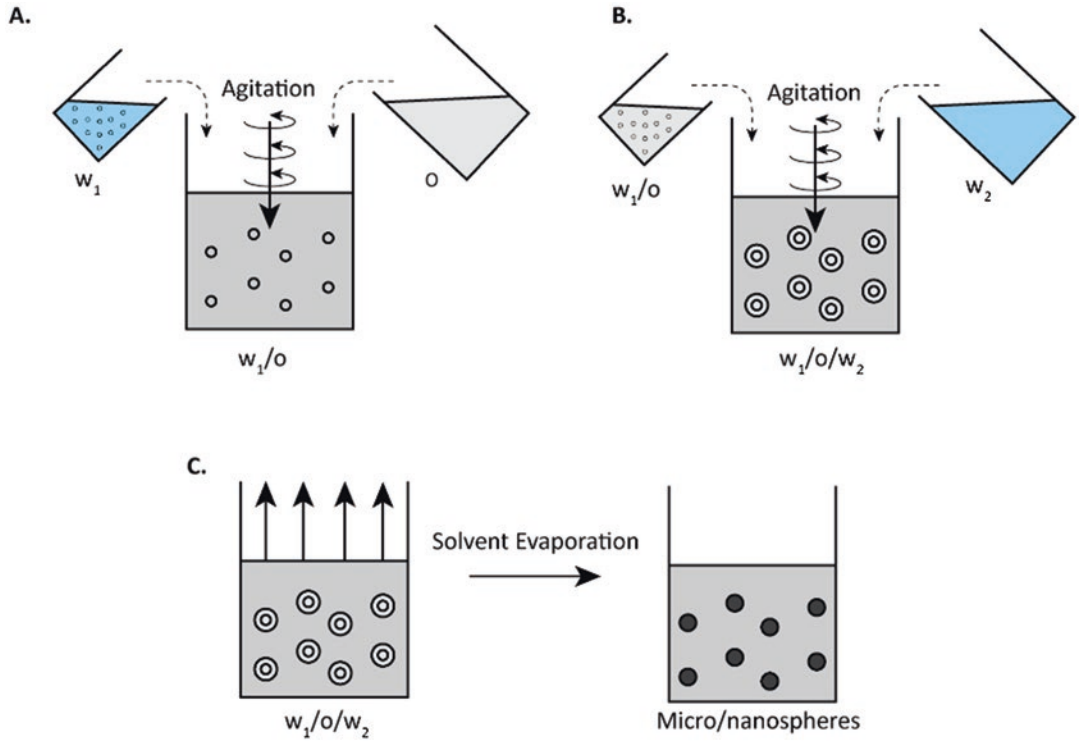

Fig. 13.3 Basic process for the creation of biodegradable micro-/nanospheres using the double solvent emulsion-evaporation technique. (**a**) The internal aqueous phase (w_1), which contains the bioactive compound of interest, is combined with polymer dissolved in an organic solvent (o) and agitated to create the primary emulsion (w_1/o). (**b**) The primary emulsion is then dispersed in the external aqueous phase (w_2) and agitated to form the double emulsion ($w_1/o/w_2$). (**c**) The double emulsion is stirred to remove the organic solvent via evaporation which solidifies the droplets into micro-/nanospheres

to evaporate off the DCM (Fig. 13.4c), and the resulting product is a mixture of both microspheres and nanospheres; the morphology of the resulting product prior to centrifugation can be seen in Fig. 13.4d. To remove the microspheres, the colloid is centrifuged at low g (<1000 × g) and the resulting supernatant aspirated and stored on ice. Once microspheres are removed, the nanospheres in the supernatant are harvested by ultracentrifuging (>50,000 × g), washing to remove non-encapsulated protein and excess PLGA, flash-frozen, and lyophilized for long-term storage (Fig. 13.4e). Figure 13.4f displays the resulting product once the microspheres and excess protein and PLGA are removed. Zeta potential (ζ) is a physical property that determines colloidal stability and hence the propensity of suspended particles to flocculate; IL-12-NS made via the homogenization method (IL-12-HNS) have a ζ of -15.1 ± 1.25 mV.

Double Solvent Emulsion-Evaporation Method with Ultrasonication

First, the primary emulsion is formed by using a microprobe sonicator set at 50 W to agitate aqueous rIL-12 in PLGA dissolved in DCM in an ice bath (Fig. 13.5a). For this application, each emulsion is formed in a glass test tube to minimize time needed to create a homogeneous mixture. The primary emulsion is then transferred to another test tube containing 1% PVA in water, and the solution is sonicated on ice for another 10 seconds at 50 W power to create the double emulsion (Fig. 13.5b). The double emulsion is

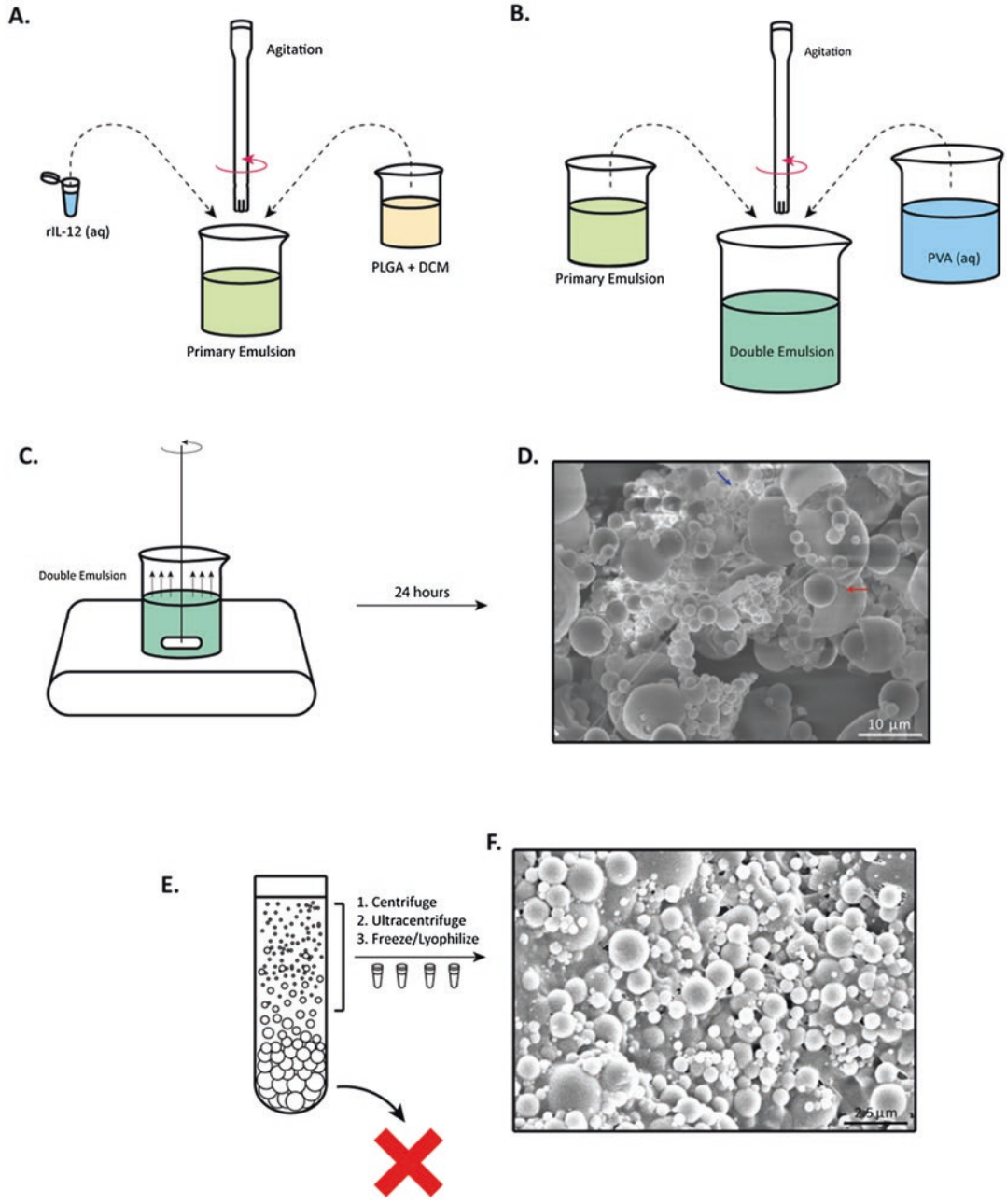

Fig. 13.4 Stepwise procedure for double solvent emulsion-evaporation method of encapsulating rIL-12 with homogenization. (**a**) To form the primary emulsion, aqueous rIL-12 is added to PLGA dissolved in DCM and homogenized for 6 minutes at 17,500 RPM. (**b**) To form the double emulsion, the primary emulsion is dispersed in an aqueous solution of 2% PVA/0.8% NaCl and homogenized for 8 minutes at 17,500 RPM. (**c**) The double emulsion is stirred overnight to evaporate off the DCM, forming the products shown by scanning electron microscopy (SEM) in (**d**), a mix of microspheres and nanospheres. The red arrow is pointing to microspheres, and the blue arrow is pointing to a next of nanospheres. (**e**) The microspheres are excluded and the nanospheres harvested via centrifugation (<1000 × g) and ultracentriguation (>50,000 × g), respectively. Following a series of washes, flash-freezing, and lyophilization, the final nanosphere product. (**f**). Scanning electron micrograph of the nanosphere product formed via the homogenization method of PLGA nanosphere synthesis

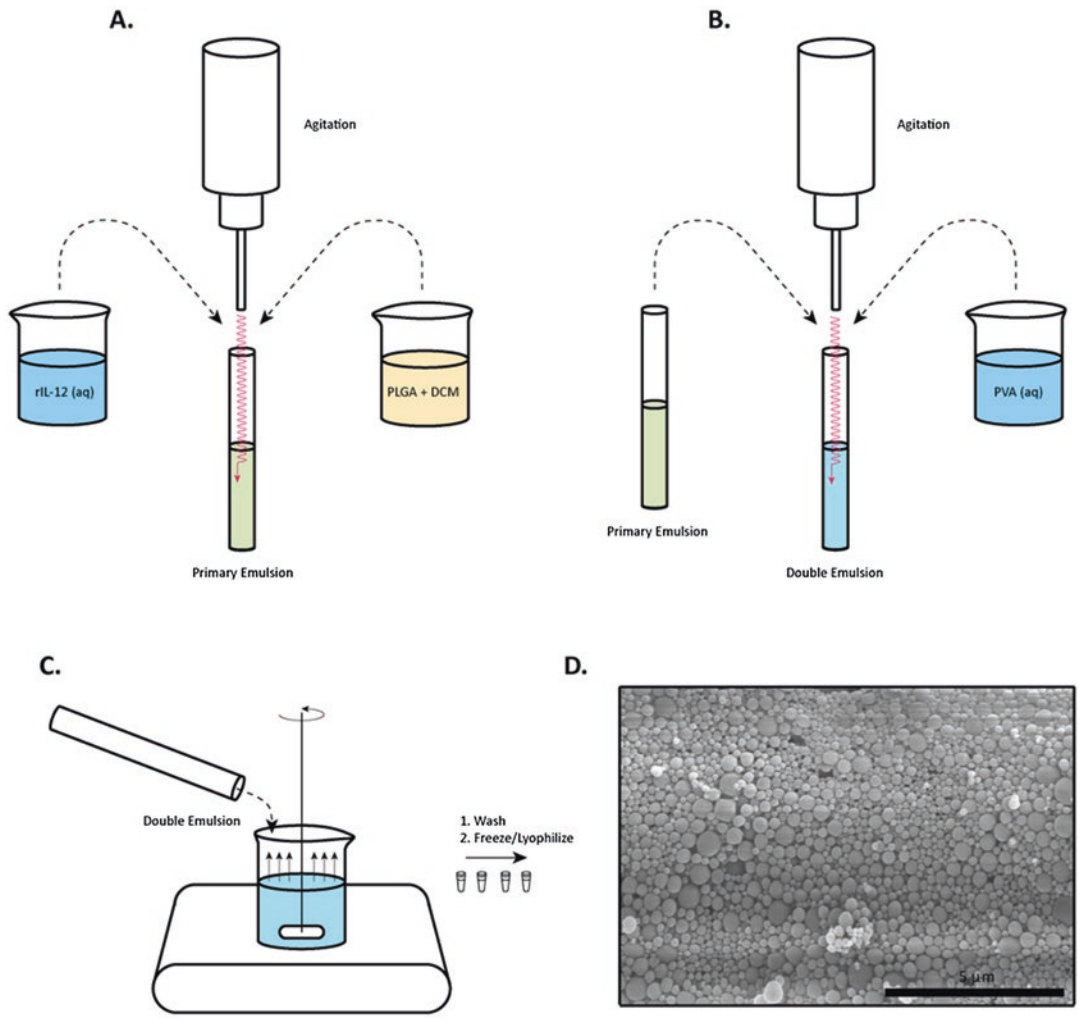

Fig. 13.5 Stepwise procedure for double solvent emulsion-evaporation method of encapsulating rIL-12 with ultrasonication. (**a**) To form the primary emulsion, aqueous rIL-12 is added to PLGA dissolved in DCM and sonicated at 50 W for 10 seconds. (**b**) To form the double emulsion, the primary emulsion is dispersed in an aqueous solution of 1% PVA and sonicated again at 50 W for 10 seconds. (**c**) The double emulsion is stirred for 3 hours to evaporate off the DCM, washed, flash-frozen, and lyophilized for long-term storage. (**d**) Scanning electron micrograph of the nanosphere product formed via the ultrasonication method of PLGA nanosphere synthesis

then stirred for at least 3 hours to evaporate the organic solvent, washed, flash-frozen, and lyophilized for long-term storage (Fig. 13.5c); a scanning electron micrograph of the final nanosphere product is shown in Fig. 13.5d. Due to the type of agitation used, the ultrasonication method does not require an extra isolation step as in the homogenization method. The zeta potential of IL-12-NS made via the ultrasonication method (IL-12-SNS) has a ζ of -36.35 ± 2.85 mV.

In Vitro IL-12 Release Characterization: Elution Study

Characterizing Nanosphere Protein Release Profile via an In Vitro Elution Study

Once a batch of IL-12-NS is synthesized, the next step is to characterize how the protein is released over time, called protein *elution*. To conduct an

in vitro elution profile, a known quantity of suspended nanospheres is shaken vigorously at 37 °C and sampled once every 24 hours. At the end of the study, the amount of biologically active protein eluted each day can be measured using enzyme-linked immunosorbent assays (ELISA) and expressed as a percent of total protein eluted throughout the entire release profile.

To understand how the encapsulated protein is released, it is important to consider the ultrastructure of a PLGA nanosphere. When the second emulsion is formed, protein becomes wound up in tiny strands of PLGA (much like a ball of string), which coalesces and precipitates into a sphere as the organic solvent is removed. During this process, protein becomes both entrapped within the polymer matrix (Fig. 13.6a, top right panel, blue arrow) and adsorbed to the outer surface (Fig. 13.6a, top left panel, red arrow), producing a characteristic biphasic elution curve shown in Fig. 13.6b. The burst phase, which normally occurs between baseline and 2 days, is due to the adsorbed protein on the surface of the nanospheres being released upon resuspension in an aqueous medium (Fig. 13.6a, bottom panel). The controlled release phase is due to entrapped protein (Fig. 13.6a, bottom panel) and is released slowly over time as the PLGA hydrolyzes via the reaction in Fig. 13.6c. The elution characteristics can be altered by varying a number of each synthesis parameter; however, an in-depth discussion of nanosphere modification is beyond the scope of this book.

Elution Profiles: Homogenization and Ultrasonication Methods

We will now compare the release profiles of IL-12-HNS and IL-12-SNS loaded with recombinant mouse (rm)IL-12. At 8 days, rmIL-12-loaded-HNS at a concentration of 1 billion particles/mL released ~3500 pg of p70 rmIL-12, or 0.7 pg per 100,000 particles. SNS, which have

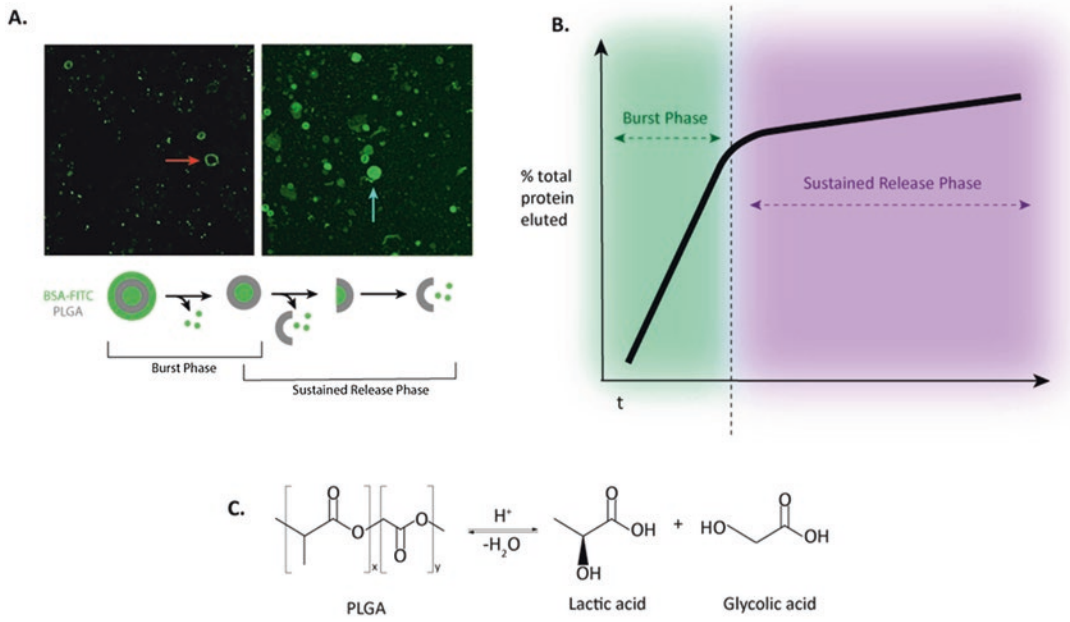

Fig. 13.6 Release characteristics of a typical protein-loaded PLGA nanosphere elution profile. (**a**) Top two panels: PLGA spheres were loaded with FITC-tagged bovine serum albumin (BSA) and imaged via confocal microscopy to show the distribution of protein both adhered to the surface (left) and entrapped within the matrix (right). Bottom panel: Schematic showing the mechanism of protein release from PLGA nanospheres creating a biphasic release profile, shown in (**b**) as a percent of total protein released. (**c**) Schematic showing the hydrolysis of PLGA into lactic and glycolic acid

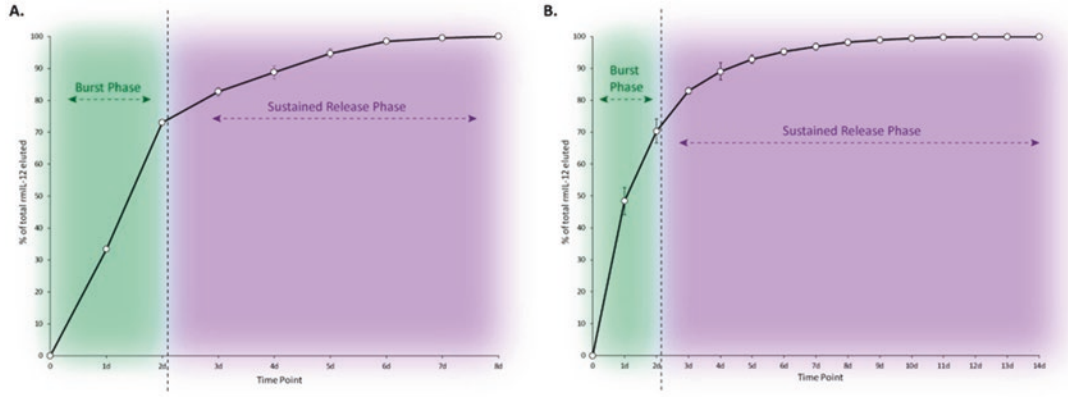

Fig. 13.7 Comparative analysis of rmIL-12-HNS and rmIL-12SNS elution profiles. (**a**) Elution of 1 billion rmIL-12-HNS/mL over the span over 8 days showing the characteristic biphasic release profile, with ~70% total IL-12 being released within the burst phase (Day 0 to Day 2) and the remainder during the sustained-release phase (Day 2 to Day 8). (**b**) Elution of 100 billion rmIL-12-SNS/mL over the span over 14 days showing the characteristic biphasic release profile, with ~70% total IL-12 being released within the burst phase (Day 0 to Day 2) and the remainder during the sustained-release phase (Day 2 to Day 14). *Y* values display the cumulative protein eluted per day/total protein eluted; each value is an average of $n = 2$ biological replicates

higher solubility and less tendency to flocculate, were suspended at a concentration of 100 billion/mL and released a total of ~230,000 pg of rmIL-12 over 14 days, averaging 0.46 pg per 100,000 particles. Therefore, although IL-12-HNS have a more efficient protein release profile per capsule, it is likely that SNS will carry more clinical impact due to their superior solubility properties and extended release profile. Figure 13.7a and b shows the release profiles as a function of cumulative protein eluted per day/total protein eluted for both HNS and SNS, respectively. Approximately, 70% of the total protein for both methods is released over the first 48 hours during the burst phase, where the remaining 30% is spread over 6 (Fig. 13.7a) and 12 (Fig. 13.7b) days during the sustained-release phase for HNS and SNS, respectively.

Conclusions and Future Perspectives

IL-12 has a long and controversial history as an agent for cancer immunotherapy. However, one central theme that has emerged over the years is that proper dosing is a key factor determining both safety and therapeutic efficacy. As of yet, the line separating what is helpful from harmful has not been established, and the number of variables with potential immunological influences (e.g., tumor type, co-morbidities, prior therapies, age, sex, etc.) further complicates the scenario. Therefore, the next step will be to conduct extensive dose escalation trials that evaluate the effects of rIL-12-NS on the systemic immunophenotype, paying careful attention to the warning signs of overstimulation. Additionally, for future attempts at IL-12 tumor immunotherapy to be successful, a reliable and efficient means of assessing immune status must be developed; as such, this project has evolved alongside that discussed in Chap. 6.

The importance of establishing systemic immunity to a malignancy is supported by earlier observations that suggest that the antitumor efficacy of rIL-12 is maximized upon systemic administration. Indeed, studies have shown that tumor-induced immunosuppression can be reversed with immunotherapy and that immunotherapy is only successful following the activation of secondary immune organs [59]. However, systemic rIL-12 delivery in humans has thus far proven difficult due to toxic loading doses being needed to ensure adequate tissue delivery. PLGA encapsulation, which effectively reduces the

amount of available rIL-12 at one time, may be a promising method for delivering controlled doses of IL-12 to tissues without massive disruption of normal homeostatic processes. In theory, unlike the destructive inflammatory syndromes induced by introducing free rIL-12 into the bloodstream, IL-12-NS-laden tissues would slowly and continually influence the sTMac to favor pathways that support cell-mediated immunity while shifting the macrophage polarization status towards an M1 bias. In turn, priming tissues for a cell-mediated immune response may increase the chances that circulating effector cells can respond appropriately when encountering tumor antigen.

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14

Discovery of Cell-Surface Vimentin (CSV) as a Sarcoma Target and Development of CSV-Targeted IL12 Immune Therapy

Izhar S. Batth and Shulin Li

Abstract

This chapter discusses a novel target of osteosarcoma (OS), cell-surface vimentin (CSV), and a novel generation of interleukin-12 (IL12), CSV-targeted IL12, for treating OS tumor metastasis. Vimentin is a known intracellular structural protein for mesenchymal cells but is also documented in tumor cells. Our recent study definitively revealed that vimentin can be translocated to the surface of very aggressive tumor cells, such as metastatic cells. This CSV property allows investigators to capture circulating tumor cells (CTCs) across any type of tumor, including OS. CTCs are known as the seeds of metastasis; therefore, targeting these cells using CSV is a logical approach for use in a metastatic OS setting. Interestingly, we found that the peptide VNTANST can bind to CSV when fused to the p40 subunit encoding the DNA of IL12. Systemic delivery of this CSV-targeted IL12 immune therapy inhibited OS metastasis and relapse in a mouse tumor model as detailed in this chapter. This CSV-targeted delivery of IL12 also reduced toxicity of IL12. In summary, this chapter details a novel approach for safe IL12 immune therapy via targeting CSV.

Keywords

IL12 · Electroporation · Gene therapy · OS · Tumor-targeting · Cytokine · Immune therapy

Introduction

Sarcomas are a mesenchymal type of cancer that can develop in the soft tissues or bones. Osteosarcomas (OS) are the most common primary bone malignancy in children and young adults, accounting for 2% of all childhood (0–14 years) cancers [1, 2]. Most importantly, the overall survival rates for OS patients have not improved meaningfully over the last 30 years. Other than leukemia and lymphoma, osteosarcomas are the most prevalent cancer diagnosis in adolescents. OS occurs most commonly at the ends of long bones, where there is a high rate of osteoblast proliferation, such as the ends of the tibia, femur, and humerus, with the lungs being the most prevalent distant metastasis location. Metastatic OS has a five-year survival rate of 19–30% [3].

The treatment for OS is underdeveloped, with surgery in combination with chemotherapy being the primary treatment approach. Treatment with

I. S. Batth · S. Li (✉)
The University of Texas MD Anderson Cancer Center, Department of Pediatrics – Research, Houston, TX, USA
e-mail: SLi4@mdanderson.org

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surgery alone only yields a 20% reduction in the appearance of metastatic disease. Therefore, chemotherapeutic approaches are necessary to improve overall survival. The most common regimens used include high-dose methotrexate in combination with doxorubicin, cisplatin, and ifosfamide, though bleomycin, cyclophosphamide, and vincristine are also used [4–6]. To improve OS survivability, researchers have targeted several genes and pathways aside from TP53 and RB1 using strategies which have demonstrated effectiveness in other tumors.

Research toward targeting OS based on the cell surface presentation of specific markers/proteins has been ongoing for many years. Disialoganglioside (GD2), a cell surface molecule, is a commonly targeted glycolipid and is considered for targeting OS because it is specific to GD2 and is safe and effective in high-risk neuroblastoma. Indeed, prevalent GD2 expression is found in osteosarcoma tissues, making it a viable therapeutic target [7]. In fact, anti-GD2 therapies have also been effective in rhabdomyosarcoma, osteosarcoma, leiomyosarcoma, liposarcoma, fibrosarcoma, small cell lung cancer, and melanoma [8]. GD2 therapeutic approaches include antibody, cytotoxic T lymphocyte (CTL), and chimeric antigen receptor (CAR) T-cell approaches, as well as antibody conjugates with radiolabels and drugs [8–12]. At present, there are five ongoing Phase 1 or 2 clinical trials regarding osteosarcoma treatment by targeting GD2 (NCT02173093, NCT01953900, NCT02502786, NCT02159443, NCT03356782).

Another category of cell surface-targeted therapy includes the vascular endothelial growth factor (VEGF) signaling pathway. The VEGF receptor (VEGFR) is a key inducer of angiogenesis, metastasis, and proliferation via the MAPK/ERK pathways. Bevacizumab, sorafenib, regorafenib, pazopanib, and cabozantinib are among the drugs capable of targeting. All except for bevacizumab are multi-targeting agents that can also inhibit signaling from platelet-derived growth factor receptor (PDGRF), fibroblast growth factor receptor (FGFR), and c-Kit, BRAF, and c-MET [13–15]. However, targeting these receptors has so far not been very fruitful; other novel drugs and OS surface receptors should be considered.

Identify Protein Mislocalization for Discovering Novel Tumor-Specific Targets

The targets described previously, including cell surface markers, are overexpressed in tumor cells compared to normal cells. Globally targeting these proteins may increase the risk of side effects. The ideal candidates may be the ones that only occur in tumor cells but are absent in normal cells, or at least rarely found in normal cells. These targets include the well-recognized fusion onco-proteins and proteins with deletion, insertion, or amino acid alterations. p53, a well-known tumor suppressor, can be found in the nucleus of normal cells, where it can facilitate DNA damage repair, cell cycle arrest, or even apoptosis if the damage incurred by the cell is too great to repair [16]. In OS, p53 is mutated in 3–7% of all patients and is included in the general umbrella of mutations associated with Li-Fraumeni syndrome (LFS) [17]. LFS is associated with multiple malignancies, and p53 autosomal dominant germline mutation is found in 70% of all LFS patients [18, 19]. Additionally, OS also presents other germline mutations in RB1 (retinoblastoma1), REQL4, WRN, BLM, and ribosomal proteins RPS19, RPL5, RPL11, RPL35a, RPS24, RPS17, and RPS7 [20]. Some of the prominent proteins with single nucleotide polymorphisms (SNPs) found in OS include tumor necrosis factor (TNFα), insulin-like growth factor 2 receptor (IGF2R), Fas receptor, and the transforming growth factor beta receptor 1 (TGFBR1). To be clear, the aforementioned receptors are not a primary cause of OS; however, germline mutations and SNPs can play a contributory role and may serve as effective targets for therapy. One of the most common gene fusions that occurs in Ewing sarcoma (ES), a primarily pediatric bone cancer, is between the genes for EWSR1 and FLI1, also denoted as EWS-FLI1 [21]. The detection of this fusion gene is one of the easiest methods of identifying this cancer, as this fusion is prevalent in

85% of all ES [22]. Furthermore, other opportunities for disease interrogation via genetic profiling are available by way of STAG2, CDKN2A, and TP53, which are present in ~20% of ES tumors.

Another class of unique targets, which is underpublished in the literature for sarcoma, is the mislocalized proteins. The mislocalization of prominent proteins and kinases in other cancer cells is not an unknown phenomenon. Several well-known receptor tyrosine kinases such as epidermal growth factor receptor (EGFR), cMet, and RON (recepteur d'origine nantais), which are normally found on the cell surface, can translocate to the nucleus in cancer cells [23–26]. Some of these may even function as transcription factors as has been previously documented [27, 28]. Conversely, tumor suppressors such as BRCA1, p53, retinoblastoma, ING1/p33, and adenomatous polyposis coli (APC) are present in the nucleus in normal cells but are mislocalized to the cytoplasm in cancer cells [29, 30]. The cytosolic concentration of p53 in normal cells is minimal due to induced degradation mediated by MDM2. However, in tumor cells, wild-type p53 levels in the cytoplasm are significantly higher while nuclear p53 is absent or markedly reduced [16, 31–34]. This cytoplasmic mislocalization of p53 in cancer cells may be associated with therapeutic resistance, tumor aggressiveness, and reduced patient survival [16]. Therefore, one of the future therapies for tumors including OS may be cell compartmentation-targeted therapy.

CSV May Serve as a Unique and Mislocalized Cell Surface Target for OS Therapy

Identification of unique intracellular targets for OS is critical for developing tumor-specific therapy, but unique cell surface targets could serve better for immune therapy, including both antibody and cell-based immune therapy. In this chapter, a novel cell surface protein—CSV—will be discussed via mislocalization.

Vimentin is a 57-kDa mesenchymal protein encoded on chromosome 10p13. It is part of the type III intermediate filament (IF) protein family and serves in a cytoskeletal structural role. This protein is present in the cytosol of cells from several tissue types including pancreatic, renal, neuronal, and immune cells (macrophages/leukocytes) [35–40]. Vimentin expression is predominantly detected in normal mesenchymal cells such as connective tissue, muscle, and central nervous system cells [41]. Vimentin is also elevated in tumor cells regardless of the origin and in epithelial cells [42].

Though vimentin is an IF protein that primarily resides in the cytosol of mesenchymal cells, its presence in other cellular compartments has been detected (Fig. 14.1). Despite lacking a nuclear localization sequence, vimentin was found to have been shuttled there by "piggybacking" on single-stranded DNA [43]. In the nucleus, vimentin interacted with lamin B to enable connection to the cellular skeleton [44].

Besides the localization in the cytosol and nucleus, this vimentin was also localized on the highly malignant tumor cell surface. This vimentin localization on the tumor cell surface (CSV) was perhaps first reported in leukemia cells [45–47]. This same protein was also discovered and published on the solid tumor cell surface from our group in 2011 [48]. This same CSV was later confirmed in sarcomas including OS [49, 50]. Taking advantage of these discoveries, a series of vimentin antibodies has been generated for binding CSV because the currently available vimentin antibody from multiple commercial sources has an extremely weak binding to CSV, suggesting that CSV may not be folded the same way as in cytosol. Plus, vimentin can form dimers, trimers, tetramers, or multimers. One high-affinity antibody for CSV detection, which is now available for commercial use, is 84–1. This antibody was successfully used for capturing circulating tumor cells (CTCs) across any type of tumor, including OS [49–55] (Fig. 14.2). Expanding on this positive detection of osteosarcoma CTCs from fresh peripheral blood, a follow-up study using cryopreserved peripheral blood mononuclear cells (PBMCs) was conducted [56]. Here, the authors demonstrated that PBMCs that were freshly cryopreserved within 2 hours of blood draw

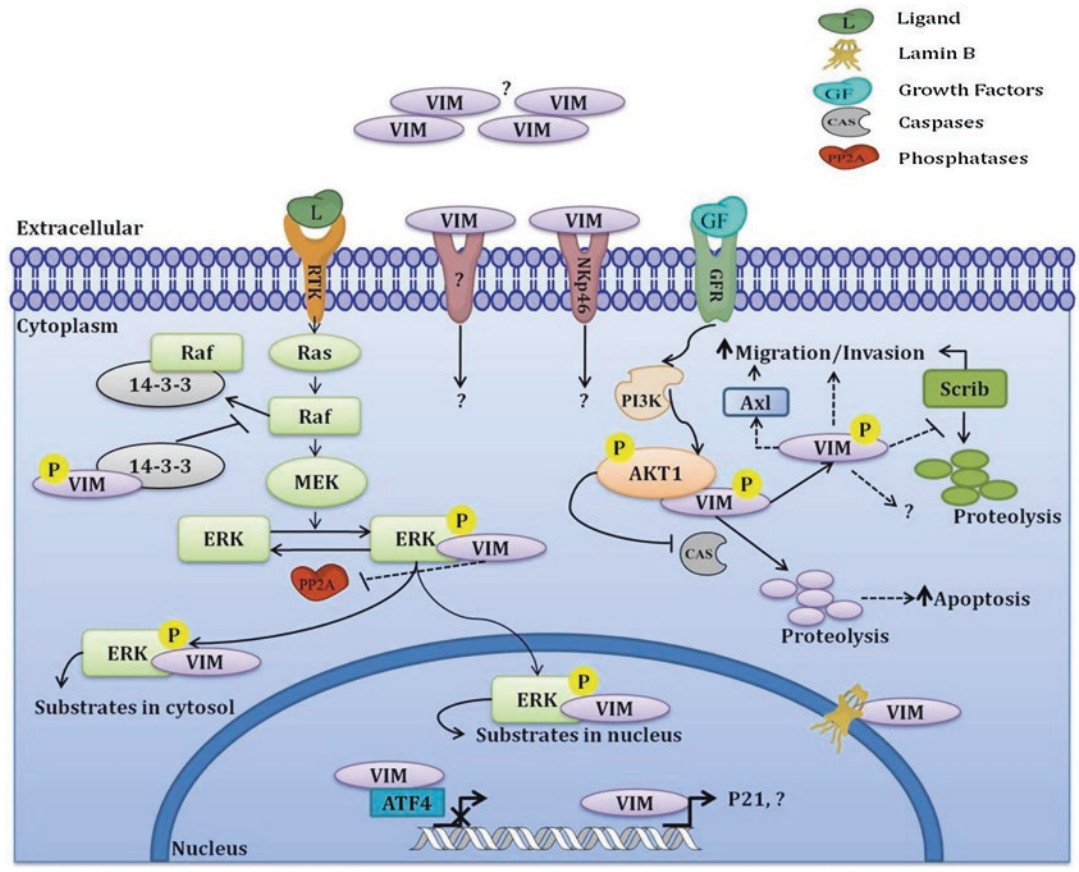

Fig. 14.1 Vimentin's role in cell signaling. In cytosol, vimentin was shown to interact with phosphorylated Erk and protect its phosphorylation by inhibiting phosphatases, which allows it to travel long distances within the cell. Also, vimentin's interaction with 14-3-3 proteins prevents the formation of the 14-3-3-Raf complex and thereby regulates several cell processes by depleting the availability of free 14-3-3. In addition, AKT1 was shown to phosphorylate vimentin and protect it from caspase-induced proteolysis; therefore, the freely available vimentin participates in processes that lead to an increased migratory and invasive capacity of the cells. In the nucleus, vimentin was shown to regulate p21 expression, while the complex formed between ATF4 and vimentin prevents active transcription by ATF4. Extracellularly, vimentin-specific receptors have not yet been identified; however, vimentin was shown to act as a specific ligand to one possible receptor, NKp46, on natural killer cells. Although reports indicate that vimentin is also secreted, neither the function of the secreted protein nor the mechanism of secretion is clear

could be later used as samples for CTC detection using the CSV-targeting 84–1 mAb. A further refinement of imaging and validation of the CTCs was discovered whereby the minimal background, false-positive staining of PBMCs could be eliminated if isolated CTCs are labeled with the CSV antibody in solution while the cells are still alive or prior to fixation. This method enables analyzing CSV-positive CTCs from cryopreserved PBMC samples when immediate processing of sample is not a viable strategy.

This CTC-based assay was heavily under our investigation on its clinical utility, including prediction of sarcoma relapse and treatment response. It might be especially useful for monitoring maintenance therapy because no visible tumors are present for patients who are under remission; CTC could become a valuable tool for

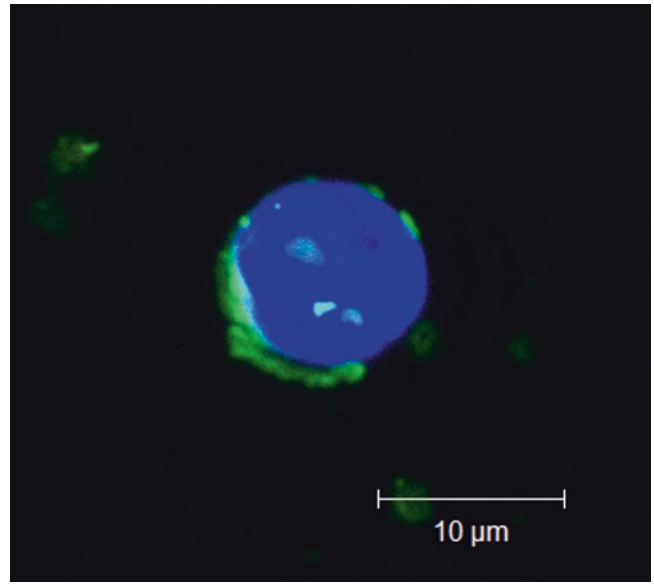

Fig. 14.2 Osteosarcoma CTC isolated from freshly drawn patient blood using CSV as a positive selection marker. Blue = nucleus; Green = CSV; Red = CD45

serving this purpose. Plus, the analysis is very simple, and only as few as 4 ml blood samples are needed for performing this assay. Though most of our publications use a manual method to perform this assay, Abnova has developed an automated system to capture CTCs using our 84–1 antibody. One caveat is that others have found CSV on the surface of macrophages, tumor endothelial cells, and neutrophils. These observations do not affect our recommendation of CSV as a valid target for OS because only a tiny number of the total neutrophils and macrophages may express CSV. Of note, expression of CSV on tumor endothelial cells may be beneficial for using CSV as a target. Indeed, some groups use CSV for inhibiting tumor angiogenesis [57–60]. Of note, the 84–1 antibody specifically bound the CSV on tumor cells' surface and did not show any false-positive binding to macrophages, neutrophils, platelets, lymphocytes, erythrocytes, or endothelial cells from the blood samples.

CSV-Targeted Linear Peptide Discovery

The discovery of CSV as a CTC identifier for sarcoma patients across types and the fact that CSV is present in six out of six PDX OS tumors strongly suggest CSV may serve as a universal target for OS. One obvious approach is to use an antibody to target CSV due to its high affinity nature and the matured manufacturing method for moving into a clinical setting. In fact, my lab has generated and characterized a panel of CSV mAbs [49, 52, 61]. Though none of these CSV mAbs were tested in OS tumor models, the hope for a CSV antibody-based therapy for OS seems low because our study in other tumor models shows low efficacy unless the CSV mAb can be co-localized into the tumor environment via co-injection [46, 48]. Therefore, to use the CSV antibody to target OS, either the antibody has to be used before visual metastatic tumor formation or intratumoral or tumor-targeting delivery methods have to be considered. One approach that is under investigation is to use CSV-targeting cell therapy, but it is in its early stage of effort. Another approach that has been used to target CSV is to discover an oligopeptide that can target CSV, which will open the door for many types of delivery. For example, the oligopeptide could be used for arming nanoparticles to carry the desired therapeutics to the OS. Likewise, the CSV-targeted oligopeptide could be also armed on the surface of therapeutic cells or fused with a therapeutic gene in the same reading frame to materialize the CSV-targeted delivery to OS.

My lab has identified one CSV-targeted oligopeptide called VNTANST back in 2011 [48]. Though the standard bench method is to screen a peptide library to define CSV interacting oligopeptides, we identified VATANST as a CSV-targeted oligopeptide backward with some coincidence. In brief, our approach is to search and collect all the peptides that bind to tumors, tumor endothelial cells, or lungs from the literature. This strategy was used because we did not know that CSV was a good target for OS when this experiment was conducted. Lung-targeted peptides were included because over 90% of metastasis occurs in the lungs for OS. In fact, the lungs are also a metastasis site for multiple other tumor types. Only tumors and lung-targeted peptides were selected because our goal was to identify one oligopeptide that is able to carry a single therapeutic molecule to the tumor site. For example, can a single CD13-targeted CDGRC oligopeptide carry a single IL12 protein to the tumor site when fused together via genetic engineering in the same reading frame? Addressing this question is important because the previous knowledge of the collected peptides mainly carry the phage or nanoparticles to the tumor site via expressing or coating an array of this tumor-targeted peptide. Defining a single peptide molecule to carry a single molecule to the tumor holds the key for tumor-targeted gene therapy. Our approach for this discovery was very tedious. In brief, these collected peptides were fused with a report gene SEAP in the same reading frame, and the distribution of these tumor-targeted SEAPs was investigated by comparing the SEAP activities across different organs including tumors. To our surprise, most of the tumor-targeted peptides only show tumor-targeted accumulation of SEAP in one or two tumor models, but only the lung-targeted peptide VNTANST consistently proved its tumor-targeting ability. To unravel the receptor of this oligopeptide, we used the synthesized VNTANST-biotin complex to capture the surface protein on the tumor cell membrane while washing the unbound proteins out. Unexpectedly, this VNTANST peptide-binding protein was vimentin following the mass spectrum analysis [48]. This result was further confirmed via an ELISA binding assay against recombinant vimentin [48]. This surprising observation was supported by publications in the literature, which showed that vimentin was present on the surface of tumor cells [47, 62, 63].

CSV-Targeted IL12 Immune Therapy for Treating Residual OS and Inhibiting Metastasis

To show that this CSV-targeted oligopeptide is able to carry therapeutic protein to the tumor site, the DNA fragment-encoding VNTANST peptide was fused to the p40 subunit encoding DNA of IL12 prior to the translational termination codon. As expected, the protein product of the administered CSV-targeted IL12 gene in the mice, also referred to as CHP-IL12 or ttIL12 [48, 64], preferred to accumulate into tumors regardless of tumor type, including OS tumors. This tumor-increased accumulation of IL12 is very critical for at least two reasons. First, presence of IL12 in blood and other organs at a high level is toxic. The primary toxicity site is the liver though other side effects are possible, such as neutropenia. Such toxicity has halted the value of IL12 for many years until the recent decade, when the IL12 gene and cell therapy are used for IL12 delivery. Even with these genes and cell therapy approaches, the clinical trial result from T-cell therapy still shows toxicity, though it is effective [65–67]. The only trial of IL12 gene therapy without this toxicity concern is intra-tumoral delivery, but intra-tumoral delivery is not practical for treating metastatic OS in lungs. Of course, intra-tumoral delivery to the primary site before surgery could be considered for inducing a systemic immune response to prevent and treat metastatic tumors, but the interest may not be high from clinicians because greater than half of patients may not need the systemic antitumor response due to the lack of metastasis. Therefore, a CSV-targeted IL12 can meet this need via systemic delivery of the encoding gene, but the protein product can be accumulated in tumors for minimizing toxicity. Indeed, administering this CSV-targeted IL12 gene significantly reduced the

toxicity in a preclinical model [48]. Interestingly, the IL12 toxicity is also genetically related because Balb/c mice are resistant to IL12 toxicity, C3H mice are very sensitive to IL12 treatment, and C57Bl/6 mice are in between. Unfortunately, no effort was conducted to decipher the genetic marker or mechanism; otherwise, this therapy could be used for the IL12 toxicity-resistant patients to address this toxicity issue. Secondly and also importantly, presence of IL12 in other organs is not effective even at a high concentration, and only presence of IL12 in tumors, even if at a relatively low concentration, may trigger both a tumor local and systemic antitumor response for inhibiting metastasis [48]. This point is often neglected by many investigators and clinicians, but it is crucial for the success of this therapy. One of the possible mechanisms is that presence of IL12 in other tissues triggers non-tumor-specific inflammation such as a high amount of IFNγ, which may induce a high amount of PD-L1 in the circulating immune cells. This PD-L1 induction may have inactivated the immune cells prior to even entry into the tumor microenvironment. Though it is scientifically logical, this mechanism remains to be validated. The other primary mechanism is that IL12 needs to be present locally in tumors to transform the immature DC to mature DC for antigen processing and presenting. This mechanism is supported by the fact that administering CSV-targeted IL12 gene therapy, in which a high amount of IL12 was found in tumors, increased the mature DC number in tumors [48].

Though this CSV-binding peptide VNTANST can be easily fused to any therapeutic genes, the fact is that only CSV-targeted IL12 fusion gene therapy has shown a superior effect against multiple types of tumors with totally different tissue origins [48]. Significantly, this same CSV-targeted IL12 fusion gene therapy is effective in preventing and treating metastasis using both a K7M3 and LM8 osteosarcoma mouse tumor model when given both before and after surgery. Either alone is not nearly as effective as the two sequential treatments (unpublished data). This result makes sense because immune therapy needs priming and boosting to induce sufficient antitumor immune response. Indeed, the CTL activity is high when two sequential administrations prior and post-surgery were given compared to a single administration. The other immune fusion genes tested include CSV-targeted IL15, IFNs, and PF4. However, CSV-targeted IL15 seems tumor model dependent, but other CSV-targeted genes did not show any merit in terms of therapeutic efficacy (unpublished data). Such a result clearly suggests that a slight change in the fusion structure between VNTANST and the therapeutic genes of interest may change its binding activity. Such a notion was supported by the fact that fusion of VNTANST-encoding DNA to the p35 unit of the IL12-encoding sequence yields the same benefit in some tumors but not in other tumors. Therefore, our recommendation is to use p40-VNTANST as the base for CSV-targeted IL12 immune therapy.

In our published data, CSV-targeted IL12-encoding DNA was administered in muscle tissues via a simple electroporation procedure [48], which is located distantly from either the primary or metastatic tumors, for expressing this targeted IL12 protein. The targeted IL12 protein can secrete from the muscle cells where manufactured and circulate to the tumor site. The CSV-targeted IL12 can be retained in the tumor cell surface via binding to CSV. In a clinical setting, electroporation of DNA into humans may not be the preferred method, though a similar type of clinical trial was initiated to test the impact of IL12 via intra-tumoral electroporation; other methods can be used, such as utility of a viral vector to arm ex vivo expanded cells (NK cells, T cells, or other types of cells) with this CSV-targeted IL12. All of which may serve the future therapy for OS really well.

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Part IV

Survivorship and Late Effects

Editorial Comments on Late Effects

Melissa M. Hudson
Survivorship Division, Department of Oncology, St. Jude Children's Research Hospital, Memphis, TN, USA

The general therapeutic strategy for osteosarcoma has remained largely unchanged since the 1980s, employing an initial period of neoadjuvant multiagent chemotherapy followed by surgical resection to achieve local tumor control and additional post-surgical adjuvant chemotherapy. The success of this approach has provided the opportunity and responsibility to characterize long-term persistent and late onset treatment-related morbidity ("late effects") that may compromise both quality and duration of survival. Because as many as one-third of newly diagnosed patients without metastatic disease will experience relapse, and prognostic factor studies have not been helpful in guiding therapeutic risk stratification, multimodality therapy for osteosarcoma typically confers substantial risks for multimorbidity among all long-term survivors that can affect both physical and psychosocial functioning.

Late effects are treatment modality specific and can be anticipated based on data from numerous observational studies performed over previous decades. For osteosarcoma survivors, relevant late health outcomes related to chemotherapy include cardiomyopathy (doxorubicin), renal dysfunction (cisplatin, high-dose methotrexate, ifosfamide), ototoxicity (cisplatin), and secondary leukemogenesis (etoposide, ifosfamide). Characterization of the prevalence and magnitude of risk associated with chemotherapy exposures has resulted in the routine integration of the cardioprotectant dexrazoxane into frontline treatment regimens and guided health surveillance recommendations during and after therapy.

Local tumor control is critical to achieving long-term survival, which means that virtually all osteosarcoma patients will experience some degree of musculoskeletal morbidity most often related to surgery and less commonly

The original version of this part was revised. The correction to this part is available at https://doi.org/10.1007/978-3-030-43032-0_17

resulting from radiation of unresectable disease in individuals with craniofacial or axial tumors. Over the years, surgical advances have reduced the need for radical procedures like amputation, but long-term functional outcomes following both amputation and limb-sparing surgeries have not been well studied. Certainly, maintaining a functional exo- or endoprosthesis requires access to rehabilitative resources across the lifespan, which may not be available to many aging survivors.

Osteosarcoma presents across an age spectrum, but most commonly in adolescents and young adults, a unique developmental group with established vulnerability to adverse psychosocial, educational, and vocational outcomes related to the cancer experience that may contribute to long-term financial hardship. As a whole, a substantial proportion of adolescent and young adult cancer patients endorse unmet emotional, practical, and informational needs. Optimizing outcomes for adolescents and young adults with osteosarcoma requires addressing psychosocial concerns during treatment planning and care transitions and facilitating access to resources to support both long-term physical and emotional health.

As therapy for osteosarcoma progresses to include immunotherapy and molecularly targeted agents, so too will the spectrum of late effects evolve. Systematic long-term follow-up of survivors treated with new agents is critical to evaluate survival benefits in the context of unintended secondary effects that invariably accompany the introduction of novel therapies. This chapter summarizes knowledge gained through late health outcomes investigations of children, adolescents, and young adults with osteosarcoma and outlines priorities for future research to assure the optimal balance of therapeutic gains and late effects.

15 Anthracycline-Induced Cardiotoxicity: Causes, Mechanisms, and Prevention

Anchit Bhagat and Eugenie S. Kleinerman

Abstract

Doxorubicin is an anthracycline and one of the more effective chemotherapy agents used in the treatment of children, adolescents, and adults with osteosarcoma. Despite its effectiveness, cardiotoxicity is a major late effect that compromises the survival and quality of life of survivors of this and other cancers. Cardiotoxicity is the inability of the heart to pump blood through the body effectively. Doxorubicin-induced cardiotoxicity is dose dependent. Additionally, the age of the patients plays a role in susceptibility with younger patients having a greater risk for cardiotoxicity and heart failure years after treatment is complete. The exact mechanism(s) responsible for doxorubicin-induced cardiotoxicity is poorly understood, and further research needs to be done to elucidate the mechanisms. This chapter summarizes the identified mechanisms that may play a role in anthracycline-induced cardiotoxicity. We will also summarize the types of cardiomyopathies that have been described in survivors treated with doxorubicin and the current recommendations for monitoring survivor for the development of cardiomyopathies. Included will be the important search for defining early biomarkers to identify patients and survivors at risk. Finally, we will summarize some of the interventions proposed for decreasing anthracycline-induced cardiotoxicity.

Keywords

Cardiotoxicity · Anthracycline · Doxorubicin · Dexrazoxane · Osteosarcoma · Chemotherapy · Pediatric cancer · Heart failure · Cardiovascular disease

Anthracyclines are among the most effective chemotherapy agents used to treat pediatric, adolescent, and adult patients with osteosarcoma [1]. In addition to osteosarcoma and other sarcomas, anthracycline-containing regimens are used to treat lymphoma, leukemia, and breast cancer, with 50–60% of childhood cancer patients receiving an anthracycline-containing regimen during treatment [1]. Some of the commonly used anthracyclines are doxorubicin, mitoxantrone, epirubicin, idarubicin, and daunorubicin. The structure of an anthracycline is composed of a tetracyclic ring that is attached to a sugar. There are also quinone and hydroquinone moieties present on adjacent rings, which permit the gain and loss of electrons.

Doxorubicin is one of the more widely used chemotherapy agents in the anthracycline family

A. Bhagat (✉) · E. S. Kleinerman
Department of Pediatrics, MD Anderson Cancer Center, Houston, TX, USA
e-mail: abhagat@mdanderson.org

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with respect to osteosarcoma. It, along with daunorubicin, was the first to be used in clinical practice. Daunorubicin was identified first from the bacterium *Streptomyces peucetius*. Doxorubicin, a derivative of daunorubicin, was identified in the 1960s and found to be a more effective antitumor agent. With regard to osteosarcoma, there is a correlation with dose intensity and patient survival. However, there is also a correlation between the dose of doxorubicin and late comorbidities which results in compromised quality of life [1]. One of the most frequently seen late effects is cardiac disease. Sarcoma survivors are at a significantly higher risk of being diagnosed with some form of cardiovascular event compared to community controls, and these events occur at a much earlier age [1]. These include vascular disease, cardiac dysfunction, myocardial infarction, arrhythmias, dyslipidemia and essential hypertension, and structural defects. It is unclear whether the mechanism(s) responsible for anthracycline-induced cardiotoxicity are the same as those that are responsible for killing tumor cells.

In this chapter, we will review the identified mechanisms of action of anthracyclines, and doxorubicin in particular, since it is one of the major chemotherapy agents used in the treatment of osteosarcoma. We will also summarize the cardiomyopathies that have been documented in survivors treated with doxorubicin and what has been described in terms of monitoring patients for heart disease. This will include investigations looking for early biomarkers to identify patients and survivors at risk. Finally, we will summarize interventions aimed at decreasing anthracycline-induced cardiotoxicity.

Mechanism of Antitumor Action

There are three proposed mechanisms by which doxorubicin acts as an antitumor agent [2]. These include (i) intercalation into DNA strands, (ii) DNA damage by topoisomerase suppression, and (iii) generation of free radical species and the subsequent cellular damage including lipid peroxidation of cell membranes.

Intercalation into DNA strands Doxorubicin has the capacity to inhibit DNA biosynthesis by DNA intercalation and independent of inhibition of DNA polymerase activity. Doxorubicin intercalates into sites containing adjacent GC base pairs. Additionally, the drug has an affinity for these sites mainly due to hydrogen bond formation between doxorubicin and guanine. This leads to the formation of Doxorubicin-DNA adducts that can activate DNA damage responses and induce cell death. This discovery has led to a few different approaches to increase the antitumor efficacy of doxorubicin. One such approach has been to combine with compounds that release formaldehyde upon hydrolysis including pivaloyloxymethyl butyrate (AN-9), butyroyloxymethyl-diethyl phosphate (AN-7), and hexamethylenetetramine (HMTA) [3]. In some instances, this combination was effective in increasing the tumor-killing capacity of Doxorubicin. This is mediated by the stabilization of the covalent bond between doxorubicin and DNA by formaldehyde, resulting in an increase in the formation of DNA adduct levels. However, contradictory data makes it difficult to establish the DNA adduct formation as the major mechanism of doxorubicin-mediated tumor cell killing [3].

DNA damage by topoisomerase suppression DNA topoisomerases are enzymes that play essential roles in the unwinding and rewinding of the DNA helix strands during replication, transcription, and recombination. The need for topoisomerase arises due to the double-helical nature of the DNA strand. In order to access information stored in DNA, the two strands of the helix must be separated temporarily. There are two main types of topoisomerase depending on whether there is a single- or double-stranded break in the DNA helix. Type I isomerase causes a single-stranded break, while Type II cuts both strands of the DNA helix [4]. For the purposes of this discussion, Type II topoisomerase, which consists of Top2α and Top2β, will be discussed. In malignant cells that are proliferating rapidly, Top2α is more highly expressed. Top2α works in an adenosine-dependent fashion by recognizing a DNA substrate (G segment) and causing a

double-stranded break which opens one of the DNA duplex. This helps facilitate the capture and entry of a second DNA piece termed the T segment through the opening in the G segment. This helps unwind the DNA strand and is followed by religation of the double-stranded break in the G segment. Failure in this process results in DNA lesions that lead to mitotic and apoptotic cell death [5].

Doxorubicin targets Top2α which is highly expressed in osteosarcoma cells [6]. Doxorubicin intercalates into DNA and prevents Top2 from binding with DNA. This leads to the suppression of the Top2-DNA cleavage complex formation with subsequent transcriptional arrest, which then leads to DNA damage and ultimately cell death.

Generation of free radical species Anthracylines including doxorubicin induce the generation of free radicals [7]. Reactive oxygen species and reactive nitrogen species are collectively termed free radicals, molecules with one or more unpaired electrons in their outer shells. Free radicals include hydroxyl ($OH^{\bullet}$), superoxide ($O_2^{\bullet-}$), nitric oxide ($NO^{\bullet}$), nitrogen dioxide ($NO_2^{\bullet}$), peroxyl ($ROO^{\bullet}$), and lipid peroxyl ($LOO^{\bullet}$) [8]. Under homeostatic conditions, these free radicals act as an integral part of the host defense system and aid in maturation of cellular structures. However, when produced in excess, these free radicals give rise to oxidative stress due to an imbalance between formation and the elimination of the free radical species. This can ultimately lead to damage to cell membranes by a process called lipid peroxidation [9, 10]. Doxorubicin has a quinone structure which allows for the drug to act as an electron acceptor which is mediated by other oxoreductive enzymes such as cytochrome P450 reductase, NADH dehydrogenase, and xanthine oxidase. This addition of the free electron facilitates the conversion of the quinone to a semiquinone free radical. Once these free radicals are generated, they then induce free-radical injury to DNA. Unlike the DNA damage associated with inhibition of topoisomerase II, this damage is not associated with proteins [11]. This DNA damage can be prevented by superoxide dismutase, catalase, and glutathione peroxidase. For this reason, it has been observed that free radicals generated by doxorubicin toxicity result in the alterations of glutathione levels which in turn have an impact on cell sensitivity to doxorubicin [11]. However, studies conducted have reported mixed findings as to whether free radical generation is one of the main mechanisms by which doxorubicin can cause damage to tumor cells.

Cardiotoxicity

Cardiovascular disease and heart failure are the most common late effects which compromise quality of life and the long-term survival for sarcoma survivors [11]. Doxorubicin-induced cardiotoxicity can be debilitating and an often-deadly consequence of successful tumor treatment. The acute damage of the juvenile heart caused by doxorubicin makes the adult heart more vulnerable to stresses over time, putting the heart at risk for ischemic damage, and predisposing to late-onset cardiomyopathies at a much earlier age. Thus, a minor ischemic event that would cause minimal or no damage in a healthy individual would result in more significant damage in a heart previously damaged by Doxorubicin. Cardiotoxicity is defined as the inability of the heart to pump blood through the body effectively. In 2014, there were 14.5 million cancer survivors [1]. This number is expected to increase to 18 million by 2020, indicating that this late effect will be seen in increasing numbers. One risk factor associated with the Doxorubicin-induced cardiotoxicity is age of the patients, with children under 4 years and adults over 65 years of age being at a higher risk of developing cardiotoxicity. Furthermore, Lipshultz SE et al. have also reported that females had more severe cardiotoxicity with more compromised contractility [1]. It is estimated that 60% of pediatric patients receive an anthracycline-containing treatment regimen and 10% of these are expected to develop symptomatic cardiomyopathy up to 15 years after completing therapy [1]. Another study reported the incidence of subclinical and overt cardiotox-

icity to be 17.9% and 6.3%, respectively, in cancer patients treated with anthracyclines after 9 years of follow-up [12]. Thus, cardiotoxicity can develop after a significant amount of time has passed after completion of treatment indicating that long-term cardiac monitoring of survivors is essential. One factor that contributes to the development of the cardiotoxicity is the dose of doxorubicin used in the cancer treatment. In patients who received more than 500 mg/m^2 of anthracyclines, a 63% prevalence of left ventricular dysfunction after 10 years of follow-up was reported, in contrast to an 18% prevalence in those who received less than 500 mg/m^2 [13]. Another study reported that in patients who received a cumulative dose of 400 mg/m^2, there was a 5% risk of developing heart failure which increased to 25% at 700 mg/m^2 [2]. This is indicative of the fact that the risk of cardiotoxicity correlates with increased drug dose. However, another separate study looking at the histopathological changes in endomyocardial biopsy specimens from patients concluded that there is no particularly safe dose of doxorubicin [2]. Unfortunately, the exact mechanism of Doxorubicin-induced cardiotoxicity is unknown but there are several proposed molecular mechanisms.

Definition and detection of cardiotoxicity Cardiotoxicity is defined by a number of different parameters. These include (1) reduction of left ventricular ejection fraction (LVEF), either global or specific in the interventricular septum; (2) symptoms or signs associated with heart failure (HF); (3) reduction in LVEF from baseline to lesser 55% in the presence of signs or symptoms of HF; or (4) a reduction in LVEF greater than or equal to 10% or an LVEF lesser than 55% without signs or symptoms of HF [14]. Left ventricular ejection fraction is defined as the central measure of left ventricular systolic function and is the fraction of volume ejected during the contraction phase(systole) of blood circulation in relation to the volume of blood in the ventricle at the end of the dilution phase (diastole). Normal LVEF for males is 52–72%, while for females, it is 54–74%. Less than 52% LVEF is considered abnormal and suggests compromised heart function [15].

There are several different methods to detect anthracycline-induced cardiotoxicity. One of the most successful methods is an endomyocardial biopsy. However, this technique has several limitations: first, it is invasive and second, with regard to the quality of the sample and whether the sample obtained by the biopsy contains damaged myocardium. It is for these reasons that noninvasive methods are preferred. One of the most widely used methods in this regard is echocardiography. Using this technique, the left ventricular ejection fraction, which is an indicator of cardiac systolic function, can be quantified. A study conducted on 1664 patients who were treated with anthracyclines as part of their breast cancer treatment showed that the absolute value of pretreatment LVEF was indicative of a later occurrence of heart failure. Despite its effectiveness in adult patients, there have seen some discrepancies regarding its predictive value in children treated with anthracyclines [16].

Some of the disadvantages of standard 2D echocardiography are the following: (i) the quality of images obtained determines the accuracy of the LVEF measurement, (ii) ventricular foreshortening can contribute toward lack of accuracy in measured LVEF, and (iii) use of mathematical models and geometrical assumptions to calculate LV volumes [16]. As a result, there have been further enhancements made to the echocardiography technique including use of contrast agents to help highlight the endocardial border and improve tracing of the end-systolic and end-diastolic volumes. Additionally, 3D echocardiography has helped with reducing analysis time and interobserver variability [16].

Monitoring Cardiotoxicity and Heart Function

Biomarkers to identify acute cardiotoxicity and predict late cardiac effects: Early detection of at-risk patients for cardiotoxicity combined with early intervention could help decrease the occurrence of heart failure in survivors. To address this need, many studies have been conducted to try to determine serum and plasma biomarkers that are

relevant to cardiotoxicity and predicting which patients are at risk for developing cardiotoxicity and heart failure [17]. Unfortunately, such suitable biomarkers have yet to be identified that both document acute heart damage and correlate with the development of heart failure. However, what is established is that elevated levels of troponin and natriuretic peptides are two biomarkers that are associated with acute coronary syndrome and heart failure, respectively [17]. We will now discuss these with regard to identifying anthracycline-induced cardiotoxicity, monitoring for heart failure, and/or predicting patients at risk for late cardiotoxicity.

Cardiac troponins Troponins are proteins that are found in skeletal, cardiac, and smooth muscles [17, 18]. Troponin T (TnT) and troponin I (TnI) are both exclusively found within cardiac myocytes. Upon damage to cardiomyocytes, troponins T and I are released into circulation. Elevated troponin levels indicate cardiac damage and LV dysfunction [17, 18]. Investigations looking at cardiac troponins with relevance to cardiotoxicity have concentrated on early onset heart damage. What has been observed is that early troponin elevation preceded changes in LVEF. In a study of 703 patients with breast cancer and lymphoma, it was seen that TnI elevation measured within 72 h as well as 1 month after chemotherapy administration had significantly greater risk of developing cardiotoxicity over a mean follow-up of 20 months [17]. Another study looked at changes in TnI and LVEF after cycles of high-dose anthracycline-containing chemotherapy in 204 patients. It was found that patients who had elevated TnI had significant reduction in their LVEF 7 months after completion of treatment [18]. From this standpoint, it suggests that looking at troponin levels at an acute stage of cardiotoxicity may be useful. However, other studies conducted found no correlation between troponin level and the development of late heart failure due to the use of anthracycline [19]. One such study looked at 150 childhood and 53 adult cancer survivors with hematologic malignancies and breast cancer, respectively [19]. It was found that there was no detectable elevation of TnT or TnI after a 2-year and 1-year follow-up confirming that there is a lack of correlation between troponin and LV dysfunction, particularly in childhood cancer survivors.

Natriuretic peptides These are peptide hormones that include atrial natriuretic peptides (ANP) and brain natriuretic peptides (BNP). These peptides promote natriuresis and help with the protection of the heart from mechanical stress and volume overload. Published literature illustrated an early association between anthracycline-induced cardiotoxicity and brain natriuretic peptides [19]. In one study, 71 breast cancer patients received 6 cycles of liposome-encapsulated doxorubicin (40–50 mg/m^2), docetaxel (50 mg/m^2), and epirubicin (90 mg/m^2) in combination with fluorouracil and cyclophosphamide [19]. Upon completion of the treatment cycle, it was observed that BNP levels were elevated and that the elevation 24 h after treatment was associated with reduction in LVEF. However, other studies failed to show a link between elevated natriuretic peptides and late-onset cardiotoxicity [19]. The reason for this discrepancy could be that elderly individuals and females have higher than normal natriuretic peptide levels [19]. Additionally, compromised renal function can increase natriuretic peptide levels. Finally, cancer itself may increase BNP levels through inflammation. Indeed, patients with metastatic disease were found to have higher levels of BNP than those without metastasis [19].

Myeloperoxidase Another potential biomarker is myeloperoxidase, a pro-inflammatory enzyme that is expressed by neutrophils. Myeloperoxidase is an indicator of oxidative stress and is induced following damage to the myocardium by reactive oxygen species generation and is part of the inflammatory response. In one study conducted on 78 breast cancer patients treated with doxorubicin as part of their chemotherapy regimen,

patients with increased MPO levels along with elevations in TnI over a 15-month time period had a 46.5% increased risk for developing cardiotoxicity [19]. However, further studies are needed to confirm this finding.

MicroRNAs Several studies have also explored microRNAs as viable biomarkers for anthracycline-induced cardiotoxicity. These are small non-coding RNA molecules that aid in regulation of gene expression. Some of the common cardiac miRNAs under investigation include miR-1, miR-133, miR-208, and miR-499. In myocardial infarction models in both humans and animals, miR-208 and miR-499 are found to be elevated [17]. These two microRNAs are also specifically found in cardiac myocytes. In a study of 33 children with anthracycline exposure, it was observed that there was elevation in plasma levels of miR-29b and miR-499, and this correlated with rise in troponin levels and increase in dose of anthracyclines [17]. In a separate study of breast cancer patients who were treated with doxorubicin, there was an increase in miR-1 that was associated with decline in LVEF [17]. The monitoring of miRNAs therefore may provide a potential new biomarker for assessing and monitoring early- and late-onset cardiotoxicity, as microRNAs are present in all body fluids, have a long half-life, and are relatively stable under extreme temperatures and pH [17].

Mechanisms of Anthracycline-Induced Cardiotoxicity

The exact mechanism(s) by which doxorubicin induces cardiotoxicity is poorly understood. This section summarizes several of the proposed mechanisms.

Oxidative stress The most frequently proposed mechanism for doxorubicin-induced cardiotoxicity is the generation of reactive oxygen species followed by lipid peroxidation [20]. Some of the common reactive oxygen species generated include superoxide(O_2^-), hydroxyl radicals (OH), hydrogen peroxide (H_2O_2), and singlet oxygen (O_2). A low level of oxidant species is necessary for normal transduction process; however, if these levels exceed the threshold level, this can be damaging to cells. The myocardium is especially vulnerable as there are lower levels of the antioxidant enzymes peroxidase, catalase, and superoxide dismutase present in the heart [20]. Peroxidase helps catalyze oxidation of substrates and uses hydrogen peroxide as the electron acceptor. This helps with elimination of the toxicity of hydrogen peroxide and oxidizes phenols, amines, and hydrocarbon oxidation products. Catalase helps with the decomposition of H_2O_2 and removal of H_2O_2, thereby protecting cells from H_2O_2 poisoning. Due to a lack of these enzymes, there is an accumulation of H_2O_2 which leads to damage [20].

One of the major subcellular targets of doxorubicin is the mitochondria [21]. The number of mitochondria in cardiomyocytes is increased by 35–40% [21]. In the mitochondria, ROS-producing enzymes transform the quinone moiety present in doxorubicin to a semi-quinone through a one electron reduction. Semiquinones can then be converted to a superoxide anion by reaction with oxygen. These superoxide anions can then be transferred to ROS or reactive nitrogen species via the redox cycle. However, high levels of these superoxide anions can produce highly reactive and toxic hydroxyl radicals during a reaction catalyzed by iron called the Fenton reaction.

As mentioned earlier, a lack of antioxidant enzymes in the myocardium means the heart is more vulnerable to oxidative stress. Furthermore, with a higher number of mitochondria in the cardiomyocytes, this leads to a higher production of ROS once doxorubicin binds to the mitochondria and initiates the production of superoxide anions which in turn can produce toxic hydroxyl radicals and in turn higher oxidative stress.

Cardiolipin, a phospholipid, is a component within inner mitochondrial membrane which doxorubicin has a high affinity for [22]. As a result, an irreversible complex is formed between doxorubicin and cardiolipin that accumulates on the inner mitochondrial membrane. This complex

can then lead to mitochondrial dysfunction through oxidation of enzymes that are catalyzed by cardiolipin. This creates a disturbance of the electron transport chain in mitochondria as proteins, including cytochrome C oxidase, cytochrome C reductase, and NADH dehydrogenase, are oxidized which subsequently leads to energy reduction and apoptosis of cells as 90% of the ATP utilized by cardiomyocytes is produced by the mitochondria [21].

One other proposed mechanism of oxidative stress is the "ROS and Iron" hypothesis [23]. As mentioned earlier, iron catalyzes the Fenton reaction that is needed to convert the superoxide anions to hydroxyl radicals. Doxorubicin can form a complex with iron which can potentially lead to excessive ROS production causing apoptosis of cardiac cells. This accumulation of iron in mitochondria is facilitated by members of the ABC protein family particularly ABC protein B8(ABC8). ABC8 has a role in mitochondrial iron homeostasis and helps with facilitating mitochondrial iron export. One study conducted showed that doxorubicin downregulated the ABC8 protein and mRNA levels which in turn affected the export of iron from the mitochondria leading to excessive accumulation of iron in the mitochondria which is toxic to the cells [23]. By contrast, levels of another protein, mitoferrin 2, were found unchanged. Mitoferrin 2 is involved in mitochondrial import of iron. The alteration of iron export while maintaining levels of iron import leads to excessive iron and to a disturbed state of iron homeostasis in mitochondria [23]. Another mechanism through which iron overload is caused is through the interaction of doxorubicin with iron-transporting and iron-binding proteins including IRP (iron regulatory protein). As mentioned earlier, Dox can form a complex with iron, and this complex reduces the amount of free iron [24]. This free iron is then unable to bind to IRP. This leads to inactive IRPs which then bind with iron-responsive elements (IREs) which in turn leads to a disruption of proteins related to iron metabolism [24]. This disruption leads to a decrease in synthesis of ferritin and upregulation of transferrin receptor (TfR) that leads to increase in iron levels which disturbs the iron homeostasis [24].

Nicotinamide adenosine dinucleotide phosphate is another enzyme that helps with generation of free radicals by the redox cycle [20]. These are a group of plasma membrane-associated enzymes that serve as a source of ROS. Similar to what has been discussed earlier, nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOX) helps with conversion of quinone moiety to a semi-quinone radical that can react with oxygen to generate hydrogen peroxide. NOXs can be activated by many stimuli including TNF-a and are pivotal in cardiac remodeling [20]. Furthermore, Nox 2 was found to be the main mediator of NOX-derived ROS.

Nitric oxide is also a major source of doxorubicin-induced oxidative stress. In normal homeostatic conditions, nitric oxide is a vasodilator that mediates heart contractions. An elevated level of NO is observed in doxorubicin-induced cardiotoxicity due to isoforms of NOS, namely, endothelial NOS, inducible NOS, and neuronal NOS [25]. Doxorubicin captures electrons from NADPH by directly binding the reductase domain of eNOS. This helps with superoxide formation. The role of eNOS was confirmed in a study conducted on eNOS knockout mice that displayed low levels of ROS and preserved myocardial function after exposure to doxorubicin [25]. Basal production of NO is needed to modulate cardiomyocyte contractility and blood flow distribution [25]. However, higher levels of NO production via INOS are associated with dilated cardiomyopathy and congestive heart failure [7].

There are multiple sources of oxidative stress in the myocardium. Reactive oxygen species that generates the oxidative stress overwhelms cardiomyocyte enzymatic defenses and alters gene expression through interaction with regulatory proteins. ROS also can affect function of G proteins via lipid peroxidation. Despite this overwhelming connection between oxidative stress and doxorubicin-induced cardiotoxicity, the experimental evidence that treating this oxidative stress will reduce doxorubicin-induced cardiotoxicity has not been conclusive.

Cardiomyocyte apoptosis Oxidative stress can activate apoptotic signaling that leads to cardiomyocyte apoptosis. In this scenario, both extrinsic and intrinsic pathways are involved. However, apoptosis can be induced via an oxidative stress-independent manner. The B-cell lymphoma 2 (Bcl 2)-Bcl-2-like protein 4 (bax) ratio is important in apoptosis and involves heat shock proteins [25]. These proteins act as molecular chaperones and stabilize other proteins involved in anti-apoptotic signaling by preventing dephosphorylation, ubiquitination, and degradation. The heat shock proteins that are important in having a role in the cardiac microenvironment include heat shock protein 27 (Hsp27), heat shock protein 10 (Hsp10), and heat shock protein 60 (Hsp60) [26]. In a study conducted by Liu et al., they found that overexpression of Hsp27 prevented doxorubicin-induced apoptosis and myocardial dysfunction [26]. In another study, overexpression of Hsp10 and Hsp60 was found to shift toward an anti-apoptotic pathway due to increased posttranslational modification of Bcl-2 protein [26]. Hsp20 aids in maintenance of Akt phosphorylation which is one of the main cell survival pathways. Heat shock proteins also act as ligands for Toll-like receptors (TLR) after being secreted into the bloodstream [26]. In addition to its effect on modification of Bcl-2, Hsp60 interacts with TLR-2, while Hsp70 interacts with TLR-4 [20]. The exact role of Toll-like receptors in doxorubicin-induced cardiotoxicity is not well-defined as yet; however, studies conducted indicate that apoptosis is initiated by TLR-2- and TLR-4-mediated signaling through pro-inflammatory NFκB post doxorubicin treatment [26].

Doxorubicin also influences caspase activity. In particular, caspase-3 activation is associated with doxorubicin administration in vivo. In one study, suppression of caspase activity in cardiomyocytes was achieved through the administration of NO donor S-nitrosyl-N-acetyl-penicillamine. In another study, blocking of volume-sensitive chloride channels prevented doxorubicin-induced caspase-3-dependent apoptosis [27]. It has also been observed that doxorubicin elevated the expression of death receptors such as tumor necrosis factor receptor 1 (TNFR1), fas cell surface (Fas) death receptor, DR4, and DR5 in cardiomyocyte. This elevated expression leads to activation of caspase cascade [20].

Calcium dysregulation Control of calcium levels is important in cardiomyocytes as calcium aids in regulating contractile activity [26]. Intracellular calcium levels are increased in Doxorubicin-induced cardiotoxicity. The ROS and H_2O_2 that are generated can alter normal calcium homeostasis in several different muscle types including the heart by disrupting normal sarcoplasmic reticulum [26]. By inhibiting the Ca^{2+} ATPase pump and reducing the expression levels of SERCA2a MRNA, the free radicals can impair Ca^{2+} metabolism [20]. Doxorubicin can also induce the release of calcium from sarcoplasmic reticulum by promoting the opening of the calcium channels [26]. Doxorubicin has also been shown to inhibit the sodium-calcium exchanger channel in the sarcolemma [26].

Caspase-12 activates apoptotic pathways, and its activation is dependent on calpain dysregulation which sends out signals of distress from sarcoplasmic reticulum [26]. Calpains are proteases that are activated by calcium. Much of the intracellular calcium in cardiomyocytes is present in the sarcoplasmic reticulum. Oxidative stress can cause calcium leakage, calpain activation, and caspase-12 cleavage. Calpains have been found to degrade titin which is a large protein and a key component of cardiac sarcomere which helps in maintaining cardiac contractility [26]. Hence, prevention of calpain activity helps maintain contractility after doxorubicin exposure [20]. Doxorubicin has also been found to enhance the sensitivity of mitochondria to intracellular calcium [26]. In a study conducted in rats, it was found that mitochondria of cells in doxorubicin-treated heart had a decreased ability to retain calcium [26].

Another possible mechanism by which doxorubicin can affect calcium intracellular concen-

trations is through regulation of its metabolism [28]. Doxorubicin can generate a toxic metabolite, namely, DOXol, which inhibits the sodium-calcium exchanger channel [28]. DOXol can also disrupt the sodium gradient that is needed for calcium to flow into sarcolemma of a cardiomyocyte. This leads to an imbalance in energetics of the myocardium and diminished systolic function. DOXol accumulation can thus contribute significantly to dysregulation of calcium homeostasis leading to myocardial damage [29].

Immune system Studies have shown that elevated levels of IL-1β, IL-6, and TNFα, all pro-inflammatory markers, are elevated following doxorubicin therapy [30]. As discussed above, doxorubicin can activate the NFκB pathway which enhances inflammatory mediators as mentioned above along with vascular endothelial growth factor (VEGF) and matrix metalloproteinases (MMP2) [30]. Phosphorylation of IκB is mediated by Iκκ and is an important step in NF-Kb activation. In one study, it was observed that there was a significant increase in Iκκα expression in heart following doxorubicin treatment and that this increase was observed after only 24 h of a single dose [30]. This indicates that doxorubicin treatment can induce a quick activation of transcription factors.

Toll-like receptors have also been shown to be involved in Doxorubicin-induced cardiotoxicity [26]. Toll-like receptors are part of a family of pattern recognition receptors that act to detect danger signals including pathogens, oxidative stress among many. Toll-like receptors belong to the interleukin-1 receptor family (IL-1).

Prevention of Doxorubicin-Induced Cardiotoxicity

Dexrazoxane Dexrazoxane is the only FDA-approved cardioprotective agent for anthracycline-induced cardiotoxicity [31]. Dexrazoxane is an adjunctive agent that can act as a free radical scavenger. It is converted into a ring-opening chelating agent and can replace iron in the doxorubicin Fe^{3+} complex and combine with iron. In this manner, dexrazoxane interferes with iron ion-mediated free radical production, weakening the cardiotoxic immune effector cells and blocking the inactivation of respiratory enzymes by iron complexes. Additionally, dexrazoxane can chelate iron and prevent ROS through non-enzymatic mechanism of Doxorubicin.

Many studies have been done to determine the efficacy of dexrazoxane in preventing doxorubicin-induced cardiotoxicity. In a group of 200 children with acute lymphoblastic leukemia (ALL) who had received a cumulative doxorubicin dose of 300 mg/m^2, blinded troponin T measurements were taken at different time points before, during, and after doxorubicin infusion [11]. These children were randomized to receive doxorubicin plus dexrazoxane or doxorubicin alone. At the end of treatment, it was found that 20% of children who had been given both doxorubicin and dexrazoxane had elevated TnT levels as opposed to 47% who only received doxorubicin [11]. Furthermore, in a 5-year follow-up, left ventricular fractional shortening was found to be lower in children who were treated with doxorubicin alone as opposed to those who were treated with both doxorubicin and dexrazoxane. In another study conducted by Cheng et al. in BALB/c mice with and without tumors, it was found that mice treated with doxorubicin plus dexrazoxane showed normal heart tissues morphologically with no characteristic inflammation or tissue injury [11].

However, a major concern with regard to use of dexrazoxane as a cardioprotectant is the potential risk of secondary malignancy [11]. It is for this reason that currently the Food and Drugs Administration (FDA) has approved the use of dexrazoxane only in women with metastatic breast cancer who received cumulative doses of 300 mg/m^2 [32]. There have been studies conducted however to disprove these findings. In one large study, in pediatric patients with acute lymphoblastic leukemia, it was observed that for children who received dexrazoxane, the occurrence of a secondary malignancy was a rare event. In the study conducted for 553 pediatric

patients with ALL, only 1 developed acute myeloid leukemia in a median follow-up period of 5 years. In another group, there was no significant difference reported in incidence of secondary malignancy in pediatric ALL patients ($n = 173$) who received dexrazoxane as compared to placebo ($n = 150$) [6]. This seems to suggest that the risk for developing secondary malignancy using dexrazoxane is low, but the FDA recommendation is that this cardioprotectant not be used for pediatric patients until further studies are done.

ACE inhibitors and beta-blockers Angiotensin-converting enzymes (ACE) are a mainstay in the treatment of heart failure [20]. Angiotensin is a peptide hormone that is involved in regulating blood pressure. Angiotensin-converting enzyme is part of the renin-angiotensin system that controls blood pressure by converting the angiotensin I to an active vasoconstrictor angiotensin II. Therefore, ACE can cause an increase in blood pressure by causing vessels to constrict. Hence ACE inhibitors, including enalapril, zofenopril, and lisinopril, have been used to treat heart failure [20]. These drugs can act as an antioxidant as it was observed that administration of enalapril helped attenuate Doxorubicin-induced cardiac dysfunction by preserving mitochondrial respiratory efficiency and reducing free radical generation [20]. However, for long-term use, the effectiveness of these drugs in childhood cancer survivors diminishes after 6–10 years [20]. Furthermore, ACE inhibitors could also have adverse side effects, and hence use of these agents for cardioprotection needs to be monitored.

Beta-blockers, including carvedilol, have been shown to preserve left ventricular function after doxorubicin treatment in patients as compared to placebo [26]. Beta-blockers work by blocking adrenaline and reducing blood pressure. Additionally, early addition of β-blockers and angiotensin-converting enzymes has been shown to improve myocardial contractility in doxorubicin-induced cardiotoxicity [26]. However, Georgakopoulos et al. demonstrated that metoprolol, a β-blocker without antioxidative properties, was not able to provide cardioprotection in lymphoma patients treated with doxorubicin [11].

Other cardioprotectants Antioxidants including resveratrol have been used in acute doxorubicin treatment to significantly decrease ROS generation which in turn improved glutathione, superoxide dismutase, and catalase activity [26]. This helps with improving cardiac function. Erythropoietin (EPO) is a cytokine that stimulated the production of red blood cells in the bone marrow. EPO can act as a cardioprotective agent against Doxorubicin-induced apoptosis [26]. Another drug sildenafil, a phosphodiesterase 5 inhibitor, has been used to attenuate cardiomyocyte apoptosis and preserve the mitochondrial membrane potential to maintain myofibril integrity and prevent left ventricular dysfunction in a mouse model of Doxorubicin-induced cardiotoxicity [26]. Pretreatment with sildenafil maintained mitochondrial integrity by augmenting cellular mechanisms mediated by NO/cyclic GMP.

Another cardioprotective agent is monoHER which is the main constituent of flavonoids Venoruton [26]. In an in vivo and ex vivo mouse model, pretreatment with monoHER protected against doxorubicin-induced cardiotoxicity and additionally did not interfere with the antitumor effect of doxorubicin [26]. However, more studies are needed to demonstrate the efficacy of this agent. There is also new evidence that cardiacα1-adrenergic receptors can protect from doxorubicin-induced cardiotoxicity by protecting cardiomyocytes [26]. Stimulation of these receptor agonists, including phenylephrine and dabuzalgron, reduces apoptosis and interstitial fibrosis and in turn decreases myocardial dysfunction caused by doxorubicin [26]. This effect was associated with anti-apoptotic proteins of the Bcl2 family and preserving mitochondrial function.

Exercise intervention Aerobic exercise has been shown to have cardioprotective effects and is rec-

ommended by the American Heart Association to promote cardiac health and in particular for cancer survivors of all ages [33]. Recently, in a study by Wang et al., it was observed that aerobic exercise was an effective intervention in mitigating acute cardiac side effects in pediatric mice treated with doxorubicin [33]. Reduction in ejection fraction and fractional shortening were found to be prevented by exercise in mice that had been treated with Doxorubicin. Additionally, it was found that doxorubicin caused a reduction in body weight and the heart: tibia size ratio in tumor-bearing mice that were treated with doxorubicin alone [33]. However, the exercise intervention administered during therapy helped mitigate this weight loss and rescued the heart: tibia size ratio. The exact mechanism(s) by which exercise had this cardioprotective role in mice treated with doxorubicin is yet to be determined. Additionally, the cardioprotective benefits of exercise when delivered after active treatment phase need to be examined.

Summary

In this chapter, we discussed the antitumor mechanisms of action of doxorubicin, one of the most used chemotherapy agents for the treatment of patients with osteosarcoma. We also focused on doxorubicin-induced cardiotoxicity, a late effect seen in sarcoma survivors. Additionally, the methods to detect and monitor heart cardiotoxicity and possible biomarkers that have been proposed for the early detection of cardiac damage were outlined. The exact mechanism(s) responsible for doxorubicin-induced cardiotoxicity is still unclear. Several mechanisms have been proposed, most notably the role of ROS. Drugs/interventions that have been used to alleviate doxorubicin-induced cardiotoxicity were also covered with exercise being a promising new alternative based on recently published studies. As doxorubicin-induced cardiotoxicity can occur several years after completion of treatment, it is important especially in the case of childhood and AYA sarcoma survivors to find a suitable intervention to mitigate this side effect while keeping the efficacy of the drug against tumor cells intact.

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16 Exercise and Physical Activity in Patients with Osteosarcoma and Survivors

Miriam B. Garcia, Kirsten K. Ness, and Keri L. Schadler

Abstract

Exercise has the potential to positively affect patients with osteosarcoma by improvement of function, mitigation of disability, and maintenance of independence and quality of life. Exercise may also directly impact cancer treatment efficacy. This chapter examines the feasibility and use of exercise or physical activity as therapy in the treatment of osteosarcoma and its survivors. It additionally presents the benefits of physical activity as treatment and rehabilitation both preoperatively (prehabilitation) and postoperatively. This chapter will also discuss barriers to exercise and physical activity for patients with osteosarcoma and its survivors, emphasizing the need for a comprehensive and cohesive support system to promote its incorporation into patient treatment plans and ensure compliance.

Keywords

Exercise · Physical activity · Osteosarcoma · Bone cancer · Survivors · Cancer treatment · Adjuvant therapy · Postoperative rehabilitation · Prehabilitation

Introduction

As limb salvage surgery, amputation, and rotationplasty are necessary in the treatment of osteosarcoma, all patients with osteosarcoma will require postoperative rehabilitation. These surgical interventions may significantly alter a patient's physical, psychological, and socioeconomic status; thus, postsurgical rehabilitation and physical activity can mitigate some of the challenging impacts on a patient's function and quality of life [1]. Physical activity and rehabilitation serve multiple purposes: restoration of function, minimization of disability, and maintenance of independence and quality of life [1]. The use of rehabilitation postsurgically is the standard of care for the treatment of osteosarcoma. However, in many cases, postsurgical rehabilitation alone falls short of returning a patient to a full life because there is a lack of emphasis on the need for continuing physical activity and exercise beyond the scope of

M. B. Garcia · K. L. Schadler (✉)
Department of Pediatric Research, MD Anderson Cancer Center, Houston, TX, USA
e-mail: klschadl@mdanderson.org

K. K. Ness
Department of Epidemiology and Cancer Control, St. Jude Children's Research Hospital, Memphis, TN, USA

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supervised physical therapy sessions. Cancer survivors report even lower levels of physical activity than the general population [2]; 27% adult survivors of childhood osteosarcoma report inactive lifestyles and 56% do not meet recommended physical activity guidelines [3]. A substantial body of the literature based on preclinical and clinical research supports the use of exercise interventions and promotion of physical activity for survivors of cancer, including osteosarcoma.

In addition to the importance of exercise in survivorship, a growing body of the literature supports the use of exercise and physical activity interventions as a critical component of care for patients actively undergoing chemotherapy and/or radiation, even prior to surgery. This chapter will review current evidence on the feasibility and beneficial effects of exercise for osteosarcoma patients and survivors.

Exercise and Physical Activity in Patients Undergoing Cancer Treatment

Feasibility

The feasibility and benefits of exercise and physical activity are not limited to postsurgical rehabilitation but have been well established in patients with various cancers, including sarcoma, breast, colorectal, lung, and pancreatic cancers [4, 5]. The majority of these studies are in adult populations; however, the number of feasibility and outcome studies of exercise in pediatric and adolescent and young adult (AYA) patients is growing. The feasibility of physical activity during cancer treatment has now been studied and proven in both the inpatient and outpatient settings, and even in terminally ill patients [5–8]. Importantly, barriers and motivation for physical activity and exercise during cancer treatment of patients have also been investigated and should be considered when recommending exercise to patients or designing an exercise intervention [9, 10].

Patients with lower extremity sarcomas treated surgically are at a 50% increased risk for activity limitations compared to their peers [11]. To address existing impairments to physical activity, preoperative rehabilitation ("prehabilitation") in patients undergoing preoperative chemotherapy prior to limb-sparing procedures and amputation has been studied [12]. Exercise increases strength and endurance and leads to skeletal muscle adaptations [13], thus reducing limitations to exercise present prior to potentially disabling surgical procedures [14], and may improve postoperative outcomes. The findings in bone tumor patients summarized here are supported by multiple studies in a variety of malignancies (lung, breast, colorectal, pancreatic) which demonstrate that prehabilitation exercise is indeed feasible [15–17].

In a study of adolescents and children with osteosarcoma, all patients randomized to receiving the physical therapy intervention were able to complete at least 50% of their scheduled prehabilitation exercise sessions, demonstrating that preoperative rehabilitation was feasible [12]. Patients did not attend sessions due to illness, a previous appointment that ran late, or unknown reasons. The intervention group consisted of 12 patients who participated in 10–12 weeks of 30–60 minute physical therapy sessions three times per week that included endurance, strengthening, and stretching exercises. Endurance exercises consisted of ambulation, with or without an assistance device, upper extremity ergometry, or video simulation of sport (boxing, bowling, tennis). Strengthening exercises could be done with or without resistance and included bicep/tricep curls, press-ups from a mat or wheelchair, calf raises, or squats [12].

Another trial similarly studied neo-adjuvant exercise in pediatric and adolescent patients with solid tumors, 38% ($n = 9/24$) of which were bone tumors, in an in-hospital setting where inpatient and outpatient participants performed three sessions per week that included 30 minutes of aerobic exercise and 30 minutes of strength training per session [18]. The sessions were supervised and individualized for the patient and held in the participant's room or in a hospital gymnasium. Sixty-eight percent of patients were able to complete ≥90% of the exercise prescribed to them,

further supporting the feasibility of exercise for patients with bone tumors undergoing therapy. Alterable and unalterable reasons for being unable to complete or adhere to the exercise prescribed included lack of transportation, extreme fatigue or treatment-associated side effects, a concurrent chemotherapy session or procedure, and/or poor functional status due to illness or infection [18]. Importantly, there were no injuries incurred related to exercise in either study [12, 18].

Patients with bone tumors are not only compliant, demonstrating feasibility, but overall satisfied with intense rehabilitation during postoperative chemotherapy [19]. However, physical activity should continue beyond an observed prescription period for patients with malignant bone tumors to continue to experience its benefits after completing the intervention [20]. A prospective, observational study of 27 adult patients with either osteosarcoma or Ewing sarcoma requiring a modular knee prosthesis surgery was conducted to report compliance to an intensive in-hospital rehabilitation and their satisfaction. Patient compliance ranged 61–100% where surgical complications and chemotherapy-related symptoms were the most prevalent causes of inability to participate in rehabilitation treatment. Patients reported their satisfaction on a Likert scale and resulted in a mean satisfaction score of 7.9/10. This study concluded that intense physiotherapy during postoperative chemotherapy in patients with bone tumors was feasible and satisfactory and should hence be promoted by care teams to their patients [19]. In a study of individualized exercise interventions performed on pediatric patients with osteosarcoma and Ewing sarcoma, patients who participated in the in-hospital intervention had improved physical activity at home; however, the positive effects at home declined after the in-hospital intervention ceased [20]. This study also noted that physical activity participation improved as patients became further removed from their surgical date, indicating that while an activity may be physically feasible, it may be better tolerated when separated by recovery time rather than performed daily [20]. Physical activity prescriptions should exceed in-hospital treatment periods and should be tailored to patient stamina to be carried on at home for osteosarcoma patients to reap its benefits beyond cancer treatment.

Exercise does not have to be limited to classic aerobic activity like brisk walking and upper extremity ergometry or weight-based strength training under direct supervision. With proper guidance and training by physical therapists, oncologists, and surgeons, patients can find therapeutic effect from alternative activities such as yoga, qigong, and tai chi or in-home and community-based exercise. Yoga, qigong, and tai chi are gentle and feasible methods of engaging in physical activity and can be individualized for multiple stages of treatment and safely performed at home or in group settings [21–24]. Community-based exercise programs are both feasible and effective in reducing barriers to exercise by helping to provide motivation, socialization, and provision of a program that is safe for individuals with cancer [25, 26]. Home-based physical activity programs for patients with cancer are feasible [16, 27–29], but have not yet been studied specific to patients with bone tumors. There is an ongoing trial for pediatric and adolescent patients with bone tumors comparing the feasibility of a home-based vs. supervised exercise program (NCT02893397), but results are not yet available. Currently, a patient with osteosarcoma who wishes to engage in home exercise should relay this desire to his or her treatment team and decide together what their unique physical activity program should include or restrict.

Clinical oncology societies worldwide are now recognizing the feasibility and therapeutic impact of exercise. The Clinical Oncology Society of Australia recently released a position statement on exercise in cancer care stating the safety and effectiveness of exercise in counteracting physical and psychological effects of cancer and its treatment [30]. Their publication provides standard of care guidelines for health professionals for the integration of exercise in cancer care in active treatment and beyond [30]. Both the British Association of Sport and Exercise Sciences and the American College of Sports Medicine have also published and

supported guidelines for cancer patients and survivors in the United Kingdom and the United States [31, 32]. Here along with the American Cancer Society, they confirm exercise feasibility in patients with cancer and survivors and recommend they follow the Physical Activity Guidelines for the general United Kingdom or American populations [31–33]. Canadian authorities in cancer care and exercise have also developed clinical practice guidelines for people with cancer providing recommendations for duration, frequency, and intensity that similarly support Canada's Physical Activity Guidelines for their general population [34].

A multidisciplinary team is essential to the success of an exercise regimen during cancer therapy and postoperative rehabilitation. Each individual patient will need his or her physical fitness needs and abilities detailed and met through goals or restrictions supported by medical staff (surgeons, oncologists, neurologists) and therapists (physical and occupational). Emotional and social goals and barriers will need to be discussed and addressed together with the patient, his or her support system, as well as psychologists, social workers, case managers, and in the case of pediatric patients, child life professionals [1].

Each member of the medical team will contribute to understanding a patient's capabilities, projected function, and physical barriers to mobility. The surgeon will guide the patient and medical team in safe movement and may place and lift motion restrictions based on postsurgical healing. The oncologist will continue treatment against osteosarcoma and address any physiologic barriers to functionality related to the treatment, such as chemotherapy-related nausea or fatigue or postoperative pain. A neurologist or neurophysiologist may assist in mitigating neuropathy, balance, and phantom pains related to surgery or tumor burden. Physical therapists will focus on maximization of function and fitness, as well as ensure that musculoskeletal, neuromuscular, integumentary, and cardiopulmonary rehabilitative needs resulting from tumor burden and treatment are being addressed [35]. Occupational therapists will aid in maximizing a patient's independence, activities of daily living, and performing activities important to the individual related to work, school, self-care, or leisure and exercise. Physical and occupational therapists will be key to preventing disability that would hinder regular performance of physical activity [1]. A case manager will also be critical to addressing a patient's physical barriers to exercise and daily activity, should special equipment be warranted to aid patients in mobilization.

Emotional and psychosocial health and support may also greatly influence a patient's motivation for exercise [10, 36]. Generally, patients consider exercise during cancer treatment as reasonable and feel positively towards regular exercise [10]. Patients have reported exercise as an occupation that helps distract them that is fun, makes time pass faster, provides a change of scenery, and improves their mood [10]. However, these same patients noted the role social support from family or treatment team played in their motivation to move [10]. Some patients would not have engaged in physical activity due to lack of consideration or internal motivation and emphasized the importance of someone prompting them to exercise [10, 36]. Patient family and friends can also influence patient level of physical activity through behavior modeling and exercising together with the patient [36–38]. Options for group classes can also provide socialization and increase external motivation. Assistance from experts in psychosocial support, including psychologists, social workers, and child life along with patient family and friends, will make a difference in a patient's level of physical activity through not only prompting physical activity but also assisting in highlighting and treating any emotional barriers to exercise such as depressed mood, mental fatigue, or difficulty coping with a new diagnosis and lifestyle changes.

The team together should assist in removing organizational restraints to engaging in any supervised physical activity or exercise requiring equipment. This may be done by providing fixed dates or flexibility with physical therapists or gym availability depending on the individual patient's preference and schedule. Implementing exercise naturally into a patient's hospitalization

routine will also provide a built-in prompt to engage in physical activity in the inpatient setting. Easy access to a patient gym, if this resource is available, may also mitigate barriers to access. Additionally, seeking aid from social work and insurance or financial assistance agencies may be necessary to facilitate transportation or access to physical therapy sessions or exercise facilities.

In summary, exercise during chemotherapy and/or radiation therapy has been shown to be feasible and safe for patients with numerous types of cancer diagnoses. At least five published clinical trials have included patients with osteosarcoma and have demonstrated that an exercise intervention during cancer treatment is feasible and safe. As evidence of potential benefits of exercise for cancer patients is rapidly building (discussed in the section below), the studies demonstrating safety and feasibility of different types of exercise interventions provide the needed foundation for beginning to utilize exercise in trials with clinical endpoints moving beyond feasibility to efficacy.

Benefits

Exercise and staying physically active has numerous benefits for patients with cancer. Some are similar to benefits obtained by the general population such as improved quality of life and physical functioning, while recent research suggests that an added benefit may be improved treatment efficacy. As with studies on the feasibility of exercise during cancer treatment, studies regarding the benefits of exercise for patients with osteosarcoma specifically are relatively sparse. However, data in patients with diverse cancer diagnoses supports the use of exercise during cancer treatments as likely to be advantageous in multiple capacities of patient care. Some of the first studies to identify the benefits of physical activity or exercise for patients undergoing treatment for cancer evaluated the association between physical activity and patient quality of life (QOL), followed by the effect of an exercise intervention on quality of life. Similar to physical activity in non-cancer populations, numerous studies have demonstrated that physical activity during cancer treatment is associated with improved QOL, though only a correlation and not a causation can be ascertained [2]. These studies are further supported by dozens of randomized controlled trials (RCTs) where participation in an exercise intervention was compared to control groups receiving standard of care, which often includes a general recommendation to exercise. In these studies, exercise during cancer treatment was found to improve factors that contribute to QOL, such as fatigue and mood. In a large meta-analysis of 56 RCTs that included a total of 4826 cancer patients actively receiving treatment, exercise interventions were shown to have a positive impact on overall health-related QOL [39]. Specifically, exercise interventions significantly reduced fatigue and improved social functioning. For some subsets of patients, exercise reduced anxiety [39]. In another recent meta-analysis, evaluation of nine studies of women with breast cancer undergoing radiation therapy found that participation in an exercise intervention had a significant impact on reducing fatigue [40]. In addition to the clear evidence that exercise during treatment reduces fatigue for patients with a variety of cancer diagnoses, older adult cancer patients who participate in exercise interventions experienced shortness of breath less frequently [41] and for cancer patients over 80, exercise during treatment correlated with less severe memory loss [41].

While few studies have evaluated the impact of exercise on quality of life specifically in patients with osteosarcoma, meta-analysis level data including studies of patients with multiple cancer types strongly supports improved QOL in patients who exercise. As QOL has been shown to have prognostic value for adults with cancer [42], the use of exercise to improve QOL merits intentional inclusion in patient care.

Quality of life is substantially impacted by physical function or lack thereof. Patients undergoing treatment for osteosarcoma experience significant loss of physical strength and function due to chemotherapy, radiation, and surgery. Exercise is well-known to maintain or build strength and function in healthy persons, and

evidence is building to suggest that exercise can also protect against functional loss in cancer patients. Physical function and fitness affect patient quality of life and have implications for comorbidities such as cardiovascular disease, which is increased in survivors of osteosarcoma; thus, improving physical strength and function and maintaining these gains are important benefits of exercise for patients with osteosarcoma.

A recent meta-analysis assessed 28 randomized control studies including a total of 3515 adult patients with cancer who participated in an exercise intervention or were in a control arm [43]. The studies included in the analysis evaluated exercise interventions that occurred during treatment (10 RCTs), following treatment (14 RCTs), or during and following treatment. This meta-analysis found that an exercise intervention significantly improved upper body muscle strength, lower body muscle strength, lower body muscle function, and aerobic fitness [43]. Larger effect sizes were seen for improved upper body muscle strength when the intervention was delivered while patients were on treatment, and supervised exercise caused significantly larger improvements in all outcomes measured compared to unsupervised exercise.

Similar findings were reported in a Cochrane Systematic Review meta-analysis of 56 RCTs, discussed in the previous section regarding QOL. In addition to demonstrating that exercise improved QOL for cancer patients, exercise was also found to improve physical functioning [39]. Moreover, a meta-analysis of 33 RCTs including aerobic exercise interventions in adults with cancer found that exercise significantly improved physical fitness from pre- to post-intervention. Interestingly, of the RCTs reviewed, some demonstrated evidence of reduced symptom burden including reduced nausea and cytopenia [44]. However, while the evidence supporting improved or maintained physical fitness and function by exercise during treatment is strong and consistent, evidence regarding the effect of exercise on symptom burden is variable and conclusions are not yet clear.

In agreement with data in adult cancer patients, a Cochrane Database Systematic Review of 6 RCTs that consisted of exercise interventions for children and young adults undergoing treatment for acute lymphoblastic leukemia also found that exercise improved physical strength and function [45]. The studies reviewed found improved 9-minute run-walk tests and improved back and leg strength combination scores for patients in the intervention groups compared to control.

While the number of studies specifically examining patients with osteosarcoma is again sparse, those that have been accomplished demonstrate that exercise can contribute to maintenance of physical function. In a study comparing 14 AYA patients with osteosarcoma who participated in an exercise intervention prior to surgery to 35 control patients who were also treated for osteosarcoma but did not participate in the exercise, the intervention group scored significantly better on the Functional Mobility Assessment evaluation and 9-minute run-walk test [12].

In addition to improving patient quality of life and physical functioning, newly emerging evidence suggests that physical activity or exercise may impact treatment efficacy. One potential benefit of exercise during cancer treatment may be an increase in treatment completion rates. While evidence in this area is still developing, a recent systematic review of 8 RCTs utilizing an exercise intervention found that in two of the eight studies examined, patients who participated in the exercise intervention had significantly better chemotherapy completion rates [46]. In addition to the potential to improve chemotherapy full-dose completion rates by reducing side effects, exercise may actually improve the efficacy of chemotherapy. There have not yet been clinical trials demonstrating improved chemotherapy efficacy by exercise, but numerous animal studies demonstrate exactly that. While these do not yet include osteosarcoma models, the preclinical evidence in a diverse number of cancer types including melanoma, pancreatic cancer [47], Ewing sarcoma [48], and breast cancer [49] in mice and rats strongly supports the potential of exercise as an adjuvant to chemotherapy.

Exercise-induced improvements in chemotherapy efficacy against solid tumors are believed

to be afforded by improved delivery of the chemotherapy to the tumor. Solid tumor vasculature is disorganized and dysfunctional. Therefore, drug delivery to tumor cells is impaired and tumor cells are often hypoxic. Exercise has been shown to significantly improve blood delivery to orthotopic prostate tumors [50] and to improve vascular structure or function in several other tumor models. Importantly, moderate aerobic exercise 5 days per week led to significantly more doxorubicin in melanoma [47] and Ewing sarcoma [48] tumors, suggesting that exercise does indeed make tumor vasculature more efficient at delivering chemotherapy to solid tumors. This correlated with significantly better efficacy of doxorubicin or cisplatin against melanoma, Ewing sarcoma, and mammary carcinoma [49] tumors in mice.

In addition to improving chemotherapy efficacy, improved blood vessel function after exercise has been shown to reduce tumor hypoxia. As radiation is more effective in oxygenated tissue, it is not unreasonable to expect that an exercise intervention would likely increase radiation efficacy. Indeed, this concept is being explored by preclinical laboratories. Preliminary evidence of exercise-induced improvements of radiation efficacy against mouse models of mammary 4T1 carcinoma and MC38 carcinoma has been reported [51] and is likely to be supported by further studies in the near future.

Though there is not yet data in osteosarcoma models, substantial preclinical evidence demonstrates the potential of exercise to improve therapeutic efficacy in solid tumors. Ongoing clinical trials will determine whether exercise improves chemotherapeutic and radiation efficacy, as well as survival outcomes for patients with several different cancer diagnoses, as predicted by animal studies.

Together, there is a sizeable amount of evidence to support the feasibility of exercise in patients with osteosarcoma undergoing cancer treatment and to indicate the likelihood of benefits for patients. Thus, support for physical activity and exercise for patients actively being treated for osteosarcoma should be included in patient care.

Exercise and Physical Activity in Survivors

Feasibility

Nearly 70% of children younger than age 14 years and 65% of AYAs 15–39 years will survive 5 years after a diagnosis of osteosarcoma (SEER) [52]. Unfortunately, treatment-related late effects are well documented in this vulnerable population as they move through adult life, where they are five times more likely than siblings to report severe, disabling, life threatening, or fatal chronic conditions [53]. These conditions are not limited to musculoskeletal problems because of tumor-related surgeries, but also include chemotherapy and/or lifestyle (inactivity, smoking, suboptimal nutrition)-related impairments in cardiac, pulmonary, autonomic, and neurosensory function [54]. Many of these conditions, like ischemic heart disease, obesity, dyslipidemia, and hypertension, are conditions that in the general population [55–57] respond favorably to optimal health behaviors like regular exercise. Thus, although osteosarcoma survivors cannot change their treatment exposures, they may be able to optimize health by engaging in regular exercise.

Unfortunately, nearly 30% of childhood osteosarcoma survivors are completely sedentary, and an additional 30% participate in less than the equivalent of 150 minutes of moderate physical activity each week [58]. Engaging in activity is difficult for anyone. In this population whose initial responses to exercise may be blunted and uncomfortable because of cancer- or treatment-related organ system dysfunction [59], it is especially difficult, possibly discouraging, and unlikely to result in meaningful behavioral change without specific guidance. Evidence to support interventions that promote regular exercise among childhood osteosarcoma survivors is scarce [60]; current standard of care is simply to encourage activity [61]. Interventions tailored to accommodate survivor specific impairments are needed.

Although additional work is needed, two preliminary studies among adult survivors of child-

hood cancer, including participants treated for osteosarcoma during childhood, demonstrate the safety and potential efficacy of exercise. One was done remotely (via telephone) and the other with tapered supervision (in a community fitness center). The first was a safety and preliminary efficacy study of a tailored aerobic and strengthening intervention, applied in sarcoma survivors, previously exposed to anthracyclines, whose cancer therapy included amputation and who developed subclinical cardiomyopathy. Five survivors (three male, mean age 38.0 ± 3.0 years) participated. This exercise prescription was 12 weeks in length and included aerobic and resistance training tailored to each participant's baseline capacity (i.e., accommodating their amputation and limited cardiac output). The aerobic component was progressed to achieve workloads of 40–70% of heart rate reserve for 20–45 minutes 3–5 days per week. The resistance component included one set of 12–15 repetitions on eight to ten exercises 2–3 days per week. All training was completed at home after a baseline demonstration. Weekly phone calls were completed by an exercise specialist to track adherence, answer questions, and adjust exercise progression. Adherence to prescribed exercise was 86%, there were no adverse events and peak oxygen uptake (exercise capacity), ejection fraction and quadriceps strength improved in all five participants an average of 10.6%, 12.6%, and 13.8%, respectively [62].

In a recently completed randomized placebo-controlled trial, designed to evaluate the effects of adding a protein supplement versus placebo to resistance training on muscle mass and strength among survivors with relative lean muscle mass at least one standard deviation below age-, sex and race-predicted values, an individually tailored intervention (to each participant's one repetition maximum (1RM) for each exercise) was employed. The intervention accommodated survivors with cardiac (N = 23) and pulmonary (N = 19) conditions and was 24 weeks in length. In-person supervision at local fitness centers was initially (weeks 1–4) twice a week, tapering to once a week in weeks 5–12, every other week in weeks 13–20, and then once a month in weeks 21–24. The exercise specialists and study coordinator were available by phone, text or e-mail to provide assistance between scheduled supervision as needed. Key card entry to the fitness facilities and participant logs were used to monitor adherence. Each training session included 5 minutes of upper body or cycle ergometer or treadmill warm-up. The first 4 weeks of the intervention included four sets (10–15 repetitions) for lower body exercises and two sets (10–15 repetitions) for upper body exercises at 60% of 1RM, progressing to 75% of 1RM (four sets lower body, two sets upper body, 8–10 repetitions). At week 5, four sets (8 repetitions) for lower body exercises and three sets (8–10 repetitions) for the upper body were performed at 75–80% of 1RM. Rest periods in between sets of one exercise were 1.5 minutes and in between exercises were 3 minutes. Intensity was adjusted after each 4-week 1RM reassessment. Workload was also increased if the participant successfully completed the maximum repetitions allowed for each exercise in three out of the four sets (or two out of the three sets for upper body exercises). Each training session ended with a 5-minute cool down period on the cycle ergometer or treadmill. Ninety-three persons were screened for eligibility; 70 met eligibility criteria (75%) and were randomized. During the study, three persons withdrew from the study, two because of medical events and one because of pregnancy. There were eight adverse events potentially related to the study. One was serious (myocardial infarction that did not occur during training); seven were not serious and included knee pain, muscle soreness, nausea, pain, and anxiety. Among the 67 survivors who completed the study, 57 (85%) were adherent to the prescribed resistance training (70% of sessions) and protein supplement or placebo consumption (81% of packets consumed). Participants were a median age of 32 (range 20–45) years and 52% male. Mean change in lean body mass improved for both groups (protein supplement 1.05 ± 2.34 kg, $p = 0.04$; placebo 0.13 ± 2.19 kg, $p = 0.74$; $p = 0.11$ for comparison of change between groups). Although there were no significant differences between groups (after adjusting for multiple comparisons) in muscle strength (1RM), both those who received the pro-

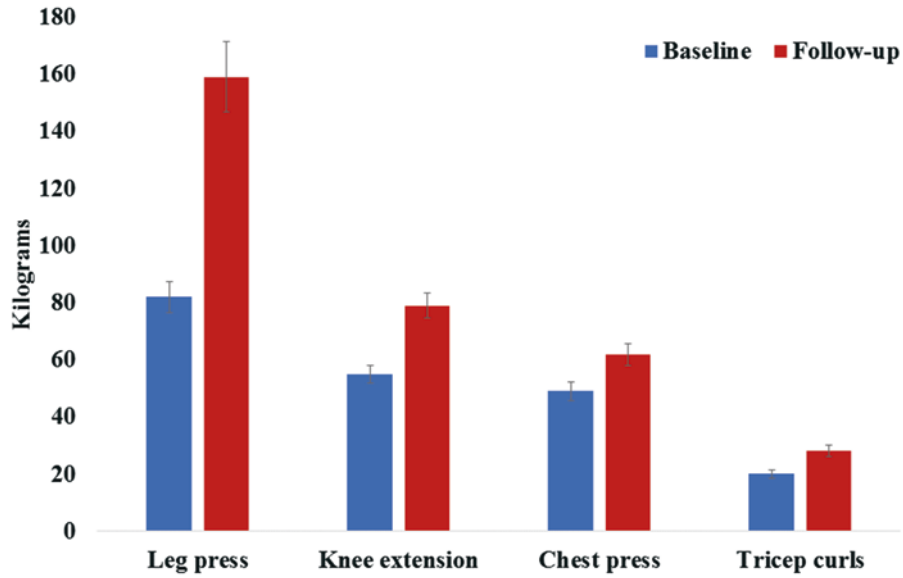

Fig. 16.1 Gains in strength over 24 weeks of resistance training

tein supplement and those who received the placebo had substantial mean gains in muscle strength. The Fig. 16.1 shows changes for four major muscle groups [63].

Benefits

Survivors of extremity sarcomas tend to be physically inactive, which may increase their morbidity and augment cancer-related late effects [64]. Survivors of osteosarcoma are at increased risk for multiple co-morbidities after completing treatment including cardiovascular, neurologic, psychologic, and orthopedic and functional abnormalities [64–66]. While the body of the literature supporting exercise and physical activity specifically in survivors of osteosarcoma is scant, there is a growing amount of support reinforcing the mitigating effects of exercise on cancer and therapeutic late effects as well as its potential to reduce non-cancer-related morbidity and mortality in cancer survivors in general [67, 68]. The role of exercise in cancer survivorship has been reviewed in depth through meta-analyses by Fong et al. and Schmitz et al. [69, 70].

Cancer survivors who engage in regular physical activity after completion of therapy may also experience several functional and physical benefits of their subsequent improved physical fitness. Clear improvements have been seen in their peak oxygen consumption, peak power output, 6-minute walk test, and strength [69–71]. Physiological benefits from exercise in cancer survivors may include improved sexual function, improved sleep, reduced incontinence, reduced pain, and improved blood pressure and cardiovascular health [72]. Cancer and therapy-related osteopenia can especially improve through strength or impact and resistance training to generate osteogenic stimuli and increase and preserve bone mass density [67]. Aerobic exercise combined with resistance training has been shown to significantly increase bone mass density in the spine, hip, and whole body of female cancer survivors [73]. Survivors may experience weight and muscle mass imbalance after treatment, developing cachexia, sarcopenia, or increased fat mass [74]. An increase in fat mass related to cancer therapies such as steroids or other hormonal therapies may mask the loss of muscle mass. These muscular changes can lead to impairments in muscle strength and

balance that fortunately can be improved with physical activity [75]. Strength and resistance training increases the synthesis of actin and myosin, thus increasing muscle mass and strength [75]. In addition to protein anabolism, exercise also has an antioxidant effect that may lower release of pro-inflammatory cytokines that can be high in cancer cachexia [67]. Physical activity has also been beneficial to survivors with chemotherapy-induced peripheral neuropathy and lymphedema [76, 77], two symptoms likely to be endured by postoperative osteosarcoma patients. Additionally, individualized exercise interventions have demonstrated improved cardiovascular and pulmonary function with simultaneous reduction in fatigue in a variety of cancer survivors [78].

Exercise has also been shown to reduce cancer-related fatigue [79] and improve survivor quality of life domains such as emotional well-being [80, 81]. Cancer-related fatigue is experienced in up to 99% of cancer survivors and can be related to treatment, depression, or inactivity and deconditioning that can at times be debilitating [81]. When compared to pharmaceutical treatment of cancer-related fatigue, exercise was superior, demonstrating significant improvement in fatigue compared to no improvement through pharmaceutical intervention [82]. Fatigue negatively impacts many aspects of a survivor's life from work, relationships, mood, and daily activities. Exercise provides an easy-accessible, non-pharmacologic tool to combat this distressing late effect of cancer and its therapies.

Cancer-related fatigue may also be related to cardiac late effects of chemotherapy. AYA patients who received anthracycline during their treatment for osteosarcoma experienced a significant decline in in their left ventricular ejection fraction, which was greater than a 10% reduction in some patients [83]. This is important due to the link between left ventricular ejection fraction decline and future congestive heart failure [84]. Fatigue and dyspnea are the two most common symptoms reported by patients with heart failure [85]. Preclinical studies have been able to demonstrate that aerobic exercise during early exposure to doxorubicin mitigated decreases in ejection and shortening fractions, thus attenuating cardiotoxicity [86]. Exercise can also improve other quality of life factors experienced earlier in life by cancer survivors, such as fear of death or loss of independence. In survivors who experience psychosocial late effects, such as anxiety, depression, or stress, physical activity has also been shown to significantly improve these symptoms compared to survivors randomized to non-exercising control groups [87]. Study groups have included patients with mixed cancer, breast, lymphoma, colorectal, prostate, and lung and participants performed a variety of exercises such as walking, cycling, strength training, swimming, elliptical training, and yoga [87, 88].

Physical activity and its impact on insulin-like growth factor-I have also been implicated in the reduction of cancer recurrence and overall mortality. Elevated concentrations of insulin-like growth factor-I (IGF-I) have been associated with an increased risk for certain cancers and their recurrence, and multiple studies have demonstrated that physical activity is associated with significantly reduced serum concentrations of IGF-I [89–92]. In a group of 832 patients with colon cancer, 47% of them had a significant improvement in disease-free survival after engaging in 18 MET hours of exercise per week [93]. Similarly, a study of women with breast cancer demonstrated the relative risk of death from breast cancer, and the risk of breast cancer recurrence was 25–40% lower in women with high levels of physical activity compared to those with low levels of physical activity. Here the most benefit was seen at a level of feasible moderate activity, e.g., 3–5 hours of walking at 2–2.9 mph [94]. Considering IGF-1 has been implicated in the pathogenesis of osteosarcoma [95], it may be hypothesized that reduced IGF-1 by exercise can reduce cancer recurrence for osteosarcoma survivors.

In cancer survivors, exercise may reduce the risk of recurrence and secondary malignancy and increase survival while increasing immune function, unlike chemotherapy, radiotherapy, and surgery, which all have immunosuppressive effects [96]. The Inverted J Hypothesis, a working the-

ory in the field of exercise immunology, proposes that regular moderate exercise can enhance the immune system and thus decrease the incidence of cancer and infection, whereas overtraining can lead to a suppressed immune system [96, 97]. Response is variable depending on chronicity, duration, and intensity; however, moderate exercise has been found to have a positive, stimulatory effect on macrophages, monocytes, neutrophils, lymphocytes, and production of certain cytokines like interleukin-6 and TNF-alpha [97]. In recent studies, statistically significant findings have been reported in survivors engaging in prescribed physical activity interventions ranging in frequency from 3 to 10 times/week, intensity from 60% to 80% maximum heart rate, and training from 30 to 60 minutes for 2–29 weeks. The subjects either walked, engaged in resistance and strength training, used cycle ergometers, or performed a combination of the three. Immunologic benefits included improved NK cell cytolytic activity, monocyte function, and an increase in circulating granulocytes with shorter periods of neutropenia [96].

Many cancer survivors may have functional limitations that prevent them from being able to achieve a moderate to vigorous level of exercise. Survivors may also benefit from light intensity activity [2, 98–100]. Participation in light activity with an active lifestyle, but not moderate to vigorous physical activity, improved physical health in colorectal cancer survivors compared to survivors with inactive lifestyles [99]. Also in colorectal cancer survivors, light physical activity has been associated with higher physical functioning, higher role functioning, and lower disability, especially in survivors with multiple comorbidities [100]. Importantly, increasing light-intensity activity in a group of mixed cancer survivors correlated with improved measures of physical function in participants who were unable to perform, initiate, or maintain moderate to vigorous intensity activity [98]. Survivors, much like non-cancer survivors, may have motivational limitations to exercise and should be urged to engage in light, necessary if not enjoyable, activity rather than remain sedentary. This can include housework, grocery shopping, a leisurely walk, visiting family and friends, crafting, gardening, or other hobbies. Patients engaging in light activity can benefit both physically and mentally from its effects. Light activity has been previously associated with lower levels of depression [101], lower plasma glucose concentrations [102], and greater physical health and function [103]. Additionally, light exercise has been shown to enhance social participation, which has been demonstrated to improve mental health in both men and women [99] and relationships in women [2]. Survivors should not feel confined to fit any specific idea of exercise, but rather be encouraged to participate in movement and perform the physical activity that interests them and has potential to engage them long term, motivates them to grow in their physical fitness, and is suitable to any unalterable functional limitations.

Summary

In summary, exercise is gaining traction as an important component of care while patients are undergoing active cancer treatment and during survivorship. The benefits of exercise include psychosocial, physical functioning, and quality of life benefits. Excitingly, benefits may also extend to improved therapeutic efficacy and reduced cancer recurrence and mortality. As professional medical organizations worldwide are publishing guidelines for exercise in the oncology setting, oncology care teams are more frequently incorporating exercise recommendations and interventions into care for patients with cancer.

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Correction to: Current Advances in Osteosarcoma

Eugenie S. Kleinerman and Richard Gorlick

Correction to:
Part IV in: E. S. Kleinerman, R. Gorlick (eds.), *Current Advances in Osteosarcoma*, Advances in Experimental Medicine and Biology 1257, https://doi.org/10.1007/978-3-030-43032-0

Unfortunately the introduction of Part IV was omitted during typesetting and the book was published without it. Now the introduction has been added.

The updated online version of this chapter can be found at:
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Index

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