

# Biology of Childhood Osteogenic Sarcoma and Potential Targets for Therapeutic Development: Meeting Summary

Richard Gorlick,<sup>1</sup> Peter Anderson,<sup>2</sup>  
 Irene Andrulis,<sup>3</sup> Carola Arndt,<sup>2</sup>  
 G. Peter Beardsley,<sup>4</sup> Mark Bernstein,<sup>5</sup>  
 Julia Bridge,<sup>6</sup> Nai-Kong Cheung,<sup>1</sup>  
 Jeffrey S. Dome,<sup>7</sup> David Ebb,<sup>8</sup> Thomas Gardner,<sup>9</sup>  
 Mark Gebhardt,<sup>8</sup> Holcombe Grier,<sup>10</sup>  
 Marc Hansen,<sup>11</sup> John Healey,<sup>1</sup> Lee Helman,<sup>12</sup>  
 Janet Hock,<sup>9</sup> Janet Houghton,<sup>7</sup> Peter Houghton,<sup>7</sup>  
 Andrew Huvos,<sup>1</sup> Chand Khanna,<sup>12</sup>  
 Mark Kieran,<sup>10</sup> Eugenie Kleinerman,<sup>13</sup>  
 Marc Ladanyi,<sup>1</sup> Ching Lau,<sup>14</sup> David Malkin,<sup>15</sup>  
 Neyssa Marina,<sup>16</sup> Paul Meltzer,<sup>1</sup> Paul Meyers,<sup>1</sup>  
 Deborah Schofield,<sup>17</sup> Cindy Schwartz,<sup>18</sup>  
 Malcolm A. Smith,<sup>22</sup> Jeffrey Toretsky,<sup>19</sup>  
 Maria Tsokos,<sup>12</sup> Leonard Wexler,<sup>1</sup>  
 Jon Wigginton,<sup>12</sup> Stephen Withrow,<sup>20</sup>  
 Mason Schoenfeldt,<sup>21</sup> and Barry Anderson<sup>22</sup>

<sup>1</sup>Memorial Sloan-Kettering Cancer Center, New York, New York; <sup>2</sup>Mayo Clinic, Rochester, Minnesota; <sup>3</sup>Mount Sinai Hospital and Research Institute, Toronto, Ontario, Canada; <sup>4</sup>Yale University School of Medicine, New Haven, Connecticut; <sup>5</sup>Hopital Sainte-Justine, Montreal, Quebec, Canada; <sup>6</sup>University of Nebraska Medical Center, Omaha, Nebraska; <sup>7</sup>St. Jude Children's Research Hospital, Memphis, Tennessee; <sup>8</sup>Massachusetts General Hospital, Boston, Massachusetts; <sup>9</sup>Indiana University Medical Center, Indianapolis, Indiana; <sup>10</sup>Dana-Farber Cancer Institute and Children's Hospital, Boston, Massachusetts; <sup>11</sup>Health Sciences Center, University of Connecticut, Farmington, Connecticut; <sup>12</sup>Pediatric Oncology Branch, National Cancer Institute, Bethesda, Maryland; <sup>13</sup>M. D. Anderson Cancer Center, Houston, Texas; <sup>14</sup>Texas Children's Cancer Center at Baylor College of Medicine, Houston, Texas; <sup>15</sup>Hospital for Sick Children, Toronto, Ontario, Canada; <sup>16</sup>Stanford University Medical Center, Stanford, California; <sup>17</sup>Children's Hospital Los Angeles, Los Angeles, California; <sup>18</sup>Johns Hopkins Hospital, Baltimore, Maryland; <sup>19</sup>Georgetown University Medical Center, Washington, DC; <sup>20</sup>College of Veterinary Medicine and Biological Sciences, Colorado State University, Ft. Collins, Colorado; <sup>21</sup>EMMES Corporation, Rockville, Maryland; and <sup>22</sup>Cancer Therapy Evaluation Program, National Cancer Institute, Rockville, Maryland

## ABSTRACT

Childhood osteogenic sarcoma (OS) is a rare bone cancer occurring primarily in adolescents. The North American pediatric cooperative groups have performed a series of clinical treatment trials in this disease over the past several decades, and biology studies of tumor tissue have been an important study component. A meeting was held in Bethesda, Maryland on November 29–30, 2001, sponsored by the NIH Office of Rare Diseases, the Children's Oncology Group, and the National Cancer Institute–Cancer Therapy Evaluation Program with the general objectives: (a) to review the current state of knowledge regarding OS biology; (b) to identify, prioritize, and support the development of biology studies of potential clinical relevance in OS; and (c) to discuss the available tissue resources and the appropriate methods for analysis of OS samples for the conduct of biology studies. This report summarizes the information presented and discussed by the meeting participants.

## INTRODUCTION

OS<sup>23</sup> biology studies are becoming increasingly important because of the clinical need for prognostic factors to stratify treatment and for new molecular targets that could indicate a role for targeted therapeutic agents in OS treatment. A meeting was held in Bethesda, Maryland, on November 29–30, 2001, sponsored by the NIH Office of Rare Diseases, Children's Oncology Group, and the National Cancer Institute–Cancer Therapy Evaluation Program with the following broad general objectives: (a) to review the current state of knowledge and share information regarding OS biology; (b) to identify, prioritize, and support the development of biology studies of potential clinical relevance in OS; and (c) to discuss the available tissue resources and the appropriate methods for analysis of OS samples for the conduct of biology studies. This article will highlight some of the information presented at the meeting.

## Pathogenesis of Osteogenic Sarcoma

**Genetic Abnormalities.** It is useful to place OS in the context of other sarcomas. In terms of oncogenetic mechanisms, there seem to be two major classes of sarcomas: (a) sarcomas such as ESFTs, with balanced karyotypes (reciprocal translocations) and alterations of tumor suppressor gene pathways in

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**Requests for reprints:** Dr. Barry Anderson, Clinical Investigations Branch, Pediatric Section, Cancer Therapy Evaluation Program, National Cancer Institute, 6130 Executive Boulevard, EPN741, Rockville, MD 20852. Phone: (301) 496-2522; E-mail: AndersonB@CTEP.NCI.NIH.gov.

<sup>23</sup> The abbreviations used are: OS, osteosarcoma; ESFT, Ewing Sarcoma Family Tumor; Rb, retinoblastoma; LOH, loss of heterozygosity; PTH, parathyroid hormone; ALT, alternative telomere lengthening; FasL, Fas ligand; IL, interleukin; Ad.mIL-12, adenoviral murine interleukin 12; MTP-PE, muramyl tripeptide phosphatidylethanolamine; MDR1, multidrug resistance gene 1; IGF, insulin-like growth factor; IGFBP-3, insulin-like growth factor binding protein 3; TK, thymidine kinase; GM-CSF, granulocyte macrophage colony-stimulating factor; COX, cyclooxygenase; VEGF, vascular endothelial growth factor.

relatively few cases; and (b) sarcomas such as OS, with complex unbalanced karyotypes, and alterations of the p53 and retinoblastoma pathways in most cases (1). Despite the complexity of the karyotype and the absence of characteristic reciprocal translocations, many recurrent, nonrandom chromosomal abnormalities are observed in OS.

A University of Nebraska Medical Center study analyzed 128 OS specimens, of which 70% were clonally abnormal (2, 3). Most demonstrated pronounced heterogeneity within the same patient. Marker chromosomes, structurally abnormal chromosomes in which no part can be identified, were detected in the majority of OS samples (58%). Ring chromosomes (7%) accompanied by multiple numerical (65%) and structural (72%) abnormalities were also prominent (2–4). There is evidence of genomic amplification (homogeneously staining regions or double min) in at least one-third of the cases. Cytogenetic abnormalities observed were both numerical and structural. Common numerical abnormalities in OS include: gain of chromosome 1; loss of chromosomes 9, 10, 13, and/or 17, and partial or complete loss of the long arm of chromosome 6 (2, 3, 5).<sup>24</sup> Frequent structural abnormalities include rearrangements of chromosomes 11, 19, and 20 (2, 3, 5, 6).<sup>24</sup>

**Tumor Suppressor Pathway Alterations.** The p53 and retinoblastoma tumor suppressor pathways are clearly involved in the pathogenesis of OS. Most OS samples have some type of combined inactivation of the retinoblastoma and p53 tumor suppressor pathways (7). In a study of 32 OS specimens, a number of loci were demonstrated to have LOH (3q, 13q, 17p, and 18q), including the locations of the *Rb* and *p53* tumor suppressor genes (8–10). The timing and sequence of these alterations, particularly relative to the development of the chromosomal complexity, and other tumor suppressor and oncogene alterations, are unclear. Evidence for the role of p53 in OS pathogenesis includes the predisposition of patients with germline *p53* mutations to develop OS (11–13).

Amplification of the 12q13 region (containing *MDM2* and *CDK4*) or *INK4A* deletion can affect both the p53 and Rb pathways, and, indeed, these alterations seldom coexist with Rb or p53 alterations (14). Because these tumors almost universally have genetic alterations that inactivate the Rb and p53 tumor suppressor pathways, gene inactivation by itself may not be a strong prognostic factor. Indeed, new data show low prognostic significance of *p53* mutations in sporadic OS. In a recent study (15), 22% of OS samples showed *p53* mutations, but there was no relationship to distant recurrence. Interestingly, *p53* status was concordant in all of the paired samples of primary and distant metastases, suggesting p53 pathway alterations may occur early in OS pathogenesis.

Genome-wide attempts have been made to identify potential tumor suppressor genes associated with the LOH in OS (16, 17). Examination of 38 chromosomal arms from OS tumor samples for LOH has found that the mean frequency of LOH is 30.79% for any chromosome arm, an unusually high mean

frequency for a childhood tumor. Moreover, several chromosome arms (3q, 13q, 17p, and 18q) underwent LOH with a frequency >2 SDs higher than the average ( $P < 0.002$ ; Ref. 16). Further mitotic mapping has identified minimal regions thought to contain candidate tumor suppressor loci on chromosomal arms 3q26.2 and 18q21.33 (9, 10). Additional analysis has suggested that other chromosomal regions may also harbor tumor suppressor loci important in OS tumorigenesis including chromosome arms 5q, 6q, 10q, 11p15q, 16p, and 22q (18).

OS is thought to be a tumor of osteoprogenitor cells. These are multipotential, hormone-responsive stromal cells in the periosteum and marrow, which are capable of differentiating into many lineages depending on their environmental cytokines (19). Published studies suggest that p53 may have a role in the normal development and physiology of bone (20). Failures in skull growth and delayed longitudinal bone growth have been described *in utero* in p53 null mice. An extensive characterization of bone, including hormonal responsiveness to PTH, was performed in p53 null mice (21). The p53 null mice had smaller, thinner bones than their wild-type counterparts; these changes were associated with decreased surface bone formation. Premature closure of sutures at the base of the skull was associated with loss of symmetry in the maxilla, suggesting a key role for p53 in normal development of the craniofacial complex. Daily PTH administration, which has anabolic effects on bone mass and strength, promoted OS formation in rats after 18 months of treatment in a 2-year oncogenicity study (21). When challenged with PTH for a few days, expression of the AP-1 complex of genes, specifically *c-fos* and *fra-2*, was blunted in p53-null mice, whereas *in vitro* induction of osteoclast differentiation was enhanced (22). Because *p53* is a key regulatory gene determining apoptosis, apoptosis was investigated in bone cells isolated from long bones of *p53* intact and *p53* null mice, using substrates degraded by caspases as surrogate markers for apoptosis. Confirming previous data (23), bone cell lysates from intact mice degraded DEVD substrate, which recognizes the apoptotic caspases 2, 3, and 7, and showed no significant activity with substrates recognizing caspases 1, 4, 5, 6, 8, or 9. In contrast, bone cell protein lysates from *p53*-null mice exhibited substantial activity on caspase substrates recognizing caspase 1 activity and only minimal activity on DEVD substrate. This suggests that in the absence of p53, bone cells switch to an alternate pathway that activates caspase 1 (interleukin-converting enzyme- $\beta$ ) rather than caspases 2, 3, and 7. PTH inhibited the caspase 3 activity in intact mice and the caspase 1 activity in p53 null mice. Although the p53-dependent switch from apoptotic caspase 3 activity to caspase 1 activity could indicate existence of a redundant pathway, caspase 1 is usually considered non-apoptotic, because it cleaves cytokines associated with inflammation and osteoclast induction. The implication of this change in caspase pathway on OS induction is unknown.

**Telomerase and ALT.** The complex unbalanced karyotypes that characterize OS may reflect its pathogenesis. Karyotypic complexity may reflect chromosomal fusion-bridge-breakage cycles that occur due to advanced telomere erosion. A potential etiology of this chromosomal instability is telomere dysfunction, as has been implicated in epithelial cancers (24). Telomeres are nucleoprotein structures that cap chromosome ends and serve at least three protective functions: (a) preventing

<sup>24</sup> A. A. Sandberg and J. A. Bridge. Updates on the cytogenetics and molecular genetics of bone and soft tissue tumors: osteosarcoma and related tumors. Cancer Genetics Cytogenetics, submitted, 2003.

chromosomes from being recognized as damaged DNA; (b) preventing chromosomal end-to-end fusions and recombinations; and (c) accommodating the loss of DNA that occurs with each round of replication. Normal human somatic cells have finite proliferative capacity, and it has been demonstrated that telomere length is one of the checkpoints that determines when a cell stops dividing (25, 26). As cells divide, the telomere length gradually decreases to a critical size, at which point senescence is triggered by a p53-dependent process. Human cells may bypass this checkpoint by inactivating the p53 pathways, in which case they continue to divide until telomeres become very short, chromosomal instability ensues, and apoptosis is triggered. Rare cells bypass this second checkpoint by activating mechanisms that lengthen telomeres, a central feature of cancer cells. About 85% of cancers activate an enzyme called telomerase, which lengthens telomeres, and the other 15% of cancers use a recombination-based method called ALT (27, 28). Unlike most cancers, at least 50% of OS samples are dependent on the ALT mechanism to maintain telomeres (29–31). The ALT and telomerase mechanisms are different means to the same end, but they are not equivalent. Telomerase-dependent OS cell lines have short telomeres with a minor range of length, whereas ALT-dependent OS cell lines have long telomeres with great heterogeneity in length. The ALT cell lines also have greater genetic instability and more translocations than the telomerase-positive cell lines (29). In a mouse model, five of five ALT-dependent cell lines were unable to generate macroscopic lung metastases, despite robust s.c. tumor growth (32). Upon telomerase reconstitution, all five of the cell lines formed massive pulmonary nodules after tail-vein injection, indicating diminished metastatic potential in ALT-dependent *versus* telomerase-dependent tumors. It has been hypothesized that ALT-dependent human OS have different clinical behavior than telomerase-dependent OS, but this remains to be studied.

**Viral Pathogenesis.** SV40, a DNA polyomavirus, consists of three major nonstructural proteins: (a) the small T antigen (enhances the ability of large T antigen to transform cells); (b) the smaller T antigen (function unknown); and (c) the large T antigen (assists in viral replication, interacts with p53 and Rb, and promotes cell proliferation by inhibiting p53 function; Ref. 33). A 1996 study concluded that 11 of 18 OS samples showed evidence of incorporated SV40 DNA (33); a 1998 study showed no correlation of presence of SV40 with p53 or Rb mutation status (34); and a 1997 study showed 50% of OS samples tested had incorporated SV40 DNA from each of the four regions of the viral genome (35). Although SV40 is not observed in all tumors, it may exist in select families affected by OS. It is postulated that SV40 large T antigen may inactivate the wild-type p53 allele in the osteoblasts of susceptible individuals, resulting in tumor development. In two families with Li-Fraumeni syndrome, in which family members with cancer harbored germ-line heterozygous p53 mutations, SV40 large T antigen was amplified and expressed in tumor cells that retained the wild-type p53 allele (36).

Future needs and directions in the characterization of the molecular pathology of OS include: (a) incorporating the current lists of genetic alterations into functionally related groups of genetic alterations (hyperproliferative, cell cycle control, apoptosis, and DNA damage response); (b) a better understand-

ing of the timing and relationship of common oncogenic events in OS [alterations of p53 and Rb as early preclinical events, p53 pathway alterations in OS in familial retinoblastoma (37), and retinoblastoma pathway alterations in OS in Li-Fraumeni syndrome]; (c) developing a comprehensive analysis of the p53 and retinoblastoma pathways in a single large set of OS samples by new high throughput approaches; (d) a better understanding of different “equivalent” common oncogenic events (preferential 12q13 amplification in low-grade/surface OS and preferential p53 missense mutation in adult OS); (e) a better understanding of the paradox of carcinoma-type cytogenetics in the setting of a younger age range; and (f) defining the biological/genetic subsets of OS according to telomerase status and karyotypic complexity.

**Cell Death/Cytokine Pathways.** The Fas cell death pathway has been implicated as having a role in determining chemosensitivity and metastatic behavior in a variety of tumors including OS, ESFT, and neuroblastoma (38, 39). Fas is a type I transmembrane protein that regulates immune cell apoptosis, but Fas is expressed in many different cells and tissues, and is functional in cells outside of the immune system (40). Tumor cells expressing surface Fas will apoptose when FasL is presented unless a mechanism of resistance is present. Mechanisms of resistance include down-regulation of Fas, FasL, or procaspase 8 (*FLICE*), and up-regulation of apoptosis inhibitory proteins, specifically bcl-2 or *FLICE*-inhibitory protein. Some tumors express FasL on their cell surface enabling a “counter-attack” against activated T cells to escape immune surveillance (41). FasL has been shown to exist in transmembrane and soluble forms in ESFT. It has been shown in certain circumstances that ESFT cells may avoid apoptosis by down-regulating their surface FasL (by cleavage) and Fas (by internalization); however, treatment of ESFT cells with synthetic matrix metalloproteinases increased surface FasL and Fas level (in the absence of increased transcription) expression, and decreased cytoplasmic Fas (42).

Fas signaling may be involved in OS metastases (43). A cell line, SAOS-LM6, has been developed through repetitive outgrowth of the pulmonary metastases that arise after SAOS-2 tail-vein injection (44), and SAOS-LM6 has a greater tendency to form pulmonary metastatic lesions. Fas expression was examined in SAOS-LM6 to determine its role in OS metastases. Using Northern analysis, it was found that SAOS-LM6 expressed a lower level of Fas as compared with the parental SAOS-2 line, and flow cytometry demonstrated that this decreased expression was reflected in the cell surface protein (45–47). To test whether Fas affects the metastatic potential of the cell lines, Fas-transfected SAOS-LM6 cells and SAOS-2 control cells were injected into mice. The mice injected with control cells developed metastases, but the mice with Fas-transfected cells did not. This demonstrates Fas expression may be important to the metastatic potential of OS. Additional studies were undertaken to determine whether IL-12, which can up-regulate Fas expression, can also alter the metastatic potential of OS cell lines. IL-12 was transfected into SAOS-LM6 cells using coinubation with an adenoviral vector, and this reduced the metastatic potential of the transfected cells, again supporting the involvement of Fas in OS metastases. Murine IL-12 can be induced in lung tissue by intranasal administration of

Ad.mIL-12 vector. A study was conducted in which mice were injected with tumor cells, and then aerosolized Ad.mIL-12 was administered. The mice receiving the Ad.mIL-12 developed no (4 of 8 mice) or <12 (4 of 8 mice) pulmonary nodules, whereas mice treated with a control adenoviral vector developed 25 to >200 nodules in 5 of 8 mice (45–47).

The Fas pathway may explain the clinical results of a recent pediatric cooperative group clinical Phase III trial (48) in nonmetastatic OS in which patients received doxorubicin, cisplatin, and methotrexate and were randomized to treatment with ifosfamide and/or MTP-PE, or doxorubicin, cisplatin, and methotrexate alone. MTP-PE can induce IL-12 in patients through activation of macrophages. Ifosfamide may up-regulate FasL expression on OS tumor cells in a manner analogous to the *in vitro* effect of cyclophosphamide in the SAOS-LM6 and SAOS-LM6-IL-12-transfected OS cells. The combined induction of both IL-12 and FasL could result in enhanced tumor apoptosis, and this Fas-related event may explain the trend toward improved outcome observed in patients treated with both ifosfamide and MTP-PE compared with either individual agent.

Clinical investigations of IL-12 combined with IL-2 have been initiated in adult cancers (49). The rationale for expecting complementary antitumor effects by IL-12 and IL-2 is as follows: different signaling pathways are used by IL-12 *versus* IL-2; and IL-2 up-regulates IL-12 receptor expression on T and natural killer cells, and the combination has enhanced immunostimulating effects for T and/or natural killer cells (cytotoxicity, cytokine production, and proliferation). A regimen of systemic IL-12 and pulse IL-2 was investigated using murine models of renal cell (50) and mammary (51) carcinoma. The regimen was well-tolerated and resulted in complete tumor regression in 90–100% of mice with metastatic renal cell carcinoma and 50% of mice with mammary carcinoma. Systemic IL-12/pulse IL-2 inhibited tumor neovascularization and induced tumor regression via mechanisms that were dependent on CD8+ T cells, IFN- $\gamma$ , and Fas/FasL (52). Additionally, investigators observed that most mice cured of the renal cancer were resistant to rechallenge with the tumor, and the tumor became infiltrated with CD8+ T cells. The regimen was ineffective in SCID mice, and depletion of the CD8+ T cell effector cells ablated the antitumor response, suggesting the importance of an immune mechanism. The impact of IL-12/pulse IL-2 on tumor vascularization may occur via IFN- $\gamma$ -dependent induction of Fas/FasL, and ultimately, vascular endothelial cell apoptosis. With this preclinical data, Phase I studies of systemic IL-12/pulse IL-2 have been initiated in adults with advanced solid tumors. Along with these findings, the subsequent demonstration of potent therapeutic efficacy by IL-12  $\pm$  IL-2 in orthotopic models of murine neuroblastoma has provided preclinical rationale for a Phase I investigation of IL-12  $\pm$  pulse IL-2 that has now been initiated in children with persistent or refractory neuroblastoma.

**Drug Resistance Pathways.** OS tumor recurrences are likely to be at least partly due to drug resistance (53). Possible mechanisms of drug resistance include alterations in: p-glycoprotein expression, multidrug resistance protein expression, topoisomerase II, glutathione *S*-transferases, DNA repair, drug metabolism or inactivation, and reduced intracellular influx.

Drug resistance can be intrinsic (in the absence of treatment) or acquired (after treatment with chemotherapeutic agents; Ref. 54).

**P-Glycoprotein Expression.** Of the drug resistance mechanisms, p-glycoprotein expression has been the most extensively studied in OS. P-glycoprotein is an ATP-dependent efflux pump for hydrophobic substances, and its expression can result in resistance to doxorubicin and etoposide. P-glycoprotein expression in OS has been investigated as a means to identify “nonresponders” early, allow treatment modification, and potentially improve their survival. Similarly, negative p-glycoprotein expression may identify patients who are more responsive and could receive less toxic therapy.

Published studies have concluded that p-glycoprotein levels can predict poor outcome, defined as development of metastases and death, in OS (55), associating p-glycoprotein expression with an adverse outcome risk ratio of 3.37 (95% confidence interval, 1.6–7.1). A review of the many published studies of p-glycoprotein in OS (55–64) suggests that p-glycoprotein expression is associated with a poorer metastasis-free survival and poorer overall survival. However, the immunohistochemical measurement of p-glycoprotein expression in the tumor biopsy samples of patients participating in the pediatric OS Phase III study discussed previously (65), did not detect a significant difference in event-free survival (relative risk = 1.05) or risk of death (relative risk = 1.0) between patients who demonstrated antibody positivity at study entry compared with antibody-negative patients. This study concluded that p-glycoprotein expression is not useful in determining the prognosis of patients presenting with localized OS treated with cisplatin, methotrexate, doxorubicin,  $\pm$  MTP-PE,  $\pm$  ifosfamide. Furthermore, tumor histological response to neoadjuvant chemotherapy did not correlate with p-glycoprotein positivity.

Caveats of studies of multidrug resistance in OS include: (a) the decreased survival associated with p-glycoprotein-positive tumors may not reflect decreased drug accumulation; (b) the presence of p-glycoprotein does not necessarily confer resistance; and (c) its absence does not imply sensitivity. In addition, it is known that other efflux proteins such as multidrug resistance protein are present in OS. The current literature concludes that there is no correlation between p-glycoprotein status and percentage of OS tumor necrosis after induction chemotherapy. The literature also suggests that p-glycoprotein may be a sign of aggressiveness as well as a marker of drug resistance, and may be useful in identifying high-risk OS patient subsets (66).

**MDR1 Expression.** In a pilot study of 15 patients, the expression of *MDR1*, which encodes the protein p-glycoprotein, was correlated with poorer overall survival. In a larger prospective study, *MDR1* expression was examined using reverse transcription-PCR and then correlated with disease-free survival. Between 1989 and 1994, 123 newly diagnosed patients with high-grade, nonmetastatic extremity OS all received neoadjuvant chemotherapy, including doxorubicin. Although tumor size was predictive of outcome, the degree of tumor necrosis was not significant in the multivariate analysis. Patients with high levels of *MDR1* did poorly, but patients with low levels of *MDR1* also did poorly. Therefore, no association between survival and *MDR1* could be made (relative risk ratio 1.15; Ref.

63). Microarray analyses or continuous monitoring of MDR1 levels may allow a determination of the reasons patients with both very low and very high levels of MDR1 expression have a poor outcome. Other reasons for the lack of correlation between *MDR1* expression levels and disease-free survival in this study as compared with previous may include: conflicting results with immunohistochemistry due to sensitivity of technique, antibodies detecting other proteins, expression of MDR2 in OS, other mechanisms of drug resistance, and the relationship between p53 and drug resistance. Many OS specimens have very high levels of expression of both *MDR1* and *MDR2*. Wild-type p53 can inhibit *MDR1* expression (67). When progressive sets of tumor biopsies from the same patient were examined at diagnosis and definitive surgery, it was seen that if the biopsy had a p53 mutation, then the resection had a mutation. When correlating type of p53 mutation and *MDR1* expression, it was found that those who had missense mutations had higher *MDR1* expression, and those who had nonmissense p53 mutations had lower levels of MDR1.

**Antifolate Resistance.** Studies of antifolate resistance have been performed in OS (68–70). Most soft tissue sarcomas are intrinsically resistant to methotrexate secondary to impaired polyglutamylation (68). The literature suggests that the peak methotrexate level, and not the systemic exposure, correlates with therapeutic response in OS (71, 72). The peak serum level is predominantly determined by drug dose. This suggests that impairments in drug influx may be a basis of methotrexate resistance in OS. Studies of methotrexate resistance have been performed on biopsy samples to identify intrinsic mechanisms of resistance and on relapsed samples to identify acquired mechanisms. Studies have shown that impairments of drug influx secondary to decreased expression and mutations in the reduced folate carrier gene, the major membrane transporter of methotrexate into cells, is the major basis of intrinsic resistance (69). Dihydrofolate reductase, the target of methotrexate, overexpression is the major mechanism of acquired resistance (69).

Trimetrexate, a newer antifolate, has been investigated as an alternative approach for OS treatment based on its ability to enter into cells that lack a functional reduced folate carrier (73). A Phase II trial of simultaneous trimetrexate and leucovorin given orally for 21 days was conducted in relapsed OS patients. Toxicity was acceptable with myelosuppression being the major side effect, and objective responses were observed in 8% ( $n = 39$ , complete response = 1, partial response = 2, mixed response = 1, stable disease = 8; Ref. 73). A Phase II study proposal is under development by the Children's Oncology Group for patients with newly diagnosed metastatic OS. The response rate of trimetrexate and simultaneous leucovorin would be predicted to be higher in newly diagnosed patients than in the setting of a conventional Phase II trial, because dihydrofolate reductase overexpression is not as frequent at diagnosis as compared with relapse. Furthermore, trimetrexate with leucovorin would be predicted to be at least as efficacious as high-dose methotrexate with less toxicity. The lack of renal toxicity may allow chemotherapy dose intensification, because it may be possible to administer trimetrexate simultaneously with other routine chemotherapy.

**Microarrays and Preclinical OS Models.** With the goal of developing new therapeutic agents, several approaches have

been taken to identify new therapeutic targets and to develop model systems for the evaluation of new agents. These model systems include cDNA microarrays, mouse OS syngeneic models, OS murine xenografts, and spontaneous OS in canines. Each of these systems will be discussed in the following sections.

**Gene and Protein Expression.** cDNA microarray profiles of gene expression using patient tumor samples are now being used as a resource to catalogue candidate drug targets (74). Distinct cancers have distinct patterns of gene expression, and robust formal diagnostic classifications can use gene expression profiles to define novel subgroups of cancers that do not have previously defined clinicopathological correlates. As this information is compiled, the clinical application of the array data may require only a small group of genes to determine the tumor diagnosis or clinical subgroup. This information will be used to improve diagnostic categorization of tumors, to provide useful prognostic markers for outcome or likely therapeutic response, and to identify critical cancer cell pathways that can act as novel drug targets. Data analysis from a pilot study of bone tumors (ESFT and OS) demonstrates a strong cluster of ESFT-specific genes, a larger group of OS-associated genes, and a very strong difference in gene expression between the two tumors types. Whereas the OS samples demonstrated significant variability, the expression profiles of coupled primary and metastatic OS tumors were very similar. Additional analytic comparison of these tumor groups is under way to detect genes that are up-regulated or down-regulated during the metastatic process and to compare patient samples from children whose OS tumor demonstrated a good or poor response to induction chemotherapy. Lists of genes associated with survival are being generated, but the interpretation of these gene lists is just beginning. Biostatistical methods to evaluate the significance of gene expression within a cellular pathway will be needed to analyze the data being generated (75).

The expression of genes associated with the "metastatic phenotype" may be predictive of outcome in OS, given that metastatic clinical events are the proximate cause of OS-related death. However, only a small proportion of tumor cells from the gross tumor actually undergo metastasis, and tumor heterogeneity may mislead the metastatic phenotype evaluation obtained from small biopsy samples. The *c-met* proto-oncogene is of interest as the binding of *c-met* ligand can activate the tumor cell growth cycle, and start pathways involved in cell motility and the lysis of basement membranes, all components of the metastatic process. Tissue microarrays are being used to examine *c-met* expression levels in OS samples. Preliminary studies indicate that ~60% of OS tissue samples express *c-met* (76, 77). The matrix metalloproteinases are a family of proteins involved in the lysis and remodeling of the extracellular matrix in the metastatic process of OS. Expression of metalloproteinases 2, 9, and 14 has been associated with OS tumor growth and invasion.

Investigators are also looking at protein expression to monitor the molecules that directly control cellular activities. Whereas mRNA expression does not necessarily correlate with protein expression, the technical difficulty in directly monitoring protein expression makes mRNA expression profiling, comparative genomic hybridization for chromosomal gains/losses/amplifications, and spectral karyotyping to detect genetic

translocations more popular currently (78, 79).<sup>25</sup> Information from patient samples is being used to compare mRNA expression in primary OS *versus* normal osteoblasts, OS before and after chemotherapy, primary OS *versus* lung metastases, to predict response to neoadjuvant therapy, and as a prognostic factor.

**Preclinical Models of OS.** The preclinical evaluation of new agents in OS is carried out using spontaneous, syngeneic, and xenogeneic or orthotopic animal models (80). As an example, the evaluation of camptothecin, epothilones, and signaling inhibitors has been performed in OS xenograft models (81). Validation issues for these OS models include the ability of the model to prospectively identify clinically active agents, the clinical heterogeneity of the model, and the false prediction rate of the model. Some relative correlation does exist between the xenograft models and clinical experience. Irinotecan has some activity in OS xenografts, but OS is much less sensitive than rhabdomyosarcoma, neuroblastoma, or Wilms tumor models. Epothilone B has some activity, but the signaling inhibitors imitinib mesylate (Gleevec) and gefitinib (Iressa) do not have significant single agent activity.

The genetic study of OS tumor cell metastatic potential has included a syngeneic murine OS model characterized by clonally related variants (K7M2 and K12) that differ in metastatic potential (82, 83). cDNA microarray analysis has defined 59 differentially expressed genes in a comparison of K7M2 and K12 primary tumors. K7M2 overexpressed genes related to cytoskeletal motility, adherence, and angiogenesis in comparison with K12, including the cytoskeleton linker protein *Ezrin*. A tissue microarray developed from canine OS tumors demonstrated that metastases have a statistically higher level of *Ezrin* expression compared with primary tumors. *Ezrin* mutants and the regulation of the protein during metastasis are under evaluation in murine models, and phosphorylated *Ezrin* expression is being evaluated in canine OS.

Canine OS provides an *in vivo* model with parallels to human OS (84–86). OS occurs spontaneously primarily in large-size breeds (most commonly in purebred dogs, with a male preponderance) affecting the long bones. The majority of canine OS cases are stage 2b, according to the Enneking staging system (87), at presentation and of high-grade histology. Approximately 15% are stage 3 at presentation, and pulmonary metastases are common. Local control is achieved by amputation or limb-sparing surgery. The occurrence of metastatic disease is greater in the lungs than bone, as in humans, but the time to local or distant recurrence is much shorter in dogs. The dog model provides a ready source for controlled preclinical treatment studies using a spontaneously arising tumor. Accrual of dogs to clinical study is rapid, and autopsy compliance is common. For example, a Colorado State University protocol examining the role of limb-sparing surgery, chemotherapy, and radi-

ation included eligibility criteria of “localized” disease and <50% bone length involvement (88). Neoadjuvant therapy included arterial cisplatin on days 1 and 21, and radiotherapy followed by surgery. Increasing tumor necrosis (measured at surgery) correlated with a decreased local recurrence rate. Cisplatin and radiation increased the percentage of necrosis over radiation alone. Interestingly, dogs with infected limb repairs lived twice as long as dogs without infection. A study of the IGF-I inhibitor, OncoLar, randomized dogs with OS to OncoLar treatment in addition to chemotherapy and amputation. Sixty-four dogs were accrued to the study in just 8 months, but there was no difference in primary tumor necrosis, tumor cell apoptosis, or survival between the two treatment arms (89). New treatment approaches now under investigation include gemcitabine, antiangiogenic agents, gene therapy immunotherapy, and bisphosphonates. Basic biology studies are ongoing to examine the influence of IGF-I, and growth hormone in OS tumor growth and metastasis, and to evaluate growth hormone and growth hormone-releasing hormone antagonists.

### New Treatment Strategies

**Growth Hormone and IGF-I.** A series of clinical and preclinical observations may link IGF-I and OS. Adolescence is the age of the peak incidence of human OS, and the peak period of physiological growth hormone and IGF-I circulating concentrations. The prevalence of OS is greatest in dog breeds with the highest IGF-I concentrations. Furthermore, hypophysectomy is associated with antitumor activity in rodent OS due to IGF-I inhibition (90), and growth hormone blockade results in IGF-I inhibition and decreased OS growth. OS cell lines, which express IGF-I receptors, are IGF-I dependent for *in vitro* growth and survival (91). Although the circulating levels of IGF-I and IGFBP-3 among a few patients were not found to be predictive of the incidence or clinical behaviors of OS, a large prospective study of IGF-I and IGFBP-3 circulating levels is under way in OS patient serum. IGF-I levels have been investigated in ESFT using patient samples. Preliminary results demonstrate that IGF-I and IGFBP-3 levels can identify patients with the most widespread disease, but these levels are not independent predictors of prognosis. A relationship between the ratio of IGF-I and IGFBP-3 levels and prognosis has also been found (92). Blockade of IGF-I has been investigated using the sustained release sandostatin OncoLar, which can block IGF-I activity. A Phase I study of OncoLar with and without tamoxifen in relapsed OS patients demonstrated that OncoLar treatment leads to a sustained 40–50% decrease in IGF-I levels. The growth hormone level was not affected by OncoLar, and tamoxifen had no impact on measured IGF-I levels (93). There was no measurable tumor response to the OncoLar treatment. Investigators are considering combining OncoLar treatment with conventional chemotherapy hoping that a synergistic effect on tumor apoptosis may result (93).

**Samarium.** Radiation therapy by either external beam radiation (94, 95) and/or a bone-seeking isotope can be effective in OS (96, 97). Samarium is a bone-seeking radiopharmaceutical that provides therapeutic irradiation to osteoblastic bone metastases. A recent clinical study administered samarium by a 30-min central line infusion to patients with bone metastases and dosimetry was performed on days 1, 2, and 5, to estimate

<sup>25</sup> C. C. Lau, C. P. Harris, X-Y. Lu, L. Perlaky, S. Gogineni, M. Chintagumpala, J. Hicks, A. G. Huvos, P. A. Meyers, J. H. Healey, R. Gorlick, and P. H. Rao. Frequent amplification and rearrangement of chromosomal bands 6p12-p21 and 17p11.2 in osteosarcomas. *Mol. Cancer Ther.*, submitted, 2003.

tumor dose and residual radioactivity (96). Peripheral blood stem cell infusion on day 14 is necessary for patients receiving  $>3$  mCi/kg, with day 13 radioactivity estimated at  $<3.6$  mCi. Nonhematological acute toxicity includes manageable hypocalcemia during the infusion, and a "flare" reaction of bone pain within a day of samarium infusion was common, but pain control improved in all of the patients after samarium treatment.

The use of a radiation sensitizer, gemcitabine (98–100), after the samarium dose may increase the radiobiological effectiveness of this treatment. A trial using high-dose samarium day 0, gemcitabine day +1, with peripheral blood stem cell infusion day +14 is under way at Mayo Clinic. Additional means to increase bone formation and, thus, attract bone-seeking radioisotope could additionally improve the therapeutic index (101). The most effective use of samarium for control of OS bone lesions will probably be in combination with external beam radiotherapy.

**Antibody-Based Strategies for Metastatic Solid Tumors.** Murine and human-mouse chimeric antibodies are under clinical investigation as directly targeted immunotherapy and anti-idiotype vaccines. The GD2 disialoganglioside is a target antigen expressed on OS and neuroblastoma cells, and GD2 is recognized by the 3F8, 5F11, and ch14.18 monoclonal antibodies. Early clinical studies of these monoclonal antibodies included patients with relapsed OS and neuroblastoma, and a randomized Phase III study of the anti-GD2 antibody (ch14.18) in high-risk neuroblastoma patients postautologous stem cell transplant is currently underway in the Children's Oncology Group. Similarly, gp58 is a surface glycoprotein found on many pediatric tumors including OS, and clinical studies of the gp58-specific 8H9 monoclonal antibody are under way (102). In addition,  $\beta$ -glucan, a glucose polymer extracted from plants and yeasts, has been studied in murine tumor models and found to significantly enhance the antitumor effect of mouse or chimeric monoclonal antibodies, prolonging progression-free survival (103).  $\beta$ -Glucans are thought to bind to the lectin site of phagocytic and natural killer cells, thus facilitating antibody-dependent and complement-mediated tumor cell killing triggered by monoclonal antibodies. Finally, antibody vaccines designed to induce anti-idiotype anti-GD2 antibody are under clinical evaluation in children with relapsed neuroblastoma and may have applicability to OS as well.

Monoclonal antibodies have also been investigated in a pretargeting strategy using scFv-streptavidin to target the antibody fragment and therapeutic agent (radioisotopes and biologics) at the tumor. After tumor targeting of scFv-streptavidin, a clearing agent containing biotin and *N*-acetyl-galactosamine removes residual fusion proteins from the blood, significantly improving the tumor:blood drug exposure ratio. Using GD2 as a target, whole antibody or antibody-fragment alone results in a tumor:blood drug exposure ratio of  $<5:1$ , whereas the pretargeting approach gives a  $>50:1$  ratio. This targeting and clearing approach focuses a high level of active agent to the tumor and diminishes nonspecific systemic exposure. T cells can also be targeted by modifying the T cells using chimeric immune receptors (T-bodies). The expanded, modified T cells recognize a predetermined antigen and proliferate when an anti-idiotype is added (104). The applicability of these targeted approaches in OS remains unclear.

**Gene Therapy.** Gene therapy targeting an osteocalcin promoter has been explored in early phase clinical trials of prostate cancer. Osteocalcin is a noncollagenous protein produced by osteoblasts during bone mineralization. Immunohistochemical staining demonstrates osteocalcin production in osteoblastic OS (100%) and fibroblastic OS (70%). An adenoviral vector, containing the toxic TK gene, has been designed to regulate TK expression via the osteocalcin promoter (105). Transfected tumor cells are rendered sensitive to the acyclovir nucleotide analogue by the acquired TK activity, and the tumor cells die on administration of acyclovir.

Preclinical studies using the Ad-OC-TK/ACV therapy have demonstrated *in vitro* and *in vivo* growth inhibition for rat OS, including localized and metastatic OS *in vivo* models (106, 107). Phase I studies of the AD-OC-TK vector delivered by intratumoral injection in prostate patients demonstrated good tolerability of the injection and no unexpected toxicities (108).

**Inhaled GM-CSF.** Preclinical studies of melanoma in mice have demonstrated the ability of GM-CSF-transfected tumor cells to induce potent, specific, long-lasting immunity to subsequent tumor challenge. Tumors transduced by GM-CSF demonstrate dense macrophage infiltration, and locally produced GM-CSF facilitates killing of nontransduced bystander tumor cells. Aerosolized GM-CSF has been investigated for delivery of the cytokine to lung tissue affected by metastatic tumor. The aerosolized cytokine is absorbed by pulmonary lymphatics and can interact with receptor-bearing cells in bronchial and pulmonary lymph nodes.

A Phase I study of aerosolized GM-CSF in solid tumor patients at the Mayo Clinic allowed inpatient dose escalation from 60 to 240  $\mu\text{g}/\text{inhalation}$  with twice daily administration for 1 week and 1-week rest (109). Among the 6 patients with stable disease, 5 continued on GM-CSF for 2–8 months after reaching the third dose level without significant toxicity. A subsequent Phase II study of 45 patients included 8 OS patients. Two of 8 OS patients with gross disease progressed, whereas the other 6 achieved a complete response with surgery, and 2 have been disease-free for 2.5 and 4 years. Clinical studies under consideration include administration of aerosolized GM-CSF before thoracotomy in OS patients with isolated pulmonary relapses. For relapsed patients with bilateral pulmonary nodules, a staged thoracotomy would allow examination of pulmonary nodules resected before and after treatment with GM-CSF to determine the possible biological and pathological changes associated with the aerosolized GM-CSF treatment. Given the demonstrated lack of significant toxicity, a pilot study of aerosolized GM-CSF for 6 months postsurgery in patients with nonmetastatic OS has been proposed.

**Localized Platinum.** Veterinary investigators have developed an absorbable sponge-like device to deliver slow-release cisplatin in the surgical site (110, 111). This localized treatment approach can address: (a) local recurrence; (b) marginal resections; (c) tumor close to the wound bed; and (d) the need for a localized tumor dose of platinum with minimal systemic exposure. The slow-release platinum also appears to have a radiosensitizing activity (112). A study in 80 dogs that underwent OS surgery resection and allograft placement included *i.v.* cisplatin therapy for all of the dogs, with a randomization to the platinum implant device. The local recurrence rate

was 15% among the implant cohort compared with 55% for the control group. Among those dogs with histologically incomplete tumor resection, a statistically significant decrease in the recurrence rate was found.

**Bisphosphonates.** Bisphosphonates bind strongly to hydroxyapatite on the bone surface, have direct inhibitory effects on osteoclast-mediated bone resorption, and affect osteoblast activity (113). Whereas no direct cytotoxic effect of bisphosphonates is reported, the agents can induce bone cell apoptosis, inhibit cytokine production (Ref. 114; IL-6 promotes OS cell growth and encourages angiogenesis), and inhibit metalloproteinases. The bisphosphonates can prevent bone loss and fractures, and the prevention of bone metastasis development has been demonstrated in breast cancer patients with microscopic bone marrow "metastases" (115–117). The bisphosphonates may have several potential roles in sarcoma treatment, including the prevention of: tumor metastases, osteoporosis associated with chemotherapy (118), and restricted weight bearing, periprosthetic osteolysis, and bone loss from the stress bypass effect (119–121). Although the agents have been used to treat osteogenesis imperfecta in children (114), pediatric data are limited, and the role of bisphosphonates in the prevention of metastatic tumor development or primary tumor progression remains to be defined.

**Angiogenesis and Metronomic Low-Dose Chemotherapy.** The use of low-dose chemotherapy to block tumor-associated endothelial cell proliferation has gained interest as an antiangiogenic approach to tumor treatment. Using a tolerable, chronic low dose of select chemotherapy agents, investigators believe that endothelial cell proliferation can be blocked (122) without the acquisition of drug resistance (the target cells are genetically stable). COX-2 inhibitors have been added to the chemotherapy regimen due to the expression of COX-2 in tumor neovasculature, neoplastic cells, and stromal cells (123). Mice lacking COX-2 expression demonstrate that decreased VEGF expression and tumors in such mice grow more slowly in association with reduced tumor angiogenesis. Additionally, COX-2 inhibitors decrease VEGF production and VEGF-induced endothelial cell activation. An ongoing clinical pilot study is evaluating the safety and toxicity of chronically administering celecoxib and low-dose vinblastine or cyclophosphamide in pediatric patients with recurrent solid tumors (124). As part of the current pilot study, the pharmacokinetics of celecoxib in children is being studied (125), and angiogenic growth factors (VEGF, basic fibroblast growth factor, and vascular cell adhesion molecule 1) are being evaluated as surrogate markers of angiogenesis. Dynamic magnetic resonance imaging is also being assessed as a tool for specific antiangiogenic tumor response. The treatment regimen has been well tolerated, and the surrogate markers have demonstrated a large interpatient variability (125).

## CONCLUSION

Investigators are pursuing a greater understanding of OS biology at the subcellular level by developing gene and protein expression array data that may soon provide customized information on tumor prognosis and metastatic potential, as well as indications of possible tumor targets for selective therapy. The broader employment of animal models, and especially sponta-

neous canine OS, can provide a much-needed means to study the activity of new treatment interventions at the preclinical level. Finally, a variety of local and targeted therapies are now under evaluation in the clinic. With these new treatments, investigators can envision that targeted agents, such as new small molecule inhibitors, growth factors, radionucleotides, viral gene therapy, and various monoclonal antibodies, may one day complement the systemic chemotherapy and surgery that remain the foundation of OS treatment.

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