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MEETING REPORTS

Meeting Report from the IX International Meeting on Cancer Induced Bone Disease

October 28-31, 2009 in Arlington, Virginia, USA

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- Biology and Treatment of Prostate Cancer Bone Metastasis – John M. Chirgwin
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Biology and Treatment of Prostate Cancer Bone Metastasis

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Prostate cancer is often an indolent disease of elderly men, but those who die from this tumor type almost always have skeletal metastases. Bone involvement is characterized as osteoblastic on X-ray and accompanied by very high markers of bone turnover. Recent years have seen the development of rapid autopsy programs at several major cancer centers in the US. The programs provide material for new insights into the pathophysiology of these metastases. The first two speakers at this session of the IX International Meeting on Cancer Induced Bone Disease were from centers with such programs.

Ken Pienta (University of Michigan) spoke on The Tumor as Ecosystem and presented surprising data that half of the cells in prostate cancer bone metastases are tumor-associated macrophages (TAMs). Two factors found at high concentrations in the microenvironment, CCL2 and IL-6, can recruit myeloid monocytes and stimulate their differentiation towards M2-type macrophages by inhibition of caspase 8, autophagy and apoptosis (1). TAMs of the M2 type promote tumor progression and metastasis, suppress anti-tumor immune responses and produce factors known to stimulate bone metastases, such as TGF-β and endothelin-1 (2). The abundance of TAMs in skeletal metastases of patients points to these cells as targets for therapeutic intervention. TAMs may also contribute to the well-known resistance of tumor cells in bone to standard cytotoxic drugs. M2 macrophages are a major source of VEGF and MMP9 and are attracted to bone by CCL2. Blockade of CCL2 decreased metastatic tumor burden to bone in an animal model using the PC3 prostate cancer cell line. An agent against CCL2 ligand (Centocor) is in clinical trials in men with

hormone-refractory prostate cancer, and one against its receptor CCR2 (Millenium) is in trials against all types of bone metastases. A poster (P88) from Corey *et al*. (University of Washington) showed data that an anti-CCL2 antibody inhibited growth of prostate cancer in bone. Anti-tumor effects were increased by combination with docetaxel. Anti-CCL2 therapy also had positive effects to increase bone mineral density at skeletal sites unaffected by tumor.

Robert Vessella (University of Washington) summarized on-going work on a challenging project to understand the natural history of the development of bone metastases. Prostate cancer patients, like those with other tumors, have circulating tumor cells (CTCs) that can be detected in peripheral blood, as well as disseminated tumor cells (DTCs) that can be detected in end-organs such as bone. Automated analysis of CTCs is now used to monitor responses to therapy in patients with advanced cancers originating from the prostate, breast and colon. Perhaps only 1% of CTCs become DTCs. In a large series of patients, about 2/3 had DTCs in bone marrow samples prior to radical prostatectomy. Persistence of DTC positivity after surgery (but not their presence prior to surgery) was a risk factor for recurrence (3). DTCs may lie dormant for 5 to 10 years, perhaps occupying the stem cell niche that was extensively discussed elsewhere in the meeting. DTCs are easier to detect than to study at the molecular level. The Vessella group has developed cell sorting procedures to eliminate most of the non-DTCs and enrich for EPCAM+ epithelial cells. Substantially purified populations can be wet-mounted and DTCs (fewer than 50 cells per patient) plucked for molecular analysis. The results suggest that the basic genotype of DTCs initially resembles that of the primary tumor. With advanced disease 100% of the DTCs show complex genome rearrangements. Such DTCs show signs of epithelial-to-mesenchymal transition with increased N-cadherin and decreased E-cadherin. The possible activation of DTCs into clinically significant metastases is likely to require contributions from mutated DTCs and from the bone microenvironment (4), perhaps including specific host genetic predispositions (5). Since DTCs are probably non-proliferative, the data suggest that their population is replaced by newer cells from the primary tumor as it progresses to a more lethal phenotype. Both the first and second speakers discussed the important idea of enforcing dormancy on these cells as a better way to combat bone metastases instead of the hawkish approach of trying to kill all tumor cells. Park *et al*. (University of Michigan, Poster P21) showed that treatment of mice with standard cyclophosphamide chemotherapy increased bone expression of CCL2 and IL6 and worsened bone metastasis in a nude mouse model. In a similar animal model, Delany *et al*. (University of Connecticut, Poster P46) found that the disorganized bone around the tumor contributed to resistance to radiation therapy. Clearly, treatments effective against primary tumors may not be ideal or even appropriate for the specific reduction of metastases.

Greg Clines (University of Virginia, now at University of Alabama, Birmingham) reviewed his published data (6) and that of others that tumor-produced endothelin-1 (ET-1) contributes to osteoblastic metastases by suppressing host Dkk1. Dkk1 is a secreted inhibitor that binds to LRP 5 and 6, sequestering them away from the Wnt signaling pathway. Dkk1 is also produced by tumors. It is commonly overexpressed by multiple myeloma cells, and its level of expression by prostate cancers alters their osteolytic versus osteoblastic phenotype in bone (7). Dr. Clines showed that Dkk1 expression is in part regulated through methylation of its gene promoter. Treatment of C4-2B human prostate cancer cells (which cause mixed osteolytic-osteoblastic bone responses in nude mice) with 5-aza-cytidine resulted in a dramatic increase in Dkk1 mRNA. Analysis of methylation patterns of the Dkk1 promoter across a series of bone-metastatic human tumor lines suggested that methylation partially controls Dkk1 transcription, but there must be additional regulatory factors responsible for turning Dkk1 on or off. Dkk1 action is also negatively regulated by a binding protein, kremen, on the surface of target cells. Dkk1 and ET-1 may be centrally important in

prostate cancer bone metastases. Fradet *et al*. (Lyon, France; Poster P17) described a new experimental model of prostate bone metastases. A rare osteoblastic variant of the normally osteolytic PC3 cell line was isolated. PC3c cells had increased expression of ET-1 and osteoprotegerin and decreased Dkk1, supporting a role for ET-1 and Dkk1 in the osteoblastic phenotype characteristic of prostate cancers metastatic to the skeleton. Antagonists of the endothelin A receptor (from Abbott and AstraZeneca) have been in a series of clinical trials in which the primary study endpoints were not reached. However, these trials have shown benefits not designated as trial endpoints. It has been unclear whether the fault lies with trial design, inadequacy of the specific drugs or whether the endothelin axis is just not the right target. A recent prostate cancer patient study with ZD4054 showed a significant increase in overall survival in men in the treatment arms (8), suggesting that the endothelin axis may prove to be of central importance in osteoblastic bone metastases. ET-1 and some other bone-active factors are nociceptive neurotransmitters, and bone pain is a tremendous problem for patients with bone metastases. Animal models of cancer bone pain are technically challenging, but we look forward to more presentation on this subject at future meetings.

Conflict of Interest: None reported.

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Cancer-Associated Fibroblasts

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For many years tumor development was thought to be due to cell-autonomous changes in tumor cells, dictated by the genetic make-up of the cancer cells. Although these genetic changes are of critical importance, there is increasing evidence supporting the role of the host microenvironment in promoting tumor development and progression; indeed it has been suggested that metastatic and invasive traits can be acquired through interactions of tumor cells with tumor stroma. It is now well-accepted that cancer cells themselves can directly alter their adjacent stroma to form a permissive and supportive environment for tumor progression. Alterations can include changes in extracellular matrix composition, angiogenesis and fibroblasts/stromal cells. This “reactive stroma” is so-called due to the similarities between the stroma surrounding tumors and reactive stroma found in wound healing. In a normal wound healing response, myofibroblasts respond through migration, proliferation and secretion of critical factors necessary for epithelial cells to respond and become “reactive.” Cancer-associated fibroblasts typically exhibit a similar myofibroblast-like phenotype, although there is increasing evidence to suggest that distinct sub-populations exist, which may differ in their role in tumor progression.

There is an emerging role for cancer-associated fibroblasts or reactive stroma in promoting carcinogenesis in solid tumors through paracrine signaling. As discussed by Simon Hayward (Vanderbilt University), the role of cancer-associated fibroblasts in promoting tumor progression is complex and dependent upon both the heterogeneity of the stromal compartment and the multiple interacting chemokine and cytokine pathways that regulate paracrine signaling. To investigate the role of prostatic cancer-associated fibroblasts *in vivo*, Dr. Hayward and colleagues utilized a tissue recombination methodology, where cancer-associated fibroblasts were isolated from human prostate tumors and recombined with the SV40T-antigen-immortalized human prostatic epithelial cell line, BPH-1. These tissue recombinants were implanted under the renal capsule, providing an *in vivo* bioassay for the effects of cancer-associated fibroblasts. The striking observation was made that the combination of the non-tumorigenic BPH-1 epithelial cells with cancer-associated fibroblasts resulted in the growth of a large carcinoma, whereas BPH-1 cells either alone or in combination with normal prostatic fibroblasts did not result in tumor development (1). In addition, although cancer-associated fibroblasts were found to induce tumor formation in the genetically-initiated but non-tumorigenic human prostate epithelium, they did not induce tumor formation from normal prostatic epithelial cells, suggesting a requirement for some form of genetic alteration in epithelial cells. Prostatic cancer-associated fibroblasts were found to secrete elevated levels of TGF-β and SDF-1, in comparison with normal prostate fibroblasts, and were found to induce expression of the SDF-1 receptor, CXCR4, in prostatic epithelial cells (2). Blockade of TGF-β signaling or CXCR4 expression in epithelial cells prevented the tumor-promoting effect of prostatic cancer-associated fibroblasts. Taken together, this suggests that TGF-β and SDF-1, secreted by cancer-associated fibroblasts, work in concert to up-regulate CXCR4 and promote tumor progression.

The role of cancer-associated fibroblasts or reactive stroma in prostate cancer progression was further discussed by David Rowley (Baylor College of Medicine). Reactive stroma has been shown to co-evolve during the early stages of prostate cancer, and is associated with the

appearance of cancer-associated fibroblasts and matrix remodeling (3). In patients with prostate cancer, the presence of reactive stroma can be graded, and is predictive of recurrence, with a significant reduction in time to recurrent disease after prostatectomy if the cancer had a high reactive stroma grade (4). Microarray analysis of the reactive stroma identified significant changes in gene expression profiles in reactive stroma as compared with normal human prostate stroma (5). Using a xenograft model system, where LNCaP prostate cancer cells are recombined with prostate stromal cells, molecular mechanisms have been identified that mediate the effect of the reactive stroma. TGF-β was found to induce a reactive stromal phenotype and blockade of TGF-β signaling in prostate stromal cells inhibited tumorigenesis and angiogenesis (3;6-8). The effect of TGF-β signaling in reactive stroma was mediated through TGF-β receptor II/SMAD3-dependent upregulation of FGF-2 expression and release. In addition, overexpression of connective tissue growth factor or ps20 in reactive stroma was found to enhance tumorigenesis and angiogenesis (9;10). The appearance of cancer-associated fibroblasts at the border between stroma and epithelial cells was also discussed, with a potential role in the homeostatic function of this epithelial barrier.

Despite increasing evidence for the role of cancer-associated fibroblasts or reactive stroma in promoting tumor progression in primary tumors, there is very little known regarding their role in bone metastases or multiple myeloma. Claire Edwards and Jessica Fowler (Vanderbilt University) presented data to demonstrate that bone marrow stromal cells isolated from myeloma-bearing mice could promote the initial establishment and progression of myeloma and the associated bone disease in mice that otherwise were not permissive for myeloma, suggesting that cancer-associated fibroblasts can promote tumor development within the bone marrow microenvironment. Although the mechanisms by which these bone marrow stromal cells promote myeloma remain to be elucidated, a potential role for bone marrow-derived adiponectin has been identified. The question was also raised as to whether changes in bone marrow stromal cells can also occur prior to the development of myeloma, and create a permissive microenvironment for the establishment and subsequent progression of myeloma. A potential role for fibroblasts in breast cancer osteolysis was also presented by Rachelle Johnson (Vanderbilt University), with fibroblasts, both cancer-associated and normal, increasing expression of the osteolytic factor PTHrP in MDA-231 breast cancer cells. Thus, it appears likely that cancer-associated fibroblasts may play important roles both in tumor growth within the bone marrow and cancer induced bone disease. A number of questions remain unanswered, including whether cancer-associated fibroblasts in bone differ from those at the primary site, whether cancer-associated fibroblasts can migrate from the primary tumor to the bone marrow, and whether cancer-associated fibroblasts promote tumor progression by directly affecting the tumor or the host microenvironment.

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Hematopoietic Stem Cells, Cancer Stem Cells, and the Hematopoietic Stem Cell Niche

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In 1889, Stephen Paget first proposed the "seed and soil" hypothesis, stating that for an organ to harbor migrating or metastatic tumor cells, there must first be congenial soil (1). Many malignancies, including leukemia, multiple myeloma, and metastatic prostate and breast cancer cells utilize the bone marrow microenvironment as a sanctuary for both dormancy and proliferation. Many aspects of the bone marrow microenvironment act as fertile "soil" for these malignant "seeds". The IX International Meeting on Cancer Induced Bone Disease included discussions about the interactions between malignant cells and the bone marrow microenvironment, with emphasis on the implications of cancer stem cells and hematopoietic effects. Within the bone marrow microenvironment, hematopoietic stem cells (HSCs) have been demonstrated to interact with osteoblasts and other cells on the endosteal surface (2-6). This microenvironment, or niche, has been demonstrated to regulate HSC homing, quiescence, and self-renewal. Similar to HSCs, other malignancies have also been proposed to contain a small population of cells that have the ability to self-renew or differentiate into cells capable of tumorigenesis (7).

As described by Laura Calvi (Rochester, NY), these cancer stem cells (CSCs) may also be regulated by the same microenvironmental factors that constitute the HSC niche. Using myelogenous leukemia as a model, Dr. Calvi demonstrated that it may be possible for aspects of the niche to induce quiescence or differentiation of CSCs. It was also suggested that CSCs may influence the expression of proteins by cells comprising the niche. As a result, it may be possible to determine new targets for treatment by further studying the interactions between cancer cells in a malignant versus a normal niche. Inaam Nakchbandi (Heidelberg, Germany) further described evidence that in addition to cellular residents of the marrow, interactions between cancer cells and extracellular matrix components, in particular fibronectin, may be important in regulating the establishment of metastases in the niche.

In order to study CSC-niche interactions, it is necessary to characterize CSCs using specific biomarkers. William Matsui (Baltimore, MD) presented data showing that a small CD138- population of multiple myeloma (MM) cells resembling normal memory B cells had high clonogenic and self-renewal capacity. While most MM cells were unable to achieve long-term proliferation, this particular CD138- population showed a marked resistance to chemotherapy, suggesting this population was more stem-like than the mature plasma cells that comprise the majority of tumor cells in MM. By examining normal stem cells for greater insights, Dr. Matsui suggested a role for Hedgehog signaling is at the crux of a cell's decision to retain stemness or to differentiate. In addition to cell surface biomarkers and signaling pathways discussed, Christel van den Hoogen (Leiden, Netherlands) presented data suggesting that aldehyde dehydrogenase (ALDH) may be a good candidate marker for use in identifying CSCs in prostate cancer (PCa). By showing that cells with characteristics of stem/progenitor cells exhibit high ALDH activity, Dr. van den Hoogen concluded that ALDH enzyme activity and expression may be related to tumorigenesis or distant bone metastases.

The specific region of the bone marrow microenvironment known as the HSC niche has been shown to support the activity of HSCs and CSCs, but it is also likely that the

same region supports metastatic tumor cells. Russell Taichman (Ann Arbor, MI) provided data to support a new concept that solid tumors directly target the osteoblastic HSC niche during metastasis to bone.

Once tumor cells have entered the marrow and taken up residence, there may be significant hematopoietic effects and immune responses. Tumor cells not only interact with bone marrow osteoblasts, but also with different populations of hematopoietic cells residing in the bone marrow microenvironment. Mahav Dhodapkar (New Haven, CT) discussed the impact of hematopoietic cells on MM in the marrow, and explored the possibility that cell-to-cell interactions with immune cells in the microenvironment may determine the progression of MM. First, Dr. Dhodapkar demonstrated the relationship between MM and SOX2 expression – known to be the marker of stem cell self-renewal. Both precursor MM (CD138-) and advanced MM (CD138+) cells express the SOX2 gene. However, T cells of the advanced MM patient fail to target SOX2. As a consequence, MM patients who have anti-SOX2 T cells have shown better survival rates. Next, it was also demonstrated that hematopoietic cells are involved in osteoclastogenesis in MM patients. IL-17 expression by T cells may be a main player in osteoclastogenesis in MM patients. In addition, cell-to-cell fusion between dendritic cells and MM cells may participate in osteoclastogenesis through CD47/TSP1 pathways.

Another set of T cells, in particular Vγ9Vδ2 T cells, also appears to suppress tumor growth. Ismahene Benzaid (Lyon, France) presented findings showing that zoledronic acid (ZOL) suppresses breast cancer growth by recruiting Vγ9Vδ2 T cells into tumor sites both *in vitro* and *in vivo*. In this study, ZOL induced the cytokine secretions (IL-6, IL-8, and MCP-1) of breast cancer by up-regulating intracellular isopentenyl pyrophosphate (IPP)/Apppl levels. Sequentially, the Vγ9Vδ2 T cells migrate toward breast cancer cells following cytokine gradients to ultimately facilitate cancer cell death by releasing interferon-γ and TNF-α. In addition to T cells, other hematopoietic populations also appear to respond to tumor cells in the bone marrow microenvironment. Xin Li (Ann Arbor, MI) demonstrated that megakaryocytes inhibit metastatic PCa growth in the bone marrow microenvironment both directly and indirectly. Direct inhibition of PCa growth by megakaryocytes was achieved through induction of apoptosis by cell-to-cell contact in co-culture. *In vivo,* smaller tumor growth lesions in bone tissues were detected by bioluminescent imaging after megakaryocyte expansion via thrombopoietin treatment, while no remarkable difference of early dissemination was observed, supporting an indirect role for tumor suppression.

In summary, many components of the marrow microenvironment that are ideally designed to protect and facilitate the growth of hematopoietic cells also provide an environment conducive to the growth of tumor cells. The HSC niche in particular appears to provide an abundance of factors that can be preyed upon by CSCs and metastatic tumor cells. Although the osteoblastic region may provide a target for malignant growth, the bone marrow microenvironment also appears to mount an immune response against tumor cells. The interplay between niche components, tumor cells, and immune cells was discussed, and provided evidence that interactions in the microenvironment are critical to disease progression. These interactions will undoubtedly be studied further, and may provide new insights regarding potential targets for therapy of cancer induced bone disease.

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Imaging and Proteomics of Bone Metastasis

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Early detection and diagnosis are critically important in the clinic for cancers that frequently metastasize to bone. One session at the IX International Meeting on Cancer Induced Bone Disease was devoted to recent advances in the development of novel imaging techniques specific to visualizing early lesions in bone. This *Meeting Report* briefly summarizes the Imaging and Proteomics of Bone Metastasis session and also some interesting poster presentations on the topic.

In the first presentation, Dr. Van der Pluijm (Leiden University, The Netherlands) highlighted that the detection of occult metastasis, particularly in the bone, is a major clinical problem in current oncology practice, and also that sensitive and reliable imaging methods are urgently required. In special efforts toward development of new techniques to image the process of bone metastasis, multiple animal models have been developed, many of which were presented at the conference. Currently, the most common approaches utilize combinations of various tumor xenograft models (*e.g.*, inoculation of human cancer cells into immunocompromised mice by intra-cardiac, intra-venous, and intra-tibial routes) and multiple imaging modalities (*e.g.*, positron-emission tomography, computerized tomography, magnetic resonance imaging, quantum-dots, etc.). Indeed, as introduced by Dr. Van der Pluijm, several poster presentations utilized such combinatorial models to study tumor cell interactions within the microenvironment.

First, Reeves *et al.* (University of Sheffield, United Kingdom) presented a novel imaging technique to investigate prostate cancer bone metastasis *in vivo* (poster 1). The authors engrafted metatarsal bones from newborn mice into a dorsal skin fold chamber implanted on severely combined immunodeficiency (SCID) mice, followed by intra-cardiac inoculation of green fluorescent protein-labeled prostate cancer cells (PC-3). Tumor cell homing and subsequent growth was followed by multi-photon imaging, and bone volume change was measured by micro-CT (computerized tomography). This approach was particularly useful for studying tumor cell interaction with the micro-vascular endothelium, and for following sequential changes of bone and tumor growth *in vivo*. The authors demonstrated that PC-3 tumor cells rapidly increased vascular density of engrafted metatarsal bones within 7 days with no significant difference of bone volume between the PC-3 tumor-implanted group and the control group. The authors also demonstrated that zoledronic acid treatment significantly reduced tumor cell adhesion to bone. It was suggested that this novel model can allow for a better understanding of the mechanisms involved in homing, interactions and extravasation of tumor cells in the bone microenvironment.

For application of a similar approach (*i.e.*, *in vivo* multi-photon microscopy) to multiple myeloma, P. Croucher's group (University of Sheffield, United Kingdom) presented a novel murine model of multiple myeloma (poster 108), using a syngeneic murine multiple myeloma model and two-photon microscopy. Specifically, the authors injected enhanced green fluorescent protein (eGFP)-labeled multiple myeloma cells (5T33MMeGFP) intravenously into C57BLKaLwRijHsd mice. Calvariae were dissected and imaged using micro-computed tomography and single- or two-photon microscopy. In addition, calvarial bone marrow cells were analyzed by flow cytometry and by the reverse transcription

polymerase chain reaction. The authors demonstrated that tumor cells localized in calvariae as early as 18 hours post-injection. They also demonstrated that tumor cells were present in calvariae at three weeks post-injection, suggesting that the calvaria is a site of multiple myeloma tumor development in their model system. The authors speculated that their novel approach may be beneficial for understanding the biology of the calvarial multiple myeloma niche, and subsequent tumor progression.

Buijs *et al.* (Leiden University, The Netherlands) reported a new mouse model of invasive lobular carcinoma of the breast, recapitulating the multi-step process of bone metastasis and minimal residual disease after surgical removal (poster 38). The authors previously generated a mouse model resembling invasive lobular breast carcinoma by deleting p53 and E-cadherin in breast epithelium (1). The authors further luciferase-labeled the invasive lobular carcinoma cells, and injected the cells into the left heart ventricle or mammary fat pad of immunodeficient mice. This novel breast cancer metastasis murine model developed bone lesions with significantly reduced lung involvement (compared to other models such as the 4T1 model) and other soft tissue tumor burdens. Overall, their model system has several benefits including tumors that closely resemble human invasive lobular carcinoma and the development of distant metastasis to bones without an excessively large primary tumor burden that can kill the mice.

As briefly pointed out by Dr. Van der Pluijm in his talk, Dr. Y. Kang's group (Princeton University, New Jersey) recently reported a breast cancer xenograft mouse model system with a dual-luciferase reporter system for tracing both metastatic tumor burden (as determined by non-invasive *in vivo* bioluminescence imaging of constitutively expressing *Renilla* luciferase) and transforming growth factor-β (TGF-β) signaling activity (as determined by bioluminescence imaging of firefly luciferase expressed under the control of a TGF-β-responsive element). This group further engineered an inducible expression system for SMAD4, which allowed *in vivo* conditional manipulation of the TGF-β-SMAD pathway in a stage-specific manner. Interestingly, this novel *in vivo* approach revealed that reduction of TGF-β signaling early in metastasis decreased metastatic bone tumor burden but the effects become less potent according to the progression of disease (2). This innovative approach enabled real-time *in vivo* manipulation and detection of TGF-β signaling, and is further applicable for analyzing the *in vivo* dynamics of other metastasis-associated genes. However, as questioned by the audience, expression of multiple exogenous genes such as GFP, luciferase, etc. may provoke an immune response and thus affect the tumor microenvironment as well as tumor cell proliferation. This potential problem should be taken into consideration when ectopically expressing genes necessary for imaging.

In the following two short presentations chosen from submitted abstracts, clinical examples of advanced imaging techniques were presented. Shokeen *et al.* (Washington University School of Medicine, St. Louis, Missouri) reported a novel approach for imaging the pre-metastatic niche in bone. Hematopoietic progenitor cells (HPCs) that express vascular endothelial growth factor-1 (VEGFR-1) and $\alpha_4\beta_1$ integrin (also known as very late antigen 4, VLA4) have been shown to arrive at sites of metastasis and to form a 'pre-metastatic niche' prior to tumor cell arrival (3). The authors implemented a multi-level imaging strategy for the *in vivo* imaging of $\alpha_4\beta_1$ integrin-positive HPCs at pre-metastatic sites with positron emission tomography (PET) and a high-affinity ligand for $\alpha_4\beta_1$ integrin, CB-TE2A-LLP2A (4;5). For this aim, they injected $\alpha_4\beta_1$ integrin-negative human breast cancer cells (MDA-MB-231/luc) into immunocompromised mice i.c. and monitored the mice for 30 days by combined bioluminescence/microPET/microCT. HSCs expressing $\alpha_4\beta_1$ integrin have been shown to be localized to sites of bone metastasis prior to tumor cell homing, and $\alpha_4\beta_1$ integrin-HSCs are cumulatively increased after tumor cell

inoculation. This approach is currently the most advanced technique for visualizing HSC recruitment in the pre-metastatic niche, particularly with regard to the bone microenvironment.

Dr. T. Bäuerle of the German Cancer Research Center (Heidelberg, Germany) pointed out that widely used response evaluation criteria in solid tumors (RECIST) have critical limitations in quantifying treatment outcomes and also in detecting the slowly changing morphology of tumors in a clinical environment. Thus, the authors tested the effects of a novel anti-angiogenic therapy ($\alpha_v\beta_3$ integrin small molecule antagonist) on metastatic bone lesions and also attempted to visualize and quantify therapeutic effects dynamic contrast enhanced magnetic resonance imaging (DCE-MRI) and flat panel volumetric computed tomography (VCT) (6;7). MDA-MB-231 human breast cancer cells were injected into the superficial epigastric artery of nude rats (8), and randomized to receive $\alpha_v\beta_3$ antagonist or control treatment. Subsequently, hindlimb bone lesions were assessed by *in vivo* live animal imaging (DCE-MRI and VCT) at days 30, 35, 45 and 50 after tumor cell inoculation. Tumor-bearing animals treated with anti-$\alpha_v\beta_3$ showed a significant decrease in progression of both osteolytic lesions and soft tissue tumors, and morphological changes were quantitatively analyzed. The audience asked whether the DCE-MRI and VCT imaging techniques can be applicable to mouse models. Dr. Bäuerle speculated negatively here because DCE-MRI+VCT can be better used for tumors larger than 4 mm in diameter.

In summary, there are currently multiple approaches for visualizing the specific steps of metastatic cascades in the bone microenvironment, and also for detecting early metastatic lesions such as occult metastasis and pre-metastatic changes. Advances in molecular imaging techniques, including multi-photon *in vivo* microscopy and quantum-dots on top of improved CT and MRI, combined with conventional human tumor xenograft and/or syngeneic animal tumor models, provide promising new approaches allowing for a better understanding of the cellular and molecular alterations in the bone microenvironment and their interaction with tumor cells, leading to the development of novel therapeutics.

Conflict of Interest: None reported.

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Tumor-Associated Osteoclastogenesis and Angiogenesis

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Angiogenesis plays an important role in the formation and progression of cancer metastasis as well as the expansion of the primary tumor. In the case of cancer metastasis or myeloma invasion to bone, destruction of bone by enhanced bone resorption is essential for the growth of the tumor. Therefore, a stimulation of angiogenesis is required for both the enhanced formation of osteoclasts and the growth of cancer cells. In fact, increased angiogenesis has been demonstrated in the bone marrow of myeloma patients. By understanding the mechanism of the enhancement of angiogenesis in the bone marrow harboring myeloma or metastatic cancer cells, novel therapeutic approaches may be developed. In this session, mechanisms of enhanced osteoclastogenesis and increased angiogenesis by cancer cells, as well as various studies related to this topic were discussed.

Myeloma cells interact with osteoblasts and osteoclasts to form a microenvironment suitable for the growth and survival of myeloma cells. Myeloma cells stimulate osteoclastic bone resorption via secretion of MIP-1. Osteoclasts thus formed not only secrete factors such as TNF family cytokines including BAFF and APRIL to enhance myeloma cell survival (1), but also produce osteopontin (2;3). Myeloma cells constitutively produce VEGF (4), and our group (University of Tokushima) presented data showing that VEGF and osteopontin act together to stimulate angiogenesis (5). Enhancement of osteoclastic bone resorption causes a release and activation of TGF-β from the bone matrix. TGF-β is also an angiogenic factor (6), and it is proposed that increased release of active TGF-β further enhances angiogenesis around myeloma cells, and inhibits bone formation via a suppression of the terminal differentiation of osteoblasts. Thus, the myeloma bone microenvironment is characterized by a vicious cycle of bone destruction, angiogenesis and myeloma expansion. Because mature osteoblasts inhibit myeloma cell growth and survival, stimulation of the terminal differentiation of osteoblasts by an inhibitor of TGF-β action may become a new therapeutic target against multiple myeloma.

Most bone metastasis models utilize immunodeficient mice lacking T cells, and the role of T cells in bone metastases has not been adequately addressed. Thus, Pierrick Fourner *et al*. (University of Virginia) investigated the distribution of T cell populations using a syngeneic mouse model of breast cancer bone metastases by inoculating 4T1 breast cancer cells from Balb/C mice into the left cardiac ventricle of female Balb/C mice. In metastatic bone tissues, the total number of CD3+ T cells decreased, but the number of CD4+ helper T cells and γδT cells increased. Among CD4+ T cells, Th2 cells (CD4+IL-4+IFNγ+) and Th17 cells (CD4+IL-17+) decreased, while regulatory T cells (Treg; CD4+CD25+) increased in bone metastases. A subset of cytotoxic T cells that can secrete osteoclastogenic IL-17 (Tc17; CD8+IL-17+) was also present in bone metastases. These results suggest that the bone metastatic microenvironment can protect cancer cells from the immune cytotoxic response and alter the distribution of T cells to increase osteoclastogenesis, and that the T cell immune system can be another therapeutic target against bone metastases.

Osteoblasts express both ephrinB2 and EphB4, but osteoclasts express mainly the ligand ephrinB2. Forward signaling promotes osteogenesis and reverse signaling inhibits osteoclastogenesis (7). Because myeloma bone disease is characterized by an

uncoupling of osteoclastic bone resorption and osteoblastic bone formation, Shmuel Yaccoby (University of Arkansas) examined the involvement of the ephrinB2/EphB4 system on the development of myeloma bone disease. Gene and protein expression of both ephrinB2 and EphB4 in mesenchymal stem cells from myeloma patients were reduced (8). Activation of reverse signaling by EphB4-Fc inhibited NFATc1 expression, osteoclastogenesis, angiogenesis and myeloma growth, with stimulation of osteoblastogenesis. Activation of forward signaling by ephrinB2-Fc induced osteoblastic differentiation and angiogenesis but did not affect osteoclastogenesis or myeloma growth. These results suggest that down-regulation of the ephrinB2/EphB4 axis contributes to the development of myeloma bone disease, and that EphB4-Fc treatment is an effective approach to inhibit myeloma bone disease and myeloma growth.

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