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Repression of Multiple Myeloma Growth and Preservation of Bone with Combined Radiotherapy and Anti-angiogenic Agent

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Abstract

The effects of ionizing radiation, with or without the antiangiogenic agent anginex (Ax), on multiple myeloma growth were tested in a SCID-rab mouse model. Mice carrying human multiple myeloma cell-containing pre-implanted bone grafts were treated weekly with various regimens for 8 weeks. Rapid multiple myeloma growth, assessed by bioluminescence intensity (IVIS), human lambda Ig light chain level in serum (ELISA), and the volume of bone grafts (caliper), was observed in untreated mice. Tumor burden in mice receiving combined therapy was reduced to 59% (by caliper), 43% (by ELISA), and 2% (by IVIS) of baseline values after 8 weeks of treatment. Ax or radiation alone slowed but did not stop tumor growth. Four weeks after the withdrawal of the treatments, tumor burden remained minimal in mice given Ax + radiation but increased noticeably in the other three groups. Multiple myeloma suppression by Ax + radiation was accompanied by a marked decrease in the number and activity of osteoclasts in bone grafts assessed by histology. Bone graft integrity was preserved by Ax + radiation but was lost in the other three groups, as assessed by microCT imaging and radiography. These results suggest that radiotherapy, when primed by anti-angiogenic agents, may be a potent therapy for focal multiple myeloma.

Introduction

Multiple myeloma is a heterogeneous hematopoietic malignancy that results from unrestrained plasma cell proliferation (1). The intramedullary form of the disease is often associated with osteolysis mediated by the invading myeloma cells (2). Treatment of multiple myeloma has evolved from the classical chemotherapy with melphalan and steroids to bone marrow stem cell transplantation and the more recent targeted therapies (3). With the continued efforts in developing new therapies for multiple myeloma, the overall response rates have grown from 20% to 50%, and the median survival time from diagnosis

has increased from 30 months between 1985–1998 to 60 months today (4, 5). In several recent clinical trials conducted at our institution in which tandem stem cell transplantation was combined with targeted therapies, the median eventfree survival and overall survival times were further increased to 4.8 and 8.0 years, respectively, with thalidomide included in the regimen, and the projected 6-year survival rate improved to 75% with the inclusion of Bortezomib (6, 7). Despite the promising results from these clinical trials, the application of these new therapeutic strategies is still in its infancy and is available to only a small fraction of patients. Given that the current annual diagnoses of multiple myeloma are estimated at 16,000 in the U.S. and over 21,000 in Europe (8, 9), identifying new therapeutic regimens with improved efficacy, reduced toxicity, and less dependence on sophisticated treatment settings is still needed.

Progression of multiple myeloma has been associated with elevated expression of angiogenic factors (e.g. vascular endothelial growth factor and basic fibroblast growth factor) and a marked increase in microvessel density (MVD) in the tumor foci in bone marrow (10). The grade of angiogenesis at the time of diagnosis has been regarded as an important prognostic indicator for multiple myeloma patients (11). Direct evidence for the role of angiogenesis in progression of multiple myeloma and the resistance of the disease to therapy, however, is still inconclusive. For instance, on the one hand, the multifaceted compound, thalidomide, has been shown to effectively suppress tumor burden in multiple myeloma patients, in part through suppressing tumor angiogenesis (12). On the other hand, responses of multiple myeloma to the conventional chemotherapy, assessed by decreased bone marrow plasma cell infiltration, were often not accompanied by a significant decrease in MVD in the tumor foci (13). These observations suggest that angiogenesis-stimulating factors, or the lack of them, might have more complex roles in sustaining the viability and progression of multiple myeloma than acting solely on blood perfusion. This notion is supported by *in vitro* findings that proliferation and migration of either cells of human multiple myeloma cell lines or freshly isolated multiple myeloma cells were stimulated by the addition of vascular endothelial growth factor when angiogenesis was absent (14).

Radiotherapy had been shown in some early clinical trials to be effective in inducing remission of plasma cell malignancies, including multiple myeloma of both solitary and disseminated forms (15, 16). The current use of radiotherapy in multiple myeloma treatment, however, is largely limited to palliative measures or to conditioning regimens in combination with marrow cytotoxic agents prior to stem cell transplantation. The conventional mechanism for control of tumor growth by radiotherapy is that free radicals generated by ionizing radiation cause DNA damage, leading to the death of tumor cells (17). In recent years, a series of studies have shown convincingly that radiation-induced suppression of endothelial cells in solid tumors is an important mechanism of radiotherapy in cancer treatment (18, 19). This observation adds a new facet to the mechanisms through which radiotherapy controls tumor growth. Given that progression of multiple myeloma is associated with increased angiogenesis (10, 20) and the growing volume of literature demonstrating therapeutic synergy with radiotherapy and anti-angiogenic strategies (21, 22), the value of radiotherapy in multiple myeloma treatment warrants a fresh look.

In this study, we tested the efficacy of once-weekly fractionated X irradiation in controlling myeloma growth and relapse in severe combined immunodeficiency (SCID) mice implanted with rabbit long bone rudiments to provide a suitable microenvironment for human multiple myeloma cell growth (the SCID-rab mouse multiple myeloma model) (23). Our recent work has indicated that anti-angiogenic peptide anginex (Ax), a designer anti-angiogenic agent (24), sensitizes tumor endothelial cells to radiation (22) and may improve tumor oxygenation, thereby further improving tumor radiation responsiveness (25). In light of the

potential role of angiogenesis in myeloma progression, the radiosensitizing properties of Ax, administered for 3 consecutive days prior to each radiation exposure, was evaluated.

Materials and Methods

Human BN Myeloma Cell Line

We previously established the human multiple myeloma cell line BN from heparinized bone marrow aspirates from a patient with active myeloma (26). The cells, which were characterized as Epstein-Barr virus negative and non-hyperdiploid, are stably transfected with lentiviral vector (pLenti6/V5/EGFP_{Luc}) to allow the tracing of the EGFP/luciferase-expressing BN cells *in vitro* and *in vivo*. *In vitro* expansion of the BN cells was performed as described previously (26). Briefly, mononucleated marrow cells from human fetal long bones (Advanced Bioscience Resources, Alameda, CA) were used as feeder cells and cultured in flasks in low-glucose Dulbecco's modified Eagle's medium (DMEMLG) supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, UT) and antibiotics. The adherent cells expressed CD166 but not CD45 or CD34 and were capable of differentiating into lineages such as osteoblasts, characteristic of their mesenchymal origin (27). At 80% confluence, the medium was replaced with RPMI-1640 medium supplemented with 10% FBS and antibiotics, and BN cells were added to establish a BN-mesenchymal coculture with the BN cells remaining in suspension. For passaging, the BN cells were collected from coculture medium, spun to remove excess medium, and seeded in new flasks containing subconfluent mesenchymal cells.

Growth of BN Cells In Vivo

The *in vivo* focal multiple myeloma mouse model SCID-rab we established previously (23) was used for evaluation of tumor control efficacy of various treatment regimens. All experimental procedures and protocols had been approved by our Institutional Animal Care and Use Committee. SCID-rab mice were constructed as described previously (23). Briefly, rudiments of long bones of newborn New Zealand rabbits (Myrtle Rabbitry, Thompson Station, TN) were implanted subcutaneously on the right flank of 6-week-old CB.17/Icr-SCID mice (Harlan Sprague Dawley, Indianapolis, IN). Six weeks after implantation of the bone grafts, BN cells ($0.5\text{--}2 \times 10^6$ cells in 50–100 μ l of PBS) were injected directly into the marrow cavity of the implanted bone grafts through the diaphyseal opening. Longitudinal monitoring of BN cell growth was performed using the following three methods.

Determination of human Ig levels in mouse serum—Mice were bled periodically from the tail vein starting 2 weeks after inoculation of BN cells. Serum levels of the human lambda light chains secreted by the BN cells, a surrogate marker for BN tumor growth, were determined by an enzyme-linked immunosorbent assay (ELISA) using the Human Lambda-B+F Quantification Kit (Bethyl Laboratories, Inc., Montgomery, TX) following the instructions of the manufacturer.

Bioluminescence imaging of BN cells in bone grafts—Bioluminescence imaging was performed once a week starting the second week after BN cell inoculation. Mice were injected intraperitoneally with firefly D-luciferin (300 mg/kg in 150 μ l PBS; Xenogen, Corp. Alameda, CA). The luminescence signal in the bone grafts on mice in a dorsal-ventral position was detected and captured 15 min after luciferin injection under anesthesia by ketamine plus xylazine using a 26-mm-square back-thinned CCD camera attached to the IVIS Imaging System 200 Series (Xenogen Corp.). The intensity of the bioluminescence signal was analyzed using Living Image Software Version 2.50 (Xenogen) by serially quantifying peak photon flux at the selected region of interest (ROI) covering the bone graft

and corrected for total area of ROI and time lapse during which the bioluminescence signals were picked by the CCD camera and expressed as photo/s/cm²/sr.

Metric caliper measurement of BN tumor—The maximum length (L) and diameter (D) of the grafts were measured weekly with a caliper, starting 5–6 weeks after BN inoculation, and the volume was calculated using the equation: volume (mm³) = D²L/2. The average graft volume was 717 ± 57 mm³ before BN inoculation and 672 ± 62 mm³ when treatment started.

Micro-PET Imaging with [¹⁸F]-Anginex in SCID-rab BN Myeloma Tumor Xenograft

Static microPET imaging was performed using a small animal positron tomograph (microPET Focus 220, Concorde Microsystems, Knoxville, TN) 2 h after i.p. injection with 0.03 mCi (1.11 MBq) [¹⁸F]-anginex tracer in a solution of 10 mg/ml unlabeled anginex. Untreated mice with implanted bone grafts containing rapidly growing BN myeloma cells were killed humanely just before the imaging to improve the resolution of detected activity. Image acquisition via sagittal slices through the tumor region was performed for 20 min. Image reconstruction was performed with a two-dimensional ordered subsets expectation maximum (OSEM) algorithm. Decay-corrected images were used to draw regions of interest (ROIs) around the BN tumor and whole body. Activities within the tumor were normalized to the total-body activity.

Treatment of Bone Grafts Inoculated with BN Cells

About 3–4 weeks after BN cell inoculation, mice with similar sized tumors (i.e., $2.8 \times 10^7 \pm 3.5 \times 10^7$ p/s/cm²/sr of bioluminescence intensity and 102 ± 62 ng/ml human lambda Ig light chain in serum) were assigned to one of four treatment groups: control, anginex (Ax), radiation and Ax + radiation. A schematic of the treatment plan is shown in Fig 1. Ax, a 33 a.a. antiangiogenic peptide (24), or vehicle PBS, was given 3 times/week on three consecutive days at a dose of 20 mg/kg/day based on our previous experience (22). Radiation was given to the bone grafts inoculated with BN cells in 5-Gy fractions once per week 2 h after the last Ax dose each week for 8 weeks (40 Gy) using a Faxitron CP-160 X-ray generating system (Faxitron X-Ray Corp., Wheeling, IL) at a dose rate of 0.5–1.5 Gy/min with 100 kVp and 10 mA. The grafts were extended gently into the radiation field with the rest of the anesthetized mouse body shielded by custom-made cerrobend blocks. The selection of 8 weeks of treatment and 4 weeks of followup was based on our previous studies with the same multiple myeloma mouse models (28).

MicroCT Analysis of Bone Grafts

Prior to harvest, mice were anesthetized with isoflurane and killed by cervical dislocation. Engrafted bones were fixed in 95% ethanol overnight and then switched to 100% ethanol for 48 h. The bone grafts were then scanned for qualitative assessment of the microarchitecture on a μ CT 40 (Scanco Medical AG, Bassersdorf, Switzerland) using the manufacturer's software. Longitudinally sectioned images were obtained with a voxel size of 16 μ m. Semiautomated contouring was used to select an ROI extending through the whole length of the grafts, composed of 150 adjacent individual two-dimensional slices.

Histochemistry

After microCT scanning, the un-decalcified bone grafts were embedded in methyl methacrylate (MMA) and cut longitudinally into 5- μ m slices. The bone slices were stained for collagens by Masson staining, mineralized bone matrix with the von Kossa technique, and osteoclasts by tartrate-resistant acidic phosphatase (TRAP) staining using the Acid Phosphatase Leukocyte Kit (Sigma-Aldrich, St. Louis, MO).

Statistics

One-way ANOVA was performed to test the differences in the mean values between treatment groups using the Student-Newman-Keuls method. $P < 0.05$ was considered as statistically significant.

Results

In Vivo BN Growth

Typically, human Ig lambda light chains became detectable in mouse serum 1–2 weeks after BN cell inoculation, and a bioluminescence signal in bone grafts became detectable 2–3 weeks after BN cell inoculation. Both signals continued to increase linearly in untreated mice for 8–10 weeks and fluctuated afterward, suggesting nonlinear growth at later stages. The presence of BN cells outside of the bone grafts became palpable 5–6 weeks after BN cell inoculation if untreated. The volume of the BN cell-containing bone grafts increased with time in untreated mice until they reached the maximum size allowed by the IACUC, at which point the mice were euthanized.

Anginex Uptake by BN Tumors

Micro-PET imaging of untreated mice engrafted with BN tumors labeled with [^{18}F]-anginex showed that the BN tumor xenografts contained 38.4% of the total radioactivity remaining in the animal 2 h after injection (Fig. 2), indicating significant preferential BN tumor targeting of Ax at the time of irradiation.

Anginex-Enhanced Suppression of BN Tumor Growth by Radiation

IVIS imaging of tumor-derived bioluminescence signals revealed a rapid growth of the tumors (i.e. 166-fold over baseline) in the untreated mice at the end of the eighth treatment cycle (Fig. 3A). In contrast, the signals in mice treated with Ax + radiation decreased to about 2% of the baseline. The suppression of tumor growth was first observed 3 weeks into the treatment and was sustained throughout the remainder of the 8-week treatment period. Radiation alone modestly slowed the tumor growth, while anginex alone had little effect.

In agreement with the bioluminescence data, serum levels of the human lambda light chains in the control group increased by 10 ± 2 - and 43 ± 11 -fold over baseline at the end of the third and sixth treatment cycle, respectively (Fig. 4). The Ax and radiation alone groups exhibited 10- and 9-fold increases, respectively, by the end of sixth treatment cycle. In contrast, the level of the serum marker in mice given the combined treatment decreased to $39 \pm 14\%$ and $42 \pm 11\%$ of the baseline values at these two times.

The volume of the bone graft was about twice the baseline volume in the control group at the end of the eight treatment cycles (Fig. 5). An increase of 1.4- and 1.3-fold in tumor volume was observed after 8 weeks of treatment with either anginex or radiation alone. By contrast, the volume of the grafts decreased to 59% of the pretreatment value after 8 weeks of combined treatment.

Delayed Relapse of Tumor Growth after Combined Therapy

Tumor growth was monitored for 4 additional weeks after the 8-week treatments. Tumor burden in the controls continued to increase as assayed by ELISA, by caliper and by IVIS (see Figs. 3–5). The tumor burden in mice treated with either anginex or radiation alone also exhibited marked increases by the end of the 4-week posttreatment period. In contrast, in mice treated with Ax + radiation the graft/tumor remained substantially smaller compared with baseline values, indicating longlasting tumor suppression by the combined treatment.

Repression of Myeloma Growth by the Combined Therapy Was Accompanied by Bone Preservation

MicroCT imaging of bone grafts excised at week 3 of treatment revealed that a great portion of the bone graft in the control mice were eroded (Fig. 6A), coincident with the rapid increase in tumor burden assessed by bioluminescence imaging and the human light chain content in serum. In contrast, the structure of the bone grafts in mice receiving the combined therapy was largely preserved, accompanied by the noticeable suppression of the tumor-derived bioluminescence signal and light chain content in serum. Radiographs of the bone grafts at posttreatment week 4 showed that the bone structure was almost completely replaced by growing tumor in the untreated mice and was largely destroyed in mice treated with either Ax or radiation alone (Fig. 6B). In contrast, the integrity of the bone grafts was well preserved in mice treated with Ax + radiation.

To assess early events that preceded tumor progression and bone destruction, bone grafts were excised from untreated mice 2 weeks after BN cell inoculation, when neither the bioluminescence signal nor the serum lambda light chains were detectable. For comparison, grafts were also excised from mice receiving the combined treatment for 3 weeks, when the tumor-associated bioluminescence signal and serum light chain content were suppressed and the bone grafts remained largely intact. Gross examination of the sectioned bone grafts after Masson staining for collagens, the major organic extracellular matrix components of the bone, showed that both grafts were similarly intact, with loci of BN cells residing in the bone marrow cavity (Fig. 7A). At higher magnification, however, striking differences between the two grafts were revealed. Tartrate-resistant acid phosphatase (TRAP)-positive, multinucleated osteoclasts were present on a large portion of the surface of trabeculae adjacent to the BN cell loci in the untreated grafts (Fig. 7B). In contrast, in the treated grafts, the proportion of BN-cell-adjacent trabecular surface covered by osteoclasts was much smaller. Furthermore, the osteoclasts in the untreated bone grafts showed active resorption activity, as seen by presence of the resorption bays between osteoclasts and the trabecular surface. In the grafts treated with Ax + radiation, the resorption bays were sparse and shallower, indicating lower bone resorbing activity.

Discussion

Intramedullary multiple myeloma is a plasma cell malignancy with a low response rate to therapy and frequent relapse (3, 5). Sustained response to therapy has been considered to be an important prerequisite for prolonged overall survival in multiple myeloma patients (29, 30). In the present study, we demonstrated in the SCID-rab mouse multiple myeloma model that fractionated irradiation combined with the anti-angiogenic reagent anginex not only repressed *in vivo* BN myeloma cell growth throughout the treatment period but also delayed relapse after treatment. Although the sample size in the present study is small, our use of multiple parameters for tumor growth monitoring in combination with longitudinal observations strengthens the findings. These results indicate the potential therapeutic value of the combined treatment.

Radiotherapy is an effective tumor control modality, and DNA damage induced in tumor cells by radiation is the classical mechanism through which radiotherapy exerts its tumor suppression effects (17). In the presence of normal oxygen level, a great portion of the damaged DNA becomes unreparable due to the formation of oxidized side groups of the DNA helix. Under hypoxic conditions, which are often seen in solid tumors, expression of DNA base excision repair proteins such as AP endonuclease (Ape1) are up-regulated (31) and cell survival factors such as Akt are activated (32), partially through the redox control of transactivation activities of hypoxia-inducible factor (HIF)-1alpha (33). These changes result in improved DNA repair capacity and decreased radiosensitivity. Our demonstration

of consistent, albeit modest, efficacy of radiation alone in delaying BN tumor growth *in vivo* during the treatment period suggests that radiotherapy might not markedly affect the hypoxic status in the tumor microenvironment in this model. It has been noted that the microenvironment of multiple myeloma host tissue, i.e. the bone marrow, is highly hypoxic (34) and that the growth of intramedullary multiple myeloma is associated with decreased hypoxia (35) at least in part through increasing angiogenesis in the course of multiple myeloma progression (20, 36). If this is also true for the SCID-rab mouse multiple myeloma model, the development of the otherwise radiation-induced hypoxia might be offset by the intrinsic angiogenic tendency in the BN tumor and might thus play a lesser role in radiation response. Whether tumor growth in bone grafts is accompanied by increased angiogenesis and whether radiation suppression of tumor growth is associated with significant changes in tumor hypoxia status require further investigation.

To overcome tumor resistance to radiotherapy, various radiosensitization strategies have been developed. Because increased angiogenesis accompanies the progression of intramedullary myeloma (20, 36), new treatments targeting multiple myeloma vasculature have evolved (37). Our demonstration of enhancement of the radiation response by anginex indicates that preirradiation targeting of endothelial cells induces robust radiosensitivity in our multiple myeloma model. This is consistent with recent reports demonstrating that radiation-induced damage to endothelial cells and tumor vasculature was a key element leading to tumor growth delay after radiotherapy and can be independent of tumor oxygenation (18, 19, 38).

The bone microenvironment plays a pivotal role in the progression of multiple myeloma. On the one hand, multiple myeloma cells stimulate the formation and activity of osteoclasts to resorb the host bone, which in turn releases growth factors and matrix proteins that have stimulatory effects on multiple myeloma. On the other hand, multiple myeloma cells suppress the proliferation and differentiation of osteoblasts, resulting not only in imbalanced bone formation and bone resorption but more importantly in the loss of osteoblast-derived molecules (e.g. osteoprotegerin) inhibitory to osteoclasts and multiple myeloma cells (39). Osteolysis (either microscopic or radiographic) is an indicator of accelerated multiple myeloma progression as well as poor prognosis (2). Consistent with these clinical observations, we demonstrated here that the successful suppression of BN tumor growth by the combined therapy was accompanied by a remarkable preservation of the bone structure in the grafts and early inhibition of osteoclast formation/activity. However, we were unable to determine whether the preservation of the bone grafts was the result of the decreased osteolytic signals from the suppressed BN cells or whether the preserved bone and bone cells were instrumental in allowing the combined therapy to induce regression of multiple myeloma. Regardless of the sequence of the events, once-weekly fractionated irradiation in combination with the novel anti-angiogenic agent anginex appears to be a skeleton-sparing modality for intramedullary multiple myeloma.

Despite the robust response of the BN tumor to the combined therapy during the treatment period and a remarkable delay in tumor relapse 4 weeks after the end of treatment, tumor growth eventually resumed, although on a much smaller scale compared with that in the other two treatment groups. In the clinic, the success of multiple myeloma treatment not only is measured by the tumor response to treatment but also is determined by the reduction of the number of multiple myeloma cells that are resistant to the therapy. These therapy-resistant cells could be a subpopulation of the multiple myeloma cells with a unique genetic composition that renders them more resistant (40, 41). They might also arise from the parental cell pool as a consequence of treatment-induced transformation (42). In addition, the tumor stem cell theory in the context of the development of multiple myeloma has drawn increasing attention (43, 44) and has provided an alternative explanation for

multiple myeloma relapse. These theories, however, leave many critical questions unanswered. For instance, how do these cells behave among the untreated, rapidly proliferating tumor cells? If they are resistant to treatment, do these cells contribute to tumor growth during treatment the same way as they would in the event of relapse? Does the treatment-induced tumor regression signal these cells to accelerate their proliferation activity? Answers to these questions may facilitate the development of more effective treatment strategies for multiple myeloma by targeting specific cell types.

The low response rate to therapy and early recurrence are two major factors contributing to the poor prognosis of multiple myeloma patients (3, 5). We present here compelling evidence that localized radiotherapy, when primed with an angiogenesis inhibitor, anginex, is potent in delaying focal BN tumor growth as well as tumor relapse in a mouse model. Our findings demonstrate the possibility of treating localized multiple myeloma of early stage with less cytotoxic but more effective therapies. With the recent demonstration of the feasibility of using helical tomotherapy to deliver total bone marrow irradiation to multiple myeloma patients (45), the application of radiotherapy in multiple myeloma management is in a stage of resurrection. The efficacy of local radiotherapy (tomotherapy) combined with strategies targeting angiogenic processes warrants further evaluation both in animal models of multiple myeloma and in multiple myeloma patients.

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References

1. Kyle RA, Rajkumar SV. Multiple myeloma. *N Engl J Med*. 2004; 351:1860–1873. [PubMed: 15509819]
2. Epstein J, Walker R. Myeloma and bone disease: “the dangerous tango”. *Clin Adv Hematol Oncol*. 2006; 4:300–306. [PubMed: 16728940]
3. Thomas S, Alexanian R. Current treatment strategies for multiple myeloma. *Clin Lymphoma Myeloma*. 2007; 7(Suppl. 4):S139–S144. [PubMed: 17562251]
4. Jacobs P. Myeloma. *Dis Mon*. 1990; 36:317–371. [PubMed: 2189718]
5. Kyle RA, Gertz MA, Witzig TE, Lust JA, Lacy MQ, Dispenzieri A, Fonseca R, Rajkumar SV, Offord JR, Greipp PR. Review of 1027 patients with newly diagnosed multiple myeloma. *Mayo Clin Proc*. 2003; 78:21–33. [PubMed: 12528874]
6. Barlogie B, Anaissie E, van Rhee F, Pineda-Roman M, Zangari M, Shaughnessy J, Epstein J, Crowley J. The Arkansas approach to therapy of patients with multiple myeloma. *Best Pract Res Clin Haematol*. 2007; 20:761–781. [PubMed: 18070718]
7. Zangari M, van Rhee F, Anaissie E, Pineda-Roman M, Haessler J, Crowley J, Barlogie B. Eight-year median survival in multiple myeloma after total therapy 2: roles of thalidomide and consolidation chemotherapy in the context of total therapy 1. *Br J Haematol*. 2008; 141:433–444. [PubMed: 18371114]
8. Hideshima T, Raje N, Richardson PG, Anderson KC. A review of lenalidomide in combination with dexamethasone for the treatment of multiple myeloma. *Ther Clin Risk Manag*. 2008; 4:129–136. [PubMed: 18728702]
9. Armoiry X, Aulagner G, Facon T. Lenalidomide in the treatment of multiple myeloma: a review. *J Clin Pharm Ther*. 2008; 33:219–226. [PubMed: 18452408]
10. Jakob C, Sterz J, Zavrski I, Heider U, Kleeberg L, Fleissner C, Kaiser M, Sezer O. Angiogenesis in multiple myeloma. *Eur J Cancer*. 2006; 42:1581–1590. [PubMed: 16797965]
11. Pruneri G, Ponzoni M, Ferreri AJ, Decarli N, Tresoldi M, Raggi F, Baldessari C, Freschi M, Baldini L, Viale G. Microvessel density, a surrogate marker of angiogenesis, is significantly

- related to survival in multiple myeloma patients. *Br J Haematol.* 2002; 118:817–820. [PubMed: 12181051]
12. Du W, Hattori Y, Hashiguchi A, Kondoh K, Hozumi N, Ikeda Y, Sakamoto M, Hata J, Yamada T. Tumor angiogenesis in the bone marrow of multiple myeloma patients and its alteration by thalidomide treatment. *Pathol Int.* 2004; 54:285–294. [PubMed: 15086832]
 13. Kumar S, Fonseca R, Dispenzieri A, Lacy MQ, Lust JA, Witzig TE, Gertz MA, Kyle RA, Greipp PR, Rajkumar SV. Bone marrow angiogenesis in multiple myeloma: effect of therapy. *Br J Haematol.* 2002; 119:665–671. [PubMed: 12437642]
 14. Hideshima T, Chauhan D, Podar K, Schlossman RL, Richardson P, Anderson KC. Novel therapies targeting the myeloma cell and its bone marrow microenvironment. *Semin Oncol.* 2001; 28:607–612. [PubMed: 11740818]
 15. Mill WB. Radiation therapy in multiple myeloma. *Radiology.* 1975; 115:175–178. [PubMed: 47178]
 16. Nieuwenhuizen L, Biesma DH. Central nervous system myelomatosis: review of the literature. *Eur J Haematol.* 2008; 80:1–9. [PubMed: 17961180]
 17. Hall, EJ.; Giaccia, AJ. *Radiobiology for the Radiologist.* 6th. Lippincott Williams & Wilkins; Philadelphia: 2005.
 18. Garcia-Barros M, Paris F, Cordon-Cardo C, Lyden D, Rafii S, Haimovitz-Friedman A, Fuks Z, Kolesnick R. Tumor response to radiotherapy regulated by endothelial cell apoptosis. *Science.* 2003; 300:1155–1159. [PubMed: 12750523]
 19. Tsai JH, Makonnen S, Feldman M, Sehgal CM, Maity A, Lee WM. Ionizing radiation inhibits tumor neovascularization by inducing ineffective angiogenesis. *Cancer Biol Ther.* 2005; 4:1395–1400.
 20. Niemoller K, Jakob C, Heider U, Zavrski I, Eucker J, Kaufmann O, Possinger K, Sezer O. Bone marrow angiogenesis and its correlation with other disease characteristics in multiple myeloma in stage I versus stage II–III. *J Cancer Res Clin Oncol.* 2003; 129:234–238. [PubMed: 12700896]
 21. Gorski DH, Beckett MA, Jaskowiak NT, Calvin DP, Mauceri HJ, Salloum RM, Seetharam S, Koons A, Hari DM, Weichselbaum R. Blockage of the vascular endothelial growth factor stress response increases the antitumor effects of ionizing radiation. *Cancer Res.* 1999; 59:3374–3378. [PubMed: 10416597]
 22. Dings RP, Williams BW, Song CW, Griffioen AW, Mayo KH, Griffin RJ. Anginex synergizes with radiation therapy to inhibit tumor growth by radiosensitizing endothelial cells. *Int J Cancer.* 2005; 115:312–319. [PubMed: 15688384]
 23. Yata K, Yaccoby S. The SCID-rab model: a novel in vivo system for primary human myeloma demonstrating growth of CD138-expressing malignant cells. *Leukemia.* 2004; 18:1891–1897. [PubMed: 15385929]
 24. van der Schaft DW, Dings RP, de Lussanet QG, van Eijk LI, Nap AW, Beets-Tan RG, Bouma-Ter Steege JC, Wagstaff J, Mayo KH, Griffioen AW. The designer anti-angiogenic peptide anginex targets tumor endothelial cells and inhibits tumor growth in animal models. *FASEB J.* 2002; 16:1991–1993. [PubMed: 12397082]
 25. Dings RP, Loren M, Heun H, McNiel E, Griffioen AW, Mayo KH, Griffin RJ. Scheduling of radiation with angiogenesis inhibitors anginex and Avastin improves therapeutic outcome via vessel normalization. *Clin Cancer Res.* 2007; 13:3395–3402. [PubMed: 17545548]
 26. Li X, Pennisi A, Zhan F, Sawyer JR, Shaughnessy JD, Yaccoby S. Establishment and exploitation of hyperdiploid and non-hyperdiploid human myeloma cell lines. *Br J Haematol.* 2007; 138:802–811. [PubMed: 17760811]
 27. Yaccoby S, Wezeman MJ, Zangari M, Walker R, Cottler-Fox M, Gaddy D, Ling W, Saha R, Barlogie B, Epstein J. Inhibitory effects of osteoblasts and increased bone formation on myeloma in novel culture systems and a myelomatous mouse model. *Haematologica.* 2006; 91:192–199. [PubMed: 16461303]
 28. Yaccoby S, Ling W, Zhan F, Walker R, Barlogie B, Shaughnessy JD Jr. Antibody-based inhibition of DKK1 suppresses tumor-induced bone resorption and multiple myeloma growth in vivo. *Blood.* 2007; 109:2106–2111. [PubMed: 17068150]

29. Barlogie B, Zangari M, Bolejack V, Hollmig K, Anaissie E, van Rhee F, Pineda-Roman M, Mohiuddin A, Crowley J, Tricot G. Superior 12-year survival after at least 4-year continuous remission with tandem transplantations for multiple myeloma. *Clin Lymphoma Myeloma*. 2006; 6:469–474. [PubMed: 16796777]
30. Barlogie B, Anaissie E, Haessler J, van Rhee F, Pineda-Roman M, Hollmig K, Alsayed Y, Epstein J, Shaughnessy JD Jr, Crowley J. Complete remission sustained 3 years from treatment initiation is a powerful surrogate for extended survival in multiple myeloma. *Cancer*. 2008; 113:355–359. [PubMed: 18470907]
31. Fishel ML, Kelley MR. The DNA base excision repair protein Ape1/Ref-1 as a therapeutic and chemopreventive target. *Mol Aspects Med*. 2007; 28:375–395. [PubMed: 17560642]
32. Martinive P, Defresne F, Quaghebeur E, Daneau G, Crockart N, Gregoire V, Gallez B, Dessy C, Feron O. Impact of cyclic hypoxia on HIF-1 α regulation in endothelial cells—new insights for anti-tumor treatments. *FEBS J*. 2009; 276:509–518. [PubMed: 19077164]
33. Chan N, Milosevic M, Bristow RG. Tumor hypoxia, DNA repair and prostate cancer progression: new targets and new therapies. *Future Oncol*. 2007; 3:329–341. [PubMed: 17547528]
34. Parmar K, Mauch P, Vergilio JA, Sackstein R, Down JD. Distribution of hematopoietic stem cells in the bone marrow according to regional hypoxia. *Proc Natl Acad Sci USA*. 2007; 104:5431–5436. [PubMed: 17374716]
35. Asosingh K, De Raeve H, de Ridder M, Storme GA, Willems A, Van Riet I, Van Camp B, Vanderkerken K. Role of the hypoxic bone marrow microenvironment in 5T2MM murine myeloma tumor progression. *Haematologica*. 2005; 90:810–817. [PubMed: 15951294]
36. Rajkumar SV, Mesa RA, Fonseca R, Schroeder G, Plevak MF, Dispenzieri A, Lacy MQ, Lust JA, Witzig TE, Greipp PR. Bone marrow angiogenesis in 400 patients with monoclonal gammopathy of undetermined significance, multiple myeloma, and primary amyloidosis. *Clin Cancer Res*. 2002; 8:2210–2216. [PubMed: 12114422]
37. Shin DH, Chun YS, Lee DS, Huang LE, Park JW. Bortezomib inhibits tumor adaptation to hypoxia by stimulating the FIH-mediated repression of hypoxia-inducible factor-1. *Blood*. 2008; 111:3131–3136. [PubMed: 18174379]
38. Folkman J, Camphausen K. Cancer. What does radiotherapy do to endothelial cells? *Science*. 2001; 293:227–228. [PubMed: 11452105]
39. Edwards CM, Zhuang J, Mundy GR. The pathogenesis of the bone disease of multiple myeloma. *Bone*. 2008; 42:1007–1013. [PubMed: 18406675]
40. Gado K, Domjan G, Kormos L, Falus A. Functional significance of genetic abnormalities in multiple myeloma. *Haematologica*. 2002; 32:191–208. [PubMed: 12611480]
41. Zhan F, Huang Y, Colla S, Stewart JP, Hanamura I, Gupta S, Epstein J, Yaccoby S, Sawyer J, Shaughnessy JD Jr. The molecular classification of multiple myeloma. *Blood*. 2006; 108:2020–2028. [PubMed: 16728703]
42. Hallek M, Bergsagel PL, Anderson KC. Multiple myeloma: increasing evidence for a multistep transformation process. *Blood*. 1998; 91:3–21. [PubMed: 9414264]
43. Huff CA, Matsui W. Multiple myeloma cancer stem cells. *J Clin Oncol*. 2008; 26:2895–2900. [PubMed: 18539970]
44. Matsui W, Wang Q, Barber JP, Brennan S, Smith BD, Borrello I, McNiece I, Lin L, Ambinder RF, Jones RJ. Clonogenic multiple myeloma progenitors, stem cell properties, and drug resistance. *Cancer Res*. 2008; 68:190–197. [PubMed: 18172311]
45. Wong JY, Rosenthal J, Liu A, Schultheiss T, Forman S, Somlo G. Image-guided total-marrow irradiation using helical tomotherapy in patients with multiple myeloma and acute leukemia undergoing hematopoietic cell transplantation. *Int J Radiat Oncol Biol Phys*. 2009; 73:273–279. [PubMed: 18786784]

FIG. 1. Schematic of treatment plan. Panel A: Timeline of experiment. Panel B: Weekly treatment schedule.

FIG. 2.

Sagittal view of ^{18}F -PET image of a SCID-rab mouse bearing a BN myeloma tumor. The relative position of the tumor-containing bone graft in a mouse is illustrated on the left. A static scan was done for 20 min, 2 h after i.p. injection with $[^{18}\text{F}]$ -anginex. A reconstructed image is shown on the right. The BN tumor contained 38% of the total activity remaining in the animal at the time of imaging.

FIG. 3.

Effect of various treatments on BN tumor growth assessed by IVIS imaging. Treatments are as described in Fig. 1: control (Ctrl), anginex (Ax), radiation (RT), and Ax + radiation (Ax + RT). Panel A: Mean bioluminescence intensities. The SD is shown where larger than symbols. * $P < 0.05$ for Ax + radiation compared to Ax, radiation or control only. Comparisons for other data points are not shown for simplicity. Panel B: Bioluminescence images of representative mice after 6 weeks of treatment.

FIG. 4.

Effect of therapy on myeloma growth assessed by BN-cell-derived human Ig light chain contents in the mouse serum measured by ELISA. Data are the means + 1 SE (n 5–7, pooled from two separate experiments). * P < 0.05 for Ax + radiation compared to Ax, radiation or control only. Comparisons for other data are not shown for simplicity.

FIG. 5.

Effect of treatment on myeloma growth assessed by bone graft/tumor volumes calculated from caliper measurements. Data are the means $\pm$ 1 SE ($n = 5-7$, pooled from two separate experiments). * $P < 0.05$ for Ax + radiation compared to Ax, radiation or control only. Comparisons for other data are not shown for simplicity.

FIG. 6. Effects of the combined therapy on preservation of bone graft integrity. Panel A: Representative microCT images of bone grafts excised from control mice (Ctrl) and mice receiving the combined therapy (Ax + RT) for 3 weeks. Panel B: Representative radiographs of bone grafts immediately after BN cell inoculation (prior to treatment, upper panel) and at post-treatment week 4 (lower panel). Radiographs were taken using a Kodak *In Vivo* Fx Optical Imaging System (Carestream Health, Inc., Rochester, NY). Scale = 1 cm.

FIG. 7. Histological examination of bone grafts from untreated mice (Ctrl) 2 week after BN cell inoculation and from mice after three cycles of combined therapy (Ax + RT). Panel A: Masson staining for collagens (blue) in the sectioned bone grafts. Scale = 1 mm. Panel B: Tartrate-resistant acid phosphatase (TRAP) staining for osteoclasts (pink) in the bone slices. BMC: bone marrow cavity; BN: BN foci; TB: trabeculae; arrow head: OC; arrow: resorption bay. Scale = 100 μ m.