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Full Length Research Paper

Tracking of individual myeloma cell homing to the calvarial bone marrow using myeloma murine models

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ABSTRACT

Multiple myeloma (MM) is a plasma cell malignancy that causes extensive osteolytic bone disease mainly in the skull. To support our understanding of MM bone disease, preclinical mouse models have been developed. C57BL/KaLwRijHsd mice develop a high frequency of monoclonal proliferative B-cell disorders. 5T33MM and 5TGM1 are the best characterized as myeloma models and used in most recent studies in this area. In this study, a combination of high-resolution confocal microscopy and two-photon video imaging was used to examine of individual myeloma cells in the calvarial bone marrow of C57BL/KaLwRijHsd mice. This study demonstrated that myeloma cells home and reside in the calvarial bone after 3 days of injection via mouse tail. In addition, this study showed a significant increase in the number of myeloma cells that colonize and home in the interparietal bone comparing to frontal and parietal bones. This study provides evidence that the micro-anatomical site in the interparietal bone may have unique characteristics to study myeloma colonization in bone and to study anti-myeloma therapies, particularly those targeting myeloma bone disease.

Keywords: Myeloma, bone microenvironment, homing, calvariae, interparietal bone.

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INTRODUCTION

Multiple myeloma (MM) is a hematological malignancy of plasma cells, leading to bone disease. MM is the second most common hematologic cancer, representing 10% of all hematologic malignancies (British Committee for Standards in Haematology, 2001). Importantly, most patients experience severe bone pain and lesions and it is associated with anaemia, hypercalcaemia and renal failure (Dispenzieri and Kyle, 2005; Esteve and Roodman, 2007). MM affects 750,000 people worldwide, and the incidence of MM worldwide is increasing. A review of 92 cases of multiple myeloma at the King Faisal Specialist Hospital and Research Centre in Saudi Arabia demonstrated 80% of patients experienced bone pain, 92% of patients had skeletal abnormalities and the median survival time for all patients was 68 months (Khalil et al. 1991). Current treatments for MM target actively recycling cells using chemotherapy (lenalidomide and bortezomib), localized radiotherapy, and bisphosphonates. Patients often go into remission but residual disease results in further myeloma growth (Richardson et al., 2003; Harousseau, 2005; Kyle et al., 2007; Kyle and Rajkumar, 2008).

C57BL/KalwRij mice develop a high frequency of monoclonal proliferative B-cell disorders. Most of C57BL/KalwRij mice have monoclonal gammopathy of undetermined significance (MGUS) similar to humans. 5T33MM model originated from a spontaneously developed myeloma in aging mice. These cells can grow *in vitro* as well as *in vivo*. In culture, 5T33MM cells grow as non-adherent single cells or in small loosely adherent clusters. 5T33MM grow rapidly in C57BL/KaLwRij mice and are mainly localized in the BM in addition to spleen and liver. 5T33MM cells cause myeloma disease in inoculated C57BL/KaLwRij mice after 5 to 8 weeks of

injection with 10^5 marrow cells of 5T33 myeloma mice. C57BL/KalwRij mice post injected with 5T33MM cells develop high levels of tumour-related monoclonal immunoglobulins in their serum with reduction in the level of normal polyclonal immunoglobulins. In contrast to human disease, radiography and histology show that 5T33MM cells do not induce osteolytic bone lesions (Manning et al., 1992; Vanderkerken et al., 1997; Asosingh et al., 2000).

5TGM1 model was derived from the 5T33 model. 5T33MM cells were seeded in mice, and cells were then obtained from the marrow of 5T33MM-bearing mice cultured and cloned. These cells have all the features of human disease including the characteristic lytic bone lesions, in contrast to 5T33MM cells that do not induce osteolytic bone lesions in mice. 5TGM1 cells grow rapidly *in vitro* as well as *in vivo*. In culture, 5TGM1 cells grow as non-adherent single cells or in small loosely adherent clusters. C57BL/KalwRij mice injected with 5TGM1 cells develop high levels of tumour-related monoclonal immunoglobulins in their serum. Radiography and histology shows that 5TGM1 cells produce osteolytic bone lesions similar to the human disease. The bisphosphonate ibandronate has been shown significantly reduce the osteolytic bone lesions in 5TGM1-bearing mice (Garrett et al., 1997; Dallas et al., 1999).

Oyajobi et al. (2007) demonstrated the murine myeloma 5TGM1-GFP tumour was detectable in calvarial bone between 10 and 14 days post intravenous inoculation into animals suggesting that these cells home to calvarial bone marrow (Oyajobi et al., 2007). However, this study lacked the imaging capability to study the arrival of single cells in calvarial bone marrow. There are no publications that demonstrate the murine myeloma 5T33MM model can home to calvarial bone marrow in C57BL/KaLwRijHsd mice. The present studies aimed to study the homing and colonization of single myeloma cell in calvarial bones.

MATERIALS AND METHODS

Cells

5T33MM cells were a kind gift from Dr. Karin Vanderkerken (Free University Brussels, Belgium). 5TGM1 were a kind gift from Dr. Claire Edwards (University of Oxford, UK). These myeloma cells were cultured separately *in vitro* in RPMI1640 medium (Invitrogen, UK) containing 10% FCS, 100 units/ml Penicillin / 100 µg/ml of Streptomycin, 1% Na_2PO_3 and 1% non-essential amino acids (NEAA). Medium was changed every 2 days (Manning et al., 1992). 5T33MM cells and 5TGM1 cells were labelled with DiD dye (Invitrogen, UK) to easily identified myeloma cells under fluorescent microscopy.

Cell labelling with DiD dye

Myeloma cells (1×10^6 cells/ml) were re-suspended in RPMI1640 medium (Invitrogen, UK) containing 10% FCS. 5 µl of 1 µg/ml DiD (Invitrogen, UK) was added to each 1×10^6 cells/ml and incubated at 37°C for 20 min. Cells were mixed every 5 min. After 20 min, myeloma cells were centrifuged at 1000 rpm for 4 min, washed twice with PBS to remove excess DiD dye and re-constituted in RPMI medium containing 10% FCS. Figure 1 shows myeloma cells after labelling under the Zeiss 510 multiphoton laser scanning microscope.

Animals

Male C57BL/KalwRiJHsD mice, aged 5 weeks, were purchased from Harlan, Netherlands and from University of Leeds, UK. Mice were housed by University of Sheffield biological services laboratory. All animals were provided with food and water *ad libitum,* light and all procedures were carried out under personal license (40/10118).

Study plan

4 to 6 week old male C57BL/KaLwRij mice were used. Mice were handled carefully. Mice were put in rodent restrainer, a plastic that hold mice body and keep their tails free for induction, and their tails were anesthetized using EMLA™ cream 5% (AstraZeneca, UK). After 5 min, 200 µl of MM cell lines: 5T33MM and 5TGM1 (2×10^6 cells/200 µl) were inoculated into each mouse via their tail veins. 20 male C57BL/KaLwRijHsd mice were used in this experiment and separated to 4 groups with each group containing 5 mice (Figure 2). Groups 1 and 3 of mice were inoculated with 5T33MM-DiD cells (2×10^6 cells/mouse) at day 0. Groups 2 and 4 of mice were inoculated with 5TGM1-DiD cells (2×10^6 cells/mouse) at day 0. On day 3 groups 1 and 2 of mice were sacrificed. On day 21 groups 3 and 4 of mice were sacrificed. Calvariae were excised carefully and analyzed under a Zeiss 510 Multiphoton Laser Scanning Microscopy. Cells were counted using Volocity™ software, 3D image analysis (http://www.quorumtechnologies.com/image-process.html).

Multi-photon microscopy

All calvariae were analyzed using Zeiss 510 Multiphoton Laser Scanning Microscopy in the University of Sheffield biological services laboratory. A Zeiss 510 Multiphoton Laser Scanning Microscopy was used to visualise fluorescence. Images included 2D X/Y plane, 2D tiled stacks and 3D z stacks to analyze imaging in more depth. Zeiss 510 Microscope connected to the computer were used to capture images. The bone was excited at 900 nM and detected at 450 nM. Red fluorescence of DiD stain was excited at 633 nM and detected at 658 nM.

High-resolution micro–computed tomography (µCT)

High resolution µCT scanner (model 1172; Skyscan, Belgium) is a non-destructive technique that provides three-dimensional micro-structure of bone. µCT scanner was used to scan calvarial bones. Image captured every 0.35° through a 360° rotation with 4.1 pixel size. Scanned images were reconstructed using Skyscan NRecon software (version 1.5.1.3, Skyscan, Belgium).

Statistical analysis

Statistical significance was determined using Tukey's, analysed using GraphPad Prism 6 Software. Data was considered significant if $p \leq 0.05$ (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). All data were presented as the mean ± standard deviation (± SD).

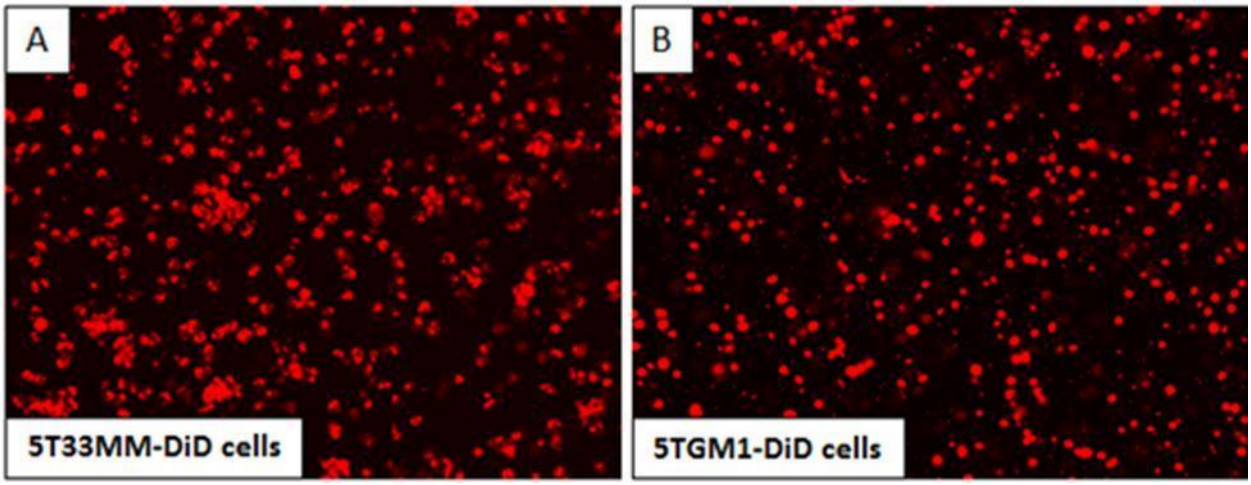

Figure 1. Zeiss 510 multiphoton laser scanning microscope analysis of myeloma cells labelled with DiD. 5T33MM-DiD cells (A) and 5TGM1-DiD cells (B) were visualised using Zeiss 510 multiphoton laser scanning microscope.

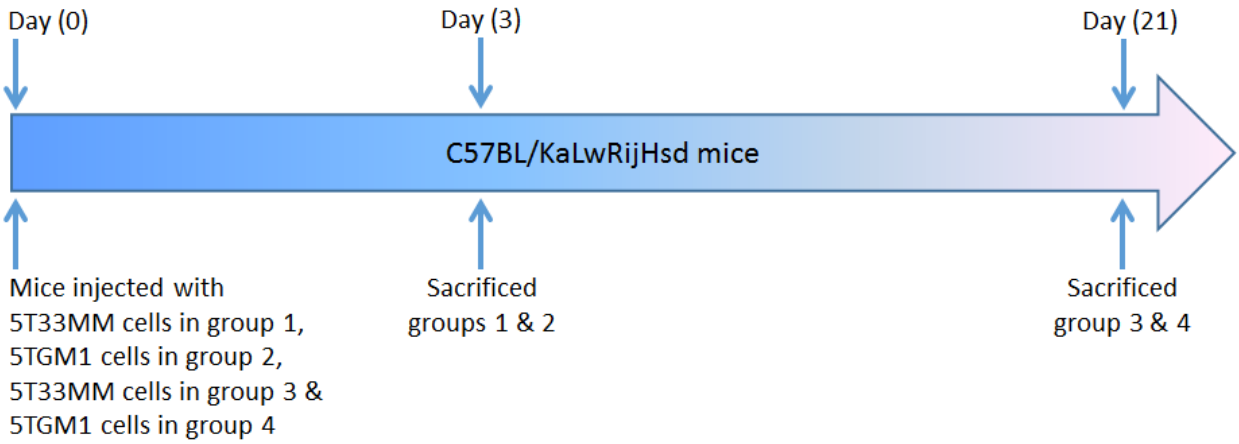

Figure 2. Experiment design to determine single cell numbers in calvarial bone marrow. 10 C57BL/KaLwRijHsd mice were injected with 200 µl of 2 × 10^6 cells/mouse 5T33MM-DiD cells at day 0 (group 1 and 3). 10 C57BL/KaLwRijHsd mice were injected with 200µl of 2 × 10^6 cells/mouse 5TGM1-DiD cells at day 0 (group 2 & 4). On day 3 groups 1 and 2 were sacrificed. On day 21 groups 3 and 4 were sacrificed. All calvarial bone were analyzed under the Zeiss 510 Multiphoton Laser Scanning Microscopy. Cells were counted using Volocity™ software.

RESULTS

Myeloma cells are detectable in calvarial BM of male C57BL/KalwRiJHsD mice using multi-photon microscopy

C57BL/KaLwRijHsd mice were inoculated with 2 × 10^6 5T33MM-DiD cells or 5TGM1-DiD cells via their tail vein. 3 and 21 days post inoculation calvariae were analyzed using multi-photon microscopy. Data show that cancer cells can be detected in calvarial bone of C57BL/KaLwRijHsd mice either by using 5T33MM or 5TGM1 models after 3 days of injection. Figure 3A shows a 3D model of mouse calvaria. The calvariae of C57BL/KaLwRijHsd mice are composed of five bones: two frontal bones, two parietal bones and one interparietal bone. Figure 3B shows the cross section of the calvarial interparietal bone. The enlarged picture demonstrates that the majority of cancer cells colonize and home close to bone surfaces, and not in the central marrow region, as commonly believed. Figure 4 shows representative photomicrographic images of frontal, parietal and interparietal bones, respectively, 3 days post 5TGM1-DiD cells inoculation.

Myeloma cells preferentially home to interparietal bone marrow

Volocity™ software was used to determine the number

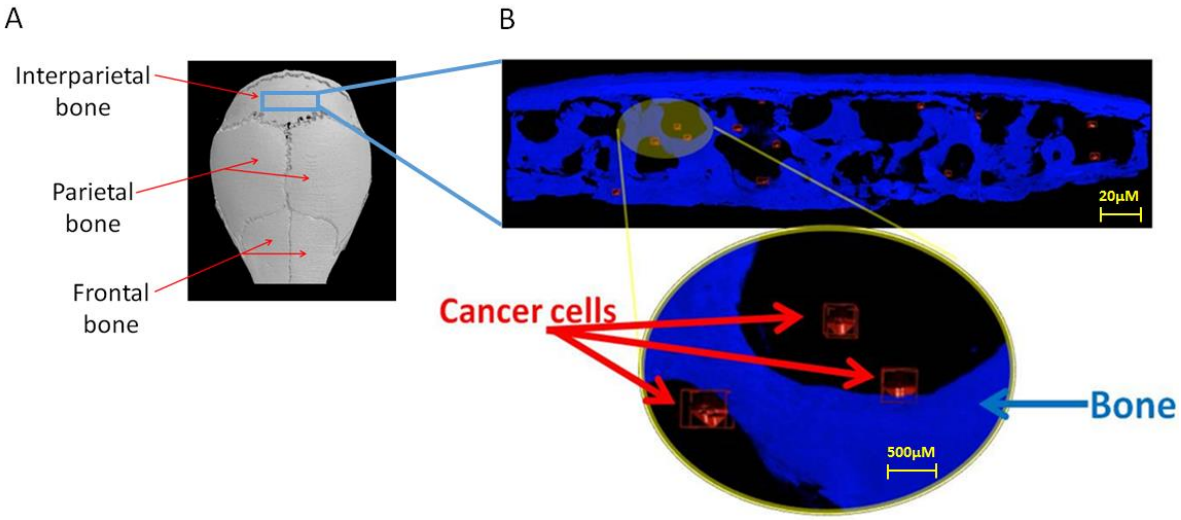

Figure 3. Individual cancer cells colonizing bone. Cells were labelled, inoculated into mice and tissue imaged using multi-photon microscopy to produce 3D images. Panel A is microCT model of a murine skull showing calvariae are composed of frontal, parietal and interparietal bones, respectively. Panels B shows cross section of the calvarial interparietal bone. Blue is bone, red is cancer cells. Enlarge picture shows the majority of cancer cells were close to bone surfaces, and not in the central marrow region (n = 5/group).

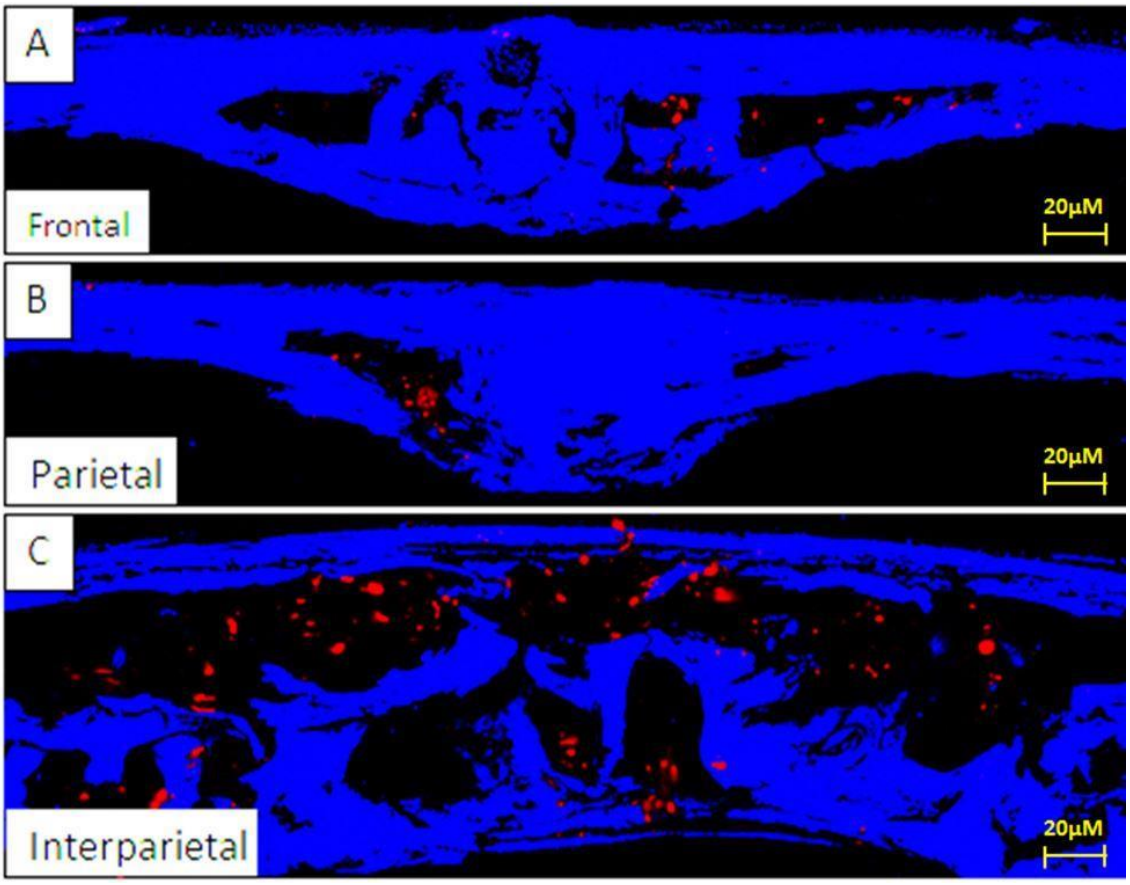

Figure 4. 5TGM1 cells are detectable in calvarial BM male C57BL/KalwRiJHsD mice. Mice were inoculated with 5TGM1-DiD cells. 3 days post inoculation mice were sacrificed and calvariae were excised. Pictures show representative photomicrographic images of interparietal, parietal and frontal bones. Blue fluorescence is the bone and red fluorescence is 5TGM1-DiD cells (n = 5/group).

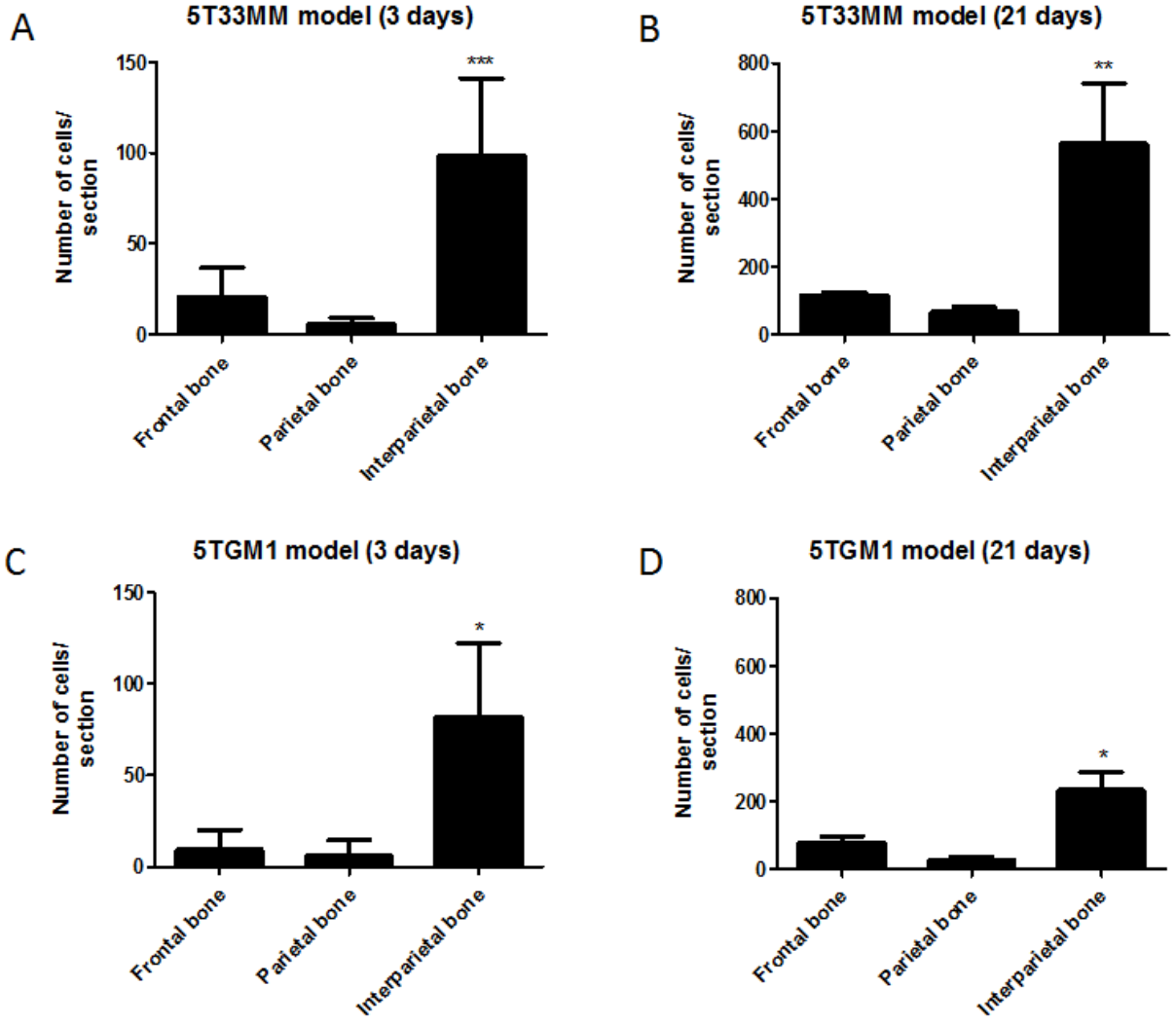

Figure 5. Increased in the number of cancer cells homed to interparietal bones. Mice were inoculated with 5T33MM-D-D or 5TGM1-DiD cells. 3 and 21 days post inoculation mice were sacrificed and calvariae were excised. Panel A&B show a significant increase in the number of 5T33MM-DiD cells in interparietal bones compared to frontal and parietal bones after 3 and 21 days, respectively. Panel C&D show a significant increase in the number of 5TGM1-DiD cells in interparietal bones compared to frontal and parietal bones after 3 and 21 days, respectively (n = 5/group).

of cancer cells in different parts of calvarial bone. Images captured by multi-photon microscopy were loaded into Volocity™ software to produce a 3D image and quantify the number of cancer cells. Figure 5A shows that there was a significant increase in the number of 5T33MM-DiD cells homed to the interparietal bones after 3 days compared to frontal bones (98.71 ± 42.48 versus 20.71 ± 15.98, $P < 0.001$) and parietal bones (98.71 ± 42.48 versus 5.57 ± 3.45, $P < 0.001$). Figure 5B shows that there was a significant increase in the number of 5T33MM-DiD cells homed to the interparietal bones after 21 days compared to frontal bones (562.00 ± 178.90 versus 117.00 ± 7.55, $P<0.01$) and parietal bones (562.00 ± 178.90 versus 67.00 ± 13.75, $P < 0.01$). The same results were found when 5TGM1 cells used. Figure 5C shows that there was a significant increase in the number of 5TGM1-DiD cells homed to the interparietal bones after 3 days compared to frontal bones (81.67 ± 40.50 versus 9.33 ± 11.02, $P < 0.05$) and parietal bones (81.67 ± 40.50 versus 6.33 ± 8.38, $P < 0.05$). Figure 5D shows that there was a significant increase in the number of 5TGM1-DiD cells homed to the interparietal bones after 21 days compared to frontal bones (233.00 ± 53.84 versus 79.67 ± 18.77, $P < 0.05$) and parietal bones (233.00 ± 53.84 versus 27.33 ± 10.41, $P < 0.05$).

DISCUSSION

Overt tumour cell growth occurs in several cancers that

arise in bone such as myeloma and those cancers that metastasise to bone such as breast and prostate. The initial spread of individual cancer cells to bone is commonly believed to occur in the marrow space providing a cancer permissive environment for overt cancer growth. However, the precise location of cancer cells that arise or metastasise in bone is unknown. Understanding the cellular and molecular mechanisms that govern arrival of cancer cells to the bone microenvironment may aid in accelerating future treatments and improve patient care. The aim of this work, using preclinical models of myeloma, was to identify and characterise the location of cancer cells arriving in bone within a few hours of arrival. The homing of individual myeloma cells in C57BL/KaLwRijHsd calvarial bone marrow was assessed using 5T33MM model and 5TGM1 model. Tumor cells may home to many tissues but there may be specific characteristics of particular bone marrows, or regions within bone marrow, that permit colonization, survival and growth of these myeloma cells (Schneider et al., 2005; van der Pluijm et al. 2005).

Multi-photon microscopy was used to determine the location of myeloma cells with respect to proximity to bone in calvariae. Xie et al. (2009) have recently used a confocal laser scanning microscope LSM 510, multi-photon microscopy, to trace the homing of GFP^+ hematopoietic stem cells (HSCs) and determine the distribution of these cells in the bone marrow (Xie et al., 2009). In addition, Lo Celso et al. (2009) used a combination of high resolution confocal microscopy and two-photon video imaging technology that allowed observation of homing of individual HSC in calvarial bone marrow of living mice in real time (Lo Celso, Fleming et al. 2009). It is only by using modern microscope that has permitted researchers to identify the stem cells in the niche.

5T33MM cells and 5TGM1 cells were labelled with DiD (Invitrogen, UK), a dye bound to phospholipid bilayer membranes of the cells (http://products.invitrogen.com/ivgn/product/V22887). To date, there is no publication demonstrating that the murine myeloma 5T33MM and 5TGM1 cells can home to calvarial bone marrow in C57BL/KaLwRijHsd mice. Previous studies demonstrated that tumour cells metastasize to skeletal sites with active bone turnover (Schneider et al., 2005; van der Pluijm et al., 2005). Data presented here showed that myeloma cells home in specific to the bone. Current treatments for MM target actively recycling cells using lenalidomide and bortezomib, chemotherapy, localised radiotherapy and bisphosphonates. Patients often go into remission but residual disease results in further myeloma growth (Richardson et al., 2003; Harousseau, 2005; Kyle et al., 2007; Kyle and Rajkumar, 2008). Myeloma cells may be protected from therapeutics due to their proximity to the bone which confer quiescence. Using modern microscope may permit researcher to study anti-myeloma therapies, particularly those targeting myeloma bone disease.

In this study, there was a significant increase in the numbers of 5T33MM and 5TGM1 myeloma cells observed in the interparietal bone marrow compared to the frontal and parietal bone marrow after 3 days. In addition, there was a significant increase in the numbers of 5T33MM and 5TGM1 myeloma cells observed in the interparietal bone marrow compared to the frontal and parietal bone marrow after 21 days. This suggests that the interparietal bone is a more favorable environment for attachment of myeloma cells. This may be because this bone offers greats access and volume for colonizing tumour cells but it may also be environmentally different from the other calvarial bones and this remains to be evaluated. The increased tumour growth in the interparietal bones may affect the increased frequency of potential colony forming cell or again may be a result of potential environmental differences stimulates growth.

Conclusion

Using modern microscopy, multiphoton microscopy, permitted us to visualized single myeloma cells in the calvarial bone marrow. This study provides evidences that the micro-anatomical site in the intraparietal bone may have unique characteristics to study myeloma colonization and growth in bone. Also, this provides platforms for pre-clinical investigations to study myeloma colonization and growth in skull (calvarial bone) and to study anti-myeloma therapies, particularly those targeting myeloma bone disease.

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