



Short Communication

Homing of Myeloma Cells into Calvarial Bone 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 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. 5TMM series of myeloma models originate from spontaneously developed MM in C57BL/KaLwRijHsd mice and have many of human features of the disease. 5T33MM and 5TGM1 are the best characterized and used in most recent studies. There are no publications that demonstrate the murine myeloma 5T33MM can home and grow in calvarial bone (skull) in C57BL/KaLwRijHsd mice. This work studied the growth of tumor in calvarial bones over the time using 5T33MM and 5TGM1 models. In summary, C57BL/KaLwRijHsd mice injected with 5T33MM or 5TGM1 provide models to study anti-myeloma therapies. I found that large myeloma tumour developed in the interparietal bones using Fluorescence ChedaLighttoolsIllumatools System imaging (LT-9900 Bright Light System). This study provides evidences that myeloma cells home into calvarial bone to study myeloma colonization and growth in bone and to study anti-myeloma therapies, particularly those targeting myeloma bone disease.

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

INTRODUCTION

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 are 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, Padmos et al. 1991). 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, Barlogie et al. 2003, Harousseau 2005, Kyle, Yee et al. 2007).

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 mainly localised in the BM in addition to spleen and liver. 5T33MM cells cause myeloma disease in inoculated C57BL/KalwRij mice after 5-8 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 5T33MM cells do not induce osteolytic bone lesions (Manning, Berger et al. 1992, Vanderkerken, De Raeve et al. 1997, Asosingh, Radl et al. 2000).

5TGM1 model was derived from the 5T33MM model. 5T33MM cells were passaged 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, Dallas et al. 1997, Dallas, Garrett et al. 1999).

In the present studies, I studied the homing and growth of tumor in calvarial bones over the time. Myeloma tumour growth was detected in calvarial bone of male C57BL/KalwRiJHsD mice using 5T33MM and 5TGM1 models. This study provides evidence that calvarial bone provides model to study anti-myeloma therapies.

MATERIALS AND METHODS

Cells

5T33MM-green fluorescent protein (GFP) cells were a kind gift from Dr. Karin Vanderkerken (Free University Brussels, Belgium). 5TGM1-GFP cells 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% Na₂PO₃ and 1% non-essential amino acids (NEAA). Medium was changed every 2 days (Manning, Berger et al. 1992).

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

4-6 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 minutes, 200 µl of MM cell lines: 5T33MM and 5TGM1 (2×10^6 cells/200 µl) were inoculated into each mouse via their tail veins. 36 male C57BL/KaLwRijHsd mice were used in this experiment and separated to 12 groups each group contain 3 mice (Figure 1). Groups 1, 4, 7 and 10 of mice used as a control, no myeloma injected. Groups 2, 5, 8, and 11 of mice were inoculated with 5T33MM-GFP cells (2×10^6 cells/ mouse) at day 0. Groups 3, 6, 9 and 12 of mice were inoculated with 5TGM1-GFP cells (2×10^6 cells/ mouse) at day 0. On day 10 groups 1, 2 and 3 of mice were sacrificed. On day 14 groups 4, 5 and 6 of mice were sacrificed. On day 17 groups 7, 8 and 9 of mice were sacrificed. On day 21 groups 10, 11 and 12 of mice were sacrificed. Calvarias were excised carefully and analyzed under the Lightools system to obtain optical images of fluorescent tumor growth in calvarial bone.

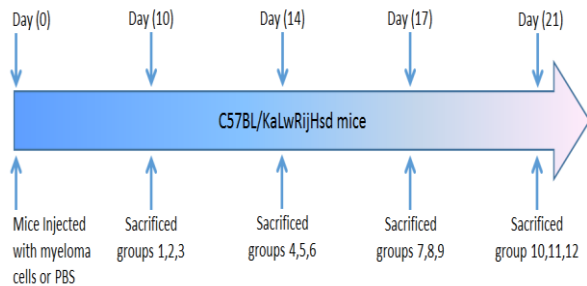


Figure 1: Experiment design to determine the tumor growth in calvarial bone.

12 C57BL/KaLwRijHsd mice were injected with 200µl of PBS as control mice at day 0 (group 1, 4, 7 & 10). 12 C57BL/KaLwRijHsd mice were injected with 200µl of 2×10^6 cells/mouse 5T33MM-GFP cells at day 0 (group 2, 5, 8 & 11). 12 C57BL/KaLwRijHsd mice were injected with 200µl of 2×10^6 cells/mouse 5TGM1-GFP cells at day 0 (group 3, 6, 9 & 12). On day 10 groups 1, 2 & 3 were sacrificed. On day 14 groups 4, 5 & 6 were sacrificed. On

day 17 groups 7, 8 & 9 were sacrificed. On day 21 groups 10, 11 & 12 were sacrificed. All calvarial bone were analysed under the light tool.

Lightools image analysis system

All calvariae were analyzed using Fluorescence ChedaLightools Illumatools System (LT-9900 Bright Light System) in the University of Sheffield biological services laboratory. The Lightools image analysis system used to visualize GFP⁺ tumour in calvarial BM. The GFP-tumour was excited at 470 nm and detected at 515 nm with a MagnaFire SP cooled color charge-coupled device (CCD) camera.

Statistical analysis

Statistical significance was determined using non-parametric Kruskal-Wallis, analysed using GraphPad Prism 6 Software. Data was considered significant if $p < 0.05$ (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). All data were presented using the standard deviation (\pm SD).

RESULTS

Myeloma tumor growth was detected in calvarial bone of male C57BL/KalwRijHsd mice

C57BL/KaLwRijHsd mice were inoculated with PBS, 2×10^6 5T33MM-GFP cells and 5TGM1-GFP cells via their tail vein. 10, 14, 17 and 21 days post inoculation calvariae were analyzed using Fluorescence Lightools Illumatools System (LT-9900 Bright Light System). Data show there is no GFP tumor in calvarial bone of C57BL/KaLwRijHsd mice either by using 5T33MM or 5TGM1 models at day 10 demonstrating that myeloma cancer cells need at least 10 days to grow tumor in the bone. Figure 2A shows there is no GFP-expression over the period of time in mice injected with PBS, a negative controls. Figure 2B shows GFP-expressing of 5T33MM tumor growth in interparietal bones, but not in frontal or parietal bones.

Figure 2C shows GFP-expressing of 5TGM1 tumour growth in frontal and interparietal bones, but not in parietal bones.

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. This study demonstrated that large 5T33MM-GFP tumors developed in the interparietal bones and not in the frontal and parietal bones after 3 weeks. In addition, I found that large 5TGM1-GFP tumors developed in the interparietal bones and in the frontal bones, not in the parietal bones after 3 weeks. The findings demonstrated that the micro-anatomical site in the interparietal bone may have unique characteristics in which to study myeloma colonisation and growth in bone.

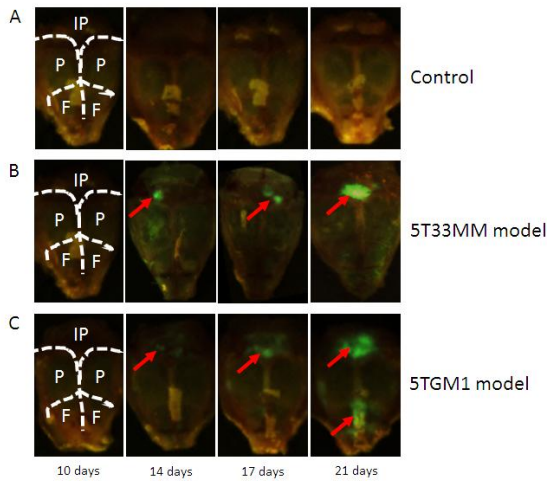


Figure 2: Myeloma tumor growth was detected in calvarial bone of male C57BL/KalwRiJHsD mice.

Murine skull showing 5 distinct bones: 2 frontal (F), 2 parietal (P) and 1 Interparietal (IP). Mice were inoculated with 5T33MM-GFP and 5TGM1-GFP cells. 10, 14, 17 & 21 days post inoculation mice were sacrificed and calvariae were excised. The results illustrated that murine myeloma 5T33MM-GFP (panel B) and 5TGM1-GFP (panel C) tumor were detectable in calvarial

bone (green). Panel A shows the PBS injected control (n=3/group).

Significant increase in tumor size over period of time

To determine the percentage of tumor growth in calvarial bone Image J software was used. The volume of tumor and the volume of whole calvaria were measured with Image J software. Percentages of tumor growth were determined by dividing the tumor volume to calvarial volume and multiply in a hundred for each mouse. Figure 3A shows a significant increase in the GFP-tumor signal in mice injected with 5T33MM cells in day 17 comparing with day 10 (8.18 ± 1.04 versus 0.0 ± 0.0 , $P < 0.01$) and day 21 comparing with day 10 (14.32 ± 4.05 versus 0.0 ± 0.0 , $P < 0.001$). Figure 3B shows a significant increase in the GFP-tumor signal in mice injected with 5TGM1 cells in day 17 comparing with day 10 (11.85 ± 3.19 versus 0.0 ± 0.0 , $P < 0.001$) and day 21 comparing with day 10 (26.39 ± 2.00 versus 0.0 ± 0.0 , $P < 0.001$).

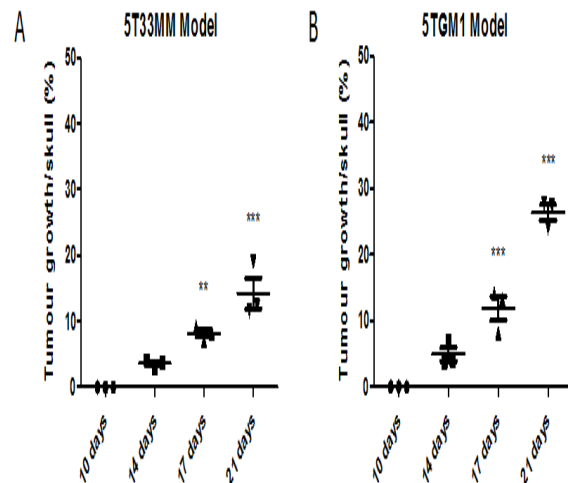


Figure 3: Percentage of tumor size in calvarial bone.

Image J software was used to determine the percentage of tumor size. Panel A shows a significant increase in the percentage of tumor size in days 17 and 21

using 5T33MM model. Panel B shows a significant increase in the percentage of tumor size in days 17 and 21 using 5TGM1 model (n=3/group).

DISCUSSION

The growth of myeloma tumour in C57BL/KaLwRijHsd calvarial bone was assessed using 5T33MM model and 5TGM1 model. This study demonstrated that 5T33MM-GFP tumour developed in the calvarial bones in days 14, 17 and 21. In addition, I found that 5TGM1-GFP tumour developed in calvarial bones in days 14, 17 and 21. Both models showed tumor cannot be detected in first 10 days after cells injected in the mice.

5T33MM and 5TGM1 models share some common features including the selective localization of myeloma cells in the bone marrow, presence of serum M-component, and expression of LFA-1, CD44, VLA-4 and VLA-5 adhesion molecules. Both models represent a model of human multiple myeloma disease and can grow in vitro and in vivo. However, mouse models do not essentially accurately reproduce human disease. Some differences were found between both models. The 5T33MM model does not induce osteolytic bone lesions however this could be due to the aggressive tumour causing rapid morbidity whereas the 5TGM1 model does induce osteolytic bone lesions similar to multiple myeloma in humans (Radl, Hollander et al. 1978, Radl, De Glopper et al. 1979, Vanderkerken, De Raeve et al. 1997). This study showed another difference between 5T33MM and 5TGM1 models, in which 5TGM1 grew in interparietal and frontal bones while 5T33MM only grew in interparietal bone and not in frontal bones. This is may be due to the 5TGM1 model being associated with an extensive osteolysis.

To date, there is no publication demonstrating that the murine myeloma 5T33MM cells can home to calvarial bone in C57BL/KaLwRijHsd mice. Previous studies demonstrated that tumour cells metastasize to skeletal sites with active bone turnover (Schneider, Kalikin et al. 2005, van der Pluijm, Que et al. 2005). In this study, myeloma cells were inoculated into C57BL/KaLwRijHsd mice for 3 weeks. Interestingly, we showed that both 5T33MM and 5TGM1 form large tumor in interparietal bones and not in frontal or parietal bones after 3 weeks. One potential explanation for this is that the interparietal bones contain higher BM area, higher bone surface area and higher bone turnover compared to frontal and parietal bones, but there is also another explanation: that there is a difference in the cellular composition, particularly in osteoblasts, between these bones.

CONCLUSION

In conclusion, this study demonstrate that inoculation of 5T33MM and 5TGM1 models into C57BL/ KaLwRijHsd mice provides an aggressive disease development as demonstrated by high tumour burden in calvarial bone. 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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