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Frequency of acute myeloid leukaemia-associated mouse chromosome 2 deletions in X-ray exposed immature haematopoietic progenitors and stem cells☆

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ABSTRACT

Exposure to ionising radiation can lead to an increased risk of cancer, particularly leukaemia. In radiation-induced acute myeloid leukaemia (rAML), a partial hemizygous deletion of mouse chromosome 2 is a common feature in several susceptible strains. The deletion is an early event detectable 24 h after exposure in bone marrow cells using cytogenetic techniques. Expanding clones of bone marrow cells with chromosome 2 deletions can be detected less than a year after exposure to ionising radiation in around half of the irradiated mice. Ultimately, 15–25% of exposed animals develop AML. It is generally assumed that leukaemia originates in an early progenitor cell or haematopoietic stem cell, but it is unknown whether the original chromosome damage occurs at a similar frequency in committed progenitors and stem cells. In this study, we monitored the frequency of chromosome 2 deletions in immature bone marrow cells (Lin^-) and haematopoietic stem cells/multipotent progenitor cells (LSK) by several techniques, fluorescent *in situ* hybridisation (FISH) and through use of a reporter gene model, flow cytometry and colony forming units in spleen (CFU-S) following *ex vivo* or *in vivo* exposure. We showed that partial chromosome 2 deletions are present in the LSK subpopulation, but cannot be detected in Lin^- cells and CFU-S12 cells. Furthermore, we transplanted irradiated Lin^- or LSK cells into host animals to determine whether specific irradiated cell populations acquire an increased proliferative advantage compared to unirradiated cells. Interestingly, the irradiated LSK subpopulation containing cells carrying chromosome 2 deletions does not appear to repopulate as well as the unirradiated population, suggesting that the chromosomal deletion does not provide an advantage for growth and *in vivo* repopulation, at least at early stages following occurrence.

1. Introduction

Exposure to ionising radiation (IR) occurs from a mixture of natural and occupational sources [1], medical treatment [2] as well as accidental exposure [3]. Epidemiological studies, including the cohort studies from survivors from the Hiroshima and Nagasaki atomic bombings, show exposure leads to an increased risk of cancer [4,5].

Acute myeloid leukaemia (AML) is one of the most common malignancies seen to occur in human populations exposed to IR [2]. A dose-dependent increase in AML incidence has been documented in the atomic bomb blast survivors [5] and it is known to occur following radiotherapy [6]. The crucial initial event for leukaemia is believed to be DNA damage with DNA double-strand breaks potentially leading to chromosomal aberrations such as deletions or fusion genes characteristic of specific leukaemias when misrepaired [7]. Through the loss of genetic material due to chromosome deletions, for example, loss-of-function mutations can occur and increase the likelihood of progression to cancer [8]. Even though tumours are generally thought to evolve from a mutation in a single cell, the "cell of origin" and the cell type sustaining the tumour, "cancer stem cells (CSC)" may not be the same [9,10]. Defining both the cell of origin and the CSC in tumours will provide crucial information to understand the molecular mechanisms behind individual cancer types, or indeed within cancer types. Furthermore,

Abbreviations: rAML, radiation-induced acute myeloid leukaemia; Lin^-, lineage negative immature bone marrow cells; LSK, Lin^- Sca-1^+ c-Kit^+; BAC-FISH, bacterial artificial chromosome-fluorescent *in situ* hybridisation; CFU-S12, colony forming unit spleen on day 12; IR, ionising radiation; CSC, cancer stem cell; del2, interstitial deletion of chromosome 2; GFP, green fluorescent protein; *Sfpi1* $^{GFP/GFP}$, homozygous for the *Sfpi1*-GFP reporter gene; *Sfpi1* $^{GFP/+}$, heterozygous for the *Sfpi1*-GFP reporter gene; BMCs, bone marrow cells; 7-AAD, 7-aminoactinomycin D; HSC, haematopoietic stem cell; IMDM, Iscove's modified Dulbecco's medium; MMP, multi-potent progenitor; MEP, myeloid-erythroid progenitor; CMP, common myeloid progenitor.

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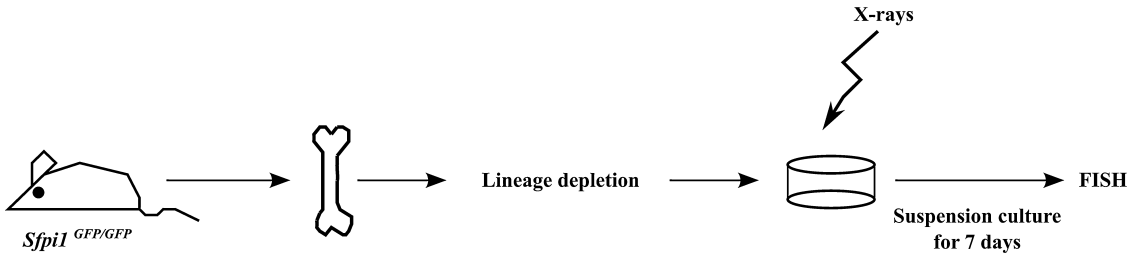

Fig. 1. Bone marrow cells were isolated from *Sfpi1*$^{GFP/GFP}$ mice and Lineage depleted. Lin$^-$ cells were put into suspension cultures, irradiated *in vitro* with a 2 Gy X-rays dose after overnight culture and then cultured for a total of 7 days. Cultures were harvested, chromosome preparations made and analysed by BAC-FISH for deletions on chromosome 2.

more accurate risk estimates can be obtained if the chromosomal aberrations in the cells are early biomarkers of late effects such as cancers.

Leukaemia is the prevailing neoplastic disorder of the haematopoietic system. It is the consequence of an acquired modification in the genome of a target cell, most probably a stem cell of the bone marrow compartment following exposure to a carcinogen, such as ionising radiation (IR). Nevertheless, leukaemia may also arise from more committed progenitors caused by mutations and/or selective expression of genes that enhance their self-renewal capabilities [11]. For example, it was demonstrated that leukaemia stem cells can be generated from committed progenitors by expressing a fusion protein encoded by a specific translocation [12].

In a mouse model of radiation-induced AML (rAML), an early event detectable 24 h after exposure is an interstitial deletion of one copy of chromosome 2 (del2) [13–15]. At 12–15 months after exposure, 50% of mice carry an expanding chromosome 2-aberrant clone [14] and eventually up to 25% of the animals develop rAML [16]. Approximately 80–90% of cases carry the partial del2 [13,17,18] and a small number of mice also carry Flt3-internal tandem duplications a common mutation in human leukaemias [19,20] as was recently reported [21]. Molecular mapping of leukaemic cell genomes in mice with rAML identified a minimal deleted region on chromosome 2, in which a potential tumour suppressor gene, *Sfpi1* was located [22,23]. The gene encodes the transcription factor PU.1, an essential and important transcription factor in haematopoiesis [24,25]. Further work has shown that PU.1 acts as a tumour suppressor in haematopoietic and myeloid cell development [26,27]. In approximately 70% of cases of rAML, the remaining copy of the *Sfpi1* gene has a point mutation in the DNA sequence coding for the DNA-binding domain of the protein [28–30]. It is currently not known at what time after irradiation this point mutation occurs. Del2 and/or point mutation of PU.1 in human AML has been less frequently reported [31,32], although heterozygous point mutations in *Sfpi1*/PU.1 have been found in 7% of cases. In these cases, it was found that the mutation leads to a reduced or aberrant function of the transcription factor [33]. PU.1 has further been shown to be of importance in certain types of AML [34,35].

Here we have conducted experiments to quantify del2 after X-irradiation using genetically modified CBA/H mice, that express green fluorescent protein (GFP) as a marker for *Sfpi1* expression (Olme *et al.* manuscript submitted) [36,37]. Immature bone marrow cells (Lin$^-$) and haematopoietic stem cells/multipotent progenitors (LSK and CFU-S12) were investigated for the frequency of *Sfpi1* loss at several early (7 days) and late (10 months) time points following *ex vivo* or *in vivo* exposure as well as assessing their ability to repopulate the bone marrow compartment with a view to have a better definition of the cell of origin of mouse rAML.

2. Materials and methods

2.1. Mice

C57BL/6 GFP expressing mice from Nutt et al. [37] and Dakic et al. [36] were re-derived onto an AML-sensitive CBA/H background at MRC (Harwell, Oxon, UK) for at least 10 generations. The mice have an IRES-GFP cassette inserted in the 3′ untranslated region of the *Sfpi1* gene. GFP is under the control of the *Sfpi1* promoter, where transcription produces a bicistronic mRNA resulting in PU.1 protein and GFP. Both CBA/H *Sfpi1*/GFP homozygous (*Sfpi1*$^{GFP/GFP}$) and CBA/H *Sfpi1*/GFP heterozygous (*Sfpi1*$^{GFP/+}$) animals were utilised in this study along with wild-type CBA/H mice as flow cytometry controls or host animals for repopulation and colony forming unit-spleen assays. All animals were bred and handled according to UK Home Office Animals (Scientific Procedures) Act 1986 and with guidance from the local ethical review committee on animal experiments. Details of the original GFP construct can be found in [36,37].

2.2. Irradiation of cells and mice

Cells were irradiated *in vitro*, either immediately post-isolation as total bone marrow cells (BMC) or as LSK or Lin$^-$ cells following 24 h culture in Stemspan (Stem Cell Technologies), with 250 kVp X rays at a dose rate of 0.5 Gy/min, 13 Amp (AGO, Reading UK).

Mice were whole body irradiated with 250 kVp X rays at a dose rate of 0.887 Gy/min, 11 Amp (MRC Radiation and Genome Stability Unit, Harwell, Oxon UK) at 3 Gy for harvest of *in vivo* irradiated donor cells, or 8.5 Gy to ablate host animals for CFU-S12 assay.

2.3. Immunomagnetic cell separation and cell sorting

To obtain Lin$^-$ or Lin$^-$ Sca-1$^+$ c-Kit$^+$ (LSK) cells, BMC were flushed from femora and tibias of 8 donor mice either exposed to 3 Gy X-rays 7–9 days beforehand or from unirradiated controls. Lin$^-$ cells were selected using the Mouse Hematopoietic Progenitor Enrichment Kit (Stem Cell Technologies, Grenoble) according to the manufacturer's instruction. Lin$^-$ cells were further sorted into LSK by staining with the following antibodies: c-Kit-PE/Allophycocyanin (APC) (BD Bioscience, Oxford) and Sca-1-PE (Biolegend, CA, USA). Unstained and single stained samples were included as controls. Samples were incubated in PBS/3% FBS for 45–60 min on ice. Following 2× washes in PBS/1%FBS 7-aminoactinomycin D (7-AAD) was added (0.25 μg/sample) to eliminate dead cells on sorting (MoFlo cell sorter (Dako Cytomation, Denmark) at Jenner Institute, Oxford). Cells were gated as 7-AAD$^-$ Sca-1$^+$ c-Kit$^+$ (Fig. 3B) and sorted into Stemspan Serum-free expansion media (SFEM) media (Stem Cell Technologies) on ice. These cells were used in repopulation assays and suspension cultures.

2.4. Suspension cultures for expansion of Lin$^-$ Sca-1$^+$ c-Kit$^+$ (LSK) or lineage depleted (Lin$^-$) cells

300 (control) and 3000 (irradiated) LSK (Fig. 3A) or Lin$^-$ (Fig. 1) cells were cultured in 35 mm Petri dishes in 2 mL SFEM (Stem Cell technologies) with the addition of recombinant murine stem cell factor 50 ng/mL (rmSCF), 100 ng/mL recombinant human interleukin-11 (rhIL-11) and 100 ng/mL recombinant human Flt3 Ligand (rhFlt3L) (all cytokines from Stem Cell Technologies), 40 μg/mL low density lipoprotein (LDL) (Sigma, UK), 100 U/mL Penicillin (Fisher Scientific, UK) and 100 μg/mL Streptomycin (Fisher Scientific). Growth was monitored by visually checking the cells under an EVOS$_{XL}$ microscope (AMG, USA). If the cell density was higher than 5×10^5 cells/mL, the culture was diluted 1:2 in fresh media. After 7–9 days, cells were harvested and the culture dish rinsed with Iscove's modified Dulbecco's medium (IMDM). A cell scraper was used to gently remove any cells that had adhered to the culture dish.