Radiation leukaemogenesis at Low Doses.

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Myeloid leukaemias feature prominently among the cancers associated with human exposures to ionising radiation. The CBA mouse model of radiation-induced acute myeloid leukaemia (AML) has been used extensively for both quantitative and mechanistic studies. Loss of genetic material from chromosome 2 (chr2) is known to be associated with most radiation-induced AMLs. AML develops in CBA mice exposed to X- or γ-radiation, after a mean latency period of 18 months, with a maximal incidence of approximately 25% at 3Gy. A strong candidate AML-suppressor gene located within the commonly deleted region of chr2 has been identified, *Sfpi1/PU.1*. This gene suffers hemizygous loss and specific point mutation within exon 5, the DNA binding Ets domain in ~ 70% of AMLs. *Sfpi1/PU.1* losses are known to occur within 24 hours of radiation exposure and in linear proportion with dose down to 100mGy (Peng et al, 2009).

In this study we focus on the implications of *Sfpi1/PU.1* loss in a panel of 6 radiation-induced (RI) AML cell lines. 3 of the 6 cell lines are from C3H mice (8016, 8709 and 7926), 2 from CBA mice (MLP3 and SA2) and the remaining (3124) from an F1 (CBA/H x C57BL/6). It is generally assumed that AMLs arise from primitive haematopoietic stem cells (HSC), although there are few studies confirming the lineage of origin of murine RI-AMLs. Cell surface marker analysis of our 6 AML cell lines confirmed that all are positive for a combination of myeloid lineage and HSC markers, suggesting a common myeloid progenitor origin. Sequencing and BAC-FISH experiments identified hemizygosity and a mutation in exon 5 of *Sfpi1/PU.1* in 5 of these cell lines, the 6th, MLP3, having a null status for the gene. In total 3 different types of point mutation are found, conferring 3 different amino-acid changes at Arginine 235, to Serine (cell lines SA2, 3124, 7926), Cysteine (8016) and Histidine (8709).

The functional consequences of the mutation type were examined with respect to gene expression. At the transcript level using the quantitative PCR (Q-PCR) ddCt method and normalising to HPRT, 8016 presents with the highest level of *Sfpi1/PU.1* expression relative to bone marrow, and 8709 with a lower level of expression (relative to bone marrow). When normalised to HPRT alone, dCt, the same expression difference trend is seen and by Student's t-tests indicate that the differences are statistically significant ($p=<0.05$). Cell lines harbouring R235S mutation present with a similar expression level as bone marrow. Thus expression levels appear to depend upon mutation type as opposed to strain of origin. Using immunocytochemistry and Western Blotting techniques protein was detectable in the three R235S cell lines, but not in those with R235C or R235H, or in MLP3. This suggests that the R235S mutation may produce a more stable or abundant protein product. Further analysis by Western blotting highlighted the presence of more than one Sfpi1/PU.1 product in SA2, 3124 and 7926, and in all positive controls samples used (spleen, transfected U2OS cells, THP cells) aside from bone marrow in which only one form was seen. Other groups such as Carey at al (1996) have reported upon the presence of multiple phosphorylated forms of Sfpi1/PU.1 in B cells, and Ford et al (1996) identified a difference in phosphorylation status between lymphoid and myeloid cells.

Bioinformatic approaches were used to analyse the predicted properties of the different mutation types. All three of the mutation types are deleterious, but specifically, the R235S mutation scores very highly as a potential novel phosphorylation site. This may contribute to the difference in levels of protein abundance detected.

We also investigated the effect of *Sfpi1/PU.1* mutation upon a number of its associated and down-stream target genes using Q-PCR. For *Gata-1*, *Gata-2*, *c-jun* and *Flt3* there was generally a reduction

in expression relative to bone marrow. *Gata-1* is massively under-expressed in all cell lines, whereas in 8016 there is a small increase in expression of *c-jun* and *Flt3*. By contrast, *Cdk6* expression was elevated in all cell lines. This gene is upregulated in murine erythroleukaemia cells (Matushansky et al 2003), but no relation between the mutation type and expression level could be informed from our data.

Further studies have revealed that some non-del2 (del2$^-$) AMLs carry internal tandem duplication (ITD) mutations in the *Flt3* gene, similar to those observed in human AML (Finnon et al, submitted). These studies indicate that 3 of 7 AMLs carry an ITD in *Flt3*. This not only provides a link to human AML, but also suggests an alternative pathway for AML development in mice in the absence of chr2 deletions.

In conclusion we postulate that the major AML pathway involves RI loss of *Sfpi1/PU.1* in a primitive myeloid precursor cell, coupled with a later point mutation in the remaining *Sfpi1/PU.1* allele, this disrupts normal *Sfpi1/PU.1* expression, and hence differentiation and proliferation in cells. The type of point mutation has a different cellular effect on expression of *Sfpi1/PU.1* and perhaps other down-stream targets genes and interacting proteins, regardless of strain of origin of mice.

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All animal experiments were carried out according to UK Home Office regulation.