Radiation-induced Chromosome 2 Breakage and the Initiation of Murine Radiation Acute Myeloid Leukaemogenesis

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INTRODUCTION

Although epidemiological studies of radiation-exposed human populations continue to provide important data on radiation oncogenesis it is recognised that a knowledge of oncogenic mechanisms is essential for meaningful extrapolation of epidemiological data to the low dose region and also for the further understanding of the biological factors that influence human sensitivity to cancer induction. In vitro cellular transformation systems that seek to mimic certain aspects of the oncogenic process\textsuperscript{12} will continue to play an important role in fundamental studies but, at present, the majority of these lack the complexity that is inherent in the whole animal and tissue responses that moderate the multistep progression towards malignancy.

Experimental animal studies on mechanisms of oncogenic initiation, whilst technically demanding, are beginning to make substantial progress. Molecular studies on rodent mammary and skin carcinogenesis\textsuperscript{2,3} following exposure to chemical agents have provided compelling evidence that codon-specific point mutations in ras proto-oncogenes are early and possibly initiating events for these neoplasms. There are also data suggesting that ras mutations are involved in early phases of murine radiation lymphomagenesis\textsuperscript{4}.

For such studies to be of greatest value in radiological protection it is important that the model animal neoplasm chosen is histopathologically similar to a human radiogenic cancer and that quantitative aspects of the inductive process have been investigated. In these respects murine models of radiation-myeloid leukaemogenesis\textsuperscript{5,6} provide an excellent starting point for mechanistic studies of radiation-leukaemogenesis.

In recognition of this Hayata and co-workers\textsuperscript{7,8} have generated an extensive cytogenetic data-base on radiation-induced acute myeloid leukaemia (AML) in a number of mouse
strains. The principal finding from these studies was that deletion/rearrangement of one copy of chromosome (ch)2 is a consistent feature of this neoplasm. These observations have been fully confirmed in other laboratories, thus providing strong evidence that specific ch2 events are a crucial element in the leukaemogenic process.

A major uncertainty that remains, however, is the status of these changes in leukaemogenesis, in essence, is ch2 deletion/rearrangement an initiating event induced directly by ionising radiation in haemopoietic target cells or does it arise spontaneously during neoplastic development and thereby make a major contribution to the 'success' of the malignant leukaemic clone? In this report we summarise a large body of cytogenetic data regarding the breakpoint of specific ch2 events in AMLs and X-irradiated marrow cells of the CBA/H mouse and argue that, in AMLs, these are leukaemia-initiating events induced directly by ionising radiation as a consequence of the expression of radiation-sensitive-sites encoded on ch2.

MATERIALS AND METHODS

CBA/H AMLs

AMLs were obtained from co-workers studying quantitative aspects of murine leukaemogenesis by X-rays or α-particles from bone-seeking radionuclides. AMLs were maintained in in vitro passage and leukaemic cells harvested according to published methods. All animal experimentation outlined here was conducted according to the UK Animals (Scientific Procedures) Act 1986.

Haemopoietic repopulation studies

In these studies the clonal repopulation of CBA/H mice by 3 Gy X-irradiated haemopoietic cells was followed by direct karyotypic analysis of proliferating cells in bone-marrow during a 3-21 day post-irradiation period. Three different experimental strategies were employed: a) in vitro 3 Gy-irradiated male donor marrow cells were transplanted into and allowed to repopulate marrow-ablated (10 Gy X-rays) female recipients; b) using a partial body marrow-ablation technique, in vivo 3 Gy-irradiated marrow in the lower body of male mice was allowed to repopulate ablated marrow in the upper body; c) marrow repopulation was studied following a 3 Gy whole body dose to male mice. Each experimental procedure contained matched unirradiated control animals and, overall, these studies involved the karyotypic analysis of 6,300 G-banded metaphases derived from marrow samples of 90 mice. These methods are described in detail elsewhere.

Karyotypic analyses

G-banded karyotypes of leukaemic cells in primary and in vivo passaged AMLs were obtained using both direct metaphase preparation and short term culture methods. In haemopoietic repopulation studies the direct cytogenetic method was used for all marrow samples. The majority of karyotypic analyses were performed using an automated karyotyping system and in all studies G-band patterns were interpreted according to Nesbitt and
RESULTS AND DISCUSSION

Acute myeloid leukaemias

Karyotypes and breakpoints in some of the AMLs studied have been presented elsewhere. Of a total of 53 CBA/H AMLs studied at primary and/or passaged phases, 51 carried ch2 abnormalities similar to those described by Hayata and co-workers. Interstitial and terminal ch2 deletion was the most common event but some AMLs carried incomplete translocations, some of which appeared to be associated with complex marker chromosomes. Loss of material from one ch2 copy, generally in an interstitial region, was evident in all AMLs. Involvement of both copies of ch2 in cytogenetic events was only evident in two AMLs. Significantly, as it now seems, both of these were ch2 → 2 non-reciprocal translocations involving the terminal regions of one of the homologues. The most striking feature of the ch2 deletions and rearrangements in AMLs was the non-random distribution of breakpoints (Fig. 1) with clear evidence of breakpoint clustering in the C2 and F regions of the chromosome. Similar clustering was also evident for ch2 breakpoints scored in AMLs of other mouse strains and appears to be a characteristic of this neoplasm. While trisomies of chs 1, 5, 6 and 15 and monosomy of ch y was present in up to 15% of AMLs, these showed considerable clonal variation; this may be contrasted with the ch2 events which, with one exception, remained stable during in vivo passage.

The cytogenetics of haemopoietic repopulation

An analysis of 3,600 haemopoietic cell metaphases derived from X-irradiation and repopulating CBA/H marrow cells yielded a total of 664 chromosome breakpoints associated with stable cytogenetic events distributed throughout the genome. While there were some differences in overall frequency of events scored in the three experimental regimens a common feature was the unexpectedly high frequency (overall ~30%) of ch2 deletions and exchanges; these were present in all mice analysed. In contrast, an analysis of 2,700 metaphases from unirradiated controls failed to detect any ch2-rearranged cells. It may be concluded therefore that ch2 deletion/rearrangement is a direct consequence of radiation damage to haemopoietic stem/progenitor cells rather than a feature of the rapid cell proliferation that occurs during the repopulation process. In these analyses it was evident that some of the ch2 events scored were probably of clonal origin, i.e. in some animals identical ch2 derivative chromosomes were present in different metaphases from the same tissue sample and, in one case, constituted ~6% of all marrow metaphases. While the scale of the cytogenetic sampling (50–100 metaphases per tissue) was insufficient to fully address the question of clonal contributions, it appears likely that a minority of X-ray induced ch2-rearranged haemopoietic cells show some form of proliferative advantage. This conclusion accords with the results of earlier studies on ch2-rearranged cells repopulating spleen and peripheral blood. It is improbable, however, that the diversity and
The distribution and concordance of chromosome 2 breaks in radiation-induced CBA/H acute myeloid leukaemias and irradiated haemopoietic cells. NSL, nominal significance level of breakpoint clustering according to Monte Carlo analyses. *+, highly significant breakpoint clustering below a 5% false positive level; +, marginally significant breakpoint clustering below a 5% false positive level; (NSL values at other bands were, AMLs 0.17–1.0; haemopoietic cells 0.09–1.0); ▲, position of possible chromosomal fragile site (see text).

**Fig. 1.** The distribution and concordance of chromosome 2 breaks in radiation-induced CBA/H acute myeloid leukaemias and irradiated haemopoietic cells. NSL, nominal significance level of breakpoint clustering according to Monte Carlo analyses. *+, highly significant breakpoint clustering below a 5% false positive level; +, marginally significant breakpoint clustering below a 5% false positive level; (NSL values at other bands were, AMLs 0.17–1.0; haemopoietic cells 0.09–1.0); ▲, position of possible chromosomal fragile site (see text).

The overall frequency of ch2 rearranged cells may be explained solely on the basis of preferential clonal expansion and it may be concluded that ch2 in CBA/H haemopoietic cells expresses radiation-induced damage at an unexpectedly high frequency. This conclusion is further supported by the highly non-random distribution of induced ch2 breaks in irradiated haemopoietic cells (Fig. 1). From these distributions it is evident that damage is preferentially expressed at certain radiation-sensitive sites (RSS) encoded on ch2.

**The specificity of chromosome 2 breakage**

Cytogenetic analysis of ch2 breakage provided a total of 63 events in radiation-induced AMLs and 198 events in irradiated and repopulating haemopoietic cells. Statistical concordance between these two chromosome breakage data sets was sought using an established random number Monte Carlo procedure. These analyses showed that, in haemopoietic cells, induced ch2 breaks formed highly significant clusters centred in the B, C1, C2, F1, F3 and G sub-regions, presumably at sites that are particularly prone to breakage and rearrangement. Statistical concordance between these sites and those in AMLs was, however, unambiguous for only the C2, F1 and F3 sub-regions (Fig. 1). These analyses, presented in detail elsewhere, substantially strengthen the argument that the ch2 deletions/rear-
rangements that characterise murine AML derive from initial radiation damage to ch2 and therefore represent initiating events for this highly radiogenic neoplasm. The lack of concordance for RSS other than C2, F1 and F3 further suggests that initial sites of preferential damage on ch2 do not contribute equally to leukaemogenic initiation, implying perhaps that critical genes for leukaemogenic initiation lie close to or between the C and F regions of the chromosome.

**Evidence on the nature of radiation-sensitive sites**

There is a continuing debate on the significance of specific sites of genomic instability, ie. chromosomal fragile sites (C-FRA) for oncogenesis; C-FRA have been identified on human and rodent chromosomes\(^{17,18,19,20}\) and some human C-FRA appear to be targets for the clastogenic action of DNA damaging agents.\(^{21}\) Fig. 1 includes the position of possible ch2 encoded murine C-FRA\(^{19,20}\) and while it seems that there may be a correspondence at B and F3 between C-FRA and RSS it is important to stress that cytogenetic correspondence alone is insufficient evidence to link C-FRA, RSS and leukaemogenic initiation. These links may however be strengthened by recent discussion on the possible role of interstitial telomere-like repeat sequences in chromosomal fragility.\(^{22}\) These tandem repeat sequences

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**Fig. 2.** The interaction of chromosome 2 radiation-sensitive sites and terminal chromosome regions in radiation-induced CBA/H acute myeloid leukaemias (a) and irradiated haemopoietic cells (b). Acute myeloid leukaemias (left to right): N36, t(2;2) (C2F1;ter) dcl2(C2E5); N383, t(2;2) (F3;ter) with loss. Irradiated haemopoietic cells (left to right): t(2;2) (B;ter); t(2;13) (F3;ter); t(2;15) (C1;ter).

del, deletion; t, translocation; ter, terminal chromosomal regions.
in their normal terminal location on chromosomes play a crucial role in the maintenance of chromosomal integrity during replication.\textsuperscript{23} Under some circumstances however these sequences are highly recombinogenic hence the speculation that, when present at interstitial chromosomal locations, they may act as fragile sites.\textsuperscript{22}

In murine AMLs some ch2 exchanges involved the interaction between RSS and telomeric chromosomal regions (eg. Fig. 2a) but the strongest evidence for such interaction was obtained in the analysis of irradiated and repopulating haemopoietic cells. These revealed a strong affinity between RSS and telomeric regions such that of 64 radiation induced ch2 exchange events, 17 were non-reciprocal and involved the interaction of ch2 RSS and chromosome termini (eg. Fig. 2b). While the distribution of interstitial telomere-like repeat sequences in the mouse genome is not known, a structural relationship between these sequences and RSS now seems plausible.

\textit{Radiation sensitive sites and human leukaemia?}

In a study of sites of preferential breakage of human chromosomes following exposure to DNA damaging agents, including ionising radiation, a correspondence between mutagen-sensitive sites (MSS) and some C-FRA was proposed.\textsuperscript{21} Although statistical concordance was not tested it was noted that breakage sites on the long(q) arm human chromosome 5 might correspond to some of those associated with the 5q\textsuperscript{-} deletions which is characteristic of human myelodysplastic conditions and some acute myeloid leukaemias.\textsuperscript{24,25} The principal significance of this preliminary observation is that 5q\textsuperscript{-} myeloid disorders and neoplasms are believed to be markedly radiogenic.\textsuperscript{26} The possible correspondence between 5q MSS and 5q\textsuperscript{-} deletion breakpoints is further strengthened by high resolution G-bandng studies on some of these myeloid disorders\textsuperscript{27} which show, in contrast to earlier low resolution studies, that breakpoints for the 5q interstitial deletion all cluster at 5q 13.3 and 5q 33.1, cytogenetically close to the MSS noted above. Although extensive human cell studies would be necessary in order to provide equivalent statistical power, these data provide an intriguing parallel to the murine cytogenetic data outlined here and raise the possibility that certain sites of preferential chromosome breakage and recombination play a role in human radiation-leukaemogenesis.

\textit{Mechanisms of radiation myeloid leukaemogenesis}

The data discussed here point towards an inductive mechanism for murine AML that is strongly influenced by the expression of radiation sensitive sites on ch2. Initially, the site-specific breakage in the F region of AMLs together with interleukin (IL)-1\beta gene mapping\textsuperscript{28} and expression\textsuperscript{29} studies suggested that this haemopoietic cytokine gene might be specifically deregulated as a consequence of closely linked ch2 rearrangement or deletion. Recent molecular studies\textsuperscript{30,31} now show this to be unlikely and, since site-specific ch2 breakage appears to be determined by RSS, the weight of evidence now tends to favour an AML initiating process involving gene losses from ch2.\textsuperscript{32,33}

The mechanisms through which gene loss or inactivation may initiate neoplastic change is an important problem in cancer genetics. Loss of one copy of a pair of autosomal
recessive 'suppressor type' genes would not obviously provide the cellular proliferative stimulus expected of an initiating event; equally, sequential loss of both genes from a single cell might, under most circumstances, be considered to be of too low a probability. A possible solution to this dilemma has been provided by recent observations on the influence of genomic imprinting\(^\text{34,35}\) on suppressor gene losses in some human tumours.\(^\text{36}\) These molecular data imply that epigenetic, imprinting-like processes are differentially affecting either mutability\(^\text{37}\) or expression\(^\text{36}\) of the two parentally derived suppressor gene copies. According to the latter hypothesis, initial mutation of the more active paternal suppressor gene copy results in a depression of gene product availability in the cell to an extent that allows inappropriate clonal proliferation. Such partially deregulated clonal expansion may then provide a mechanism whereby a pre-neoplastic cell population size is reached within which spontaneous loss of the second less active maternal gene copy may have a relatively high probability of occurrence. Loss of this second gene may then be viewed as further increasing the probability of malignant conversion.

Given the apparent involvement of ch2 deletions in murine radiation myeloid leukaemogenesis we are now investigating the possible role of genomic imprinting in the induction of this neoplasm. Preliminary evidence is consistent with preferential expression of radiation-induced ch2 RSS on one of the two autosomal copies of this chromosome in all of the 28 CBA/H mice so far examined.\(^\text{13}\) This observation provides evidence, albeit incomplete, of the direct or indirect influence of genomic imprinting on the expression of RSS and hence on leukaemogenic initiation. Extensive cytogenetic and molecular studies will however be necessary to confirm this tentative conclusion which, if correct, may have substantial implications for the cellular and molecular mechanisms that influence mammalian sensitivity to radiation leukaemogenesis.

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