The *MLL–AF9* gene fusion in mice controls myeloproliferation and specifies acute myeloid leukaemogenesis


MRC Laboratory of Molecular Biology, Division of Protein and Nucleic Acid Chemistry, Hills Road, Cambridge, CB2 2QH, UK

1Present address: Centro Regional de Hemodonacion, Servicio de Hematologia, Hospital Clinico Universitario, Ronda de Garay s/n, Murcia 30003, Spain

2Present address: Centre for Genome Research, University of Edinburgh, King’s Buildings, West Mains Road, Edinburgh EH9 3JQ, UK

3Corresponding author

The *MLL* gene from human chromosome 11q23 is involved in >30 different chromosomal translocations resulting in a plethora of different *MLL* fusion proteins. Each of these tends to associate with a specific leukaemia type, for example, *MLL–AF9* is found mainly in acute myeloid leukaemia. We have studied the role of the *MLL–AF9* gene fusion made in mouse embryonic stem cells by an homologous recombination knock-in. Acute leukaemias developed in heterozygous mice carrying this fusion as well as in chimeric mice. As with human chromosomal translocation t(9;11), the majority of cases were acute myeloid leukaemias (AMLs) involving immature myeloblasts, but a minority were acute lymphoblastic leukaemia. The AMLs were preceded by effects on haematopoietic differentiation involving a myeloproliferation resulting in accumulation of Mac-1/Gr-1 double-positive mature myeloid cells in bone marrow as early as 6 days after birth. Therefore, non-malignant expansion of myeloid precursors is the first stage of *MLL–AF9*-mediated leukaemia followed by accumulation of malignant cells in bone marrow and other tissues. Thus, the late onset of overt tumours suggests that secondary tumorigenic mutations are necessary for malignancy associated with *MLL–AF9* gene fusion and that myeloproliferation provides the pool of cells in which such events can occur. **Keywords:** ALL-1/chromosomal translocations/haematopoiesis/HRX/leukaemia

**Introduction**

Chromosomal translocations are present in many tumours and are involved in the development of the tumours which carry them (Rabbitts, 1994; Look, 1997). The cloning of the genes involved in the chromosomal translocation breakpoints in leukaemias and solid tumours of mesenchymal origin, have shown that a major molecular consequence of these aberrant chromosomes is creation of fusion genes, and therefore of fusion proteins, when the breaks occur between exons of genes on each chromosome. This was first recognized for the fusion of *BCR* and *ABL* genes resulting from the Philadelphia chromosome (Nowell and Hungerford, 1960; Rowley, 1973; de Klein et al., 1982; Bartram et al., 1983; Groffen et al., 1984). Molecular studies of these chromosomal breakpoints demonstrated that two forms of the *BCR–ABL* fusion gene result from distinct chromosomal breakage within the *BCR* gene, linking it to the same coding part of the *ABL* gene (Bartram et al., 1983; de Klein et al., 1986). More recently, complex situations have been described in subsets of leukaemias with breaks in chromosome 11, band q23 within the *MLL/HRX/ALL-1* gene (Ziemin-van der Poel et al., 1991; Djabali et al., 1992; Gu et al., 1992; McCabe et al., 1992; Tkachuk et al., 1992; Domer et al., 1993) and in sarcomas such as those with breaks within the *EWS* and *FUS/TLS* genes (Delattre et al., 1992; Crozat et al., 1993; Rabbitts et al., 1993; Aman et al., 1996; Panagopoulos et al., 1996). Under these circumstances, the respective genes become involved with a multitude of different chromosomal translocations which are predominantly associated with a particular sub-type of leukaemia or sarcoma. Furthermore, the *FUS* gene has a role in translocations in both sarcomas and in acute myeloid leukaemia (Ichikawa et al., 1994; Panagopoulos et al., 1994) depending on the cell type or on the fusion partner. These observations raise issues about possible roles of the fusion genes in tumour type, particularly the role the fusion partner might play in the specificity of the tumour phenotype.

The chromosome 11 region q23 is involved in ~10% of acute myeloid leukaemias (AMLs) and acute lymphoblastic leukaemias (ALLs), as well as in mixed lineage leukaemias and in some lymphomas. In addition, balanced chromosome 11q23 translocations are also observed in therapy-related leukaemias, especially in patients previously treated with inhibitors of topoisomerase II (Ratain et al., 1987; Pui et al., 1989; Cimino et al., 1997; Nasr et al., 1997; Rowley et al., 1997; Sobulo et al., 1997; Atlas et al., 1998; Felix, 1998). The cloning of the chromosome 11q23 breakpoint region (Ziemin-van der Poel et al., 1991) revealed the *MLL/HRX/ALL-1* gene (Djabali et al., 1992; Gu et al., 1992; McCabe et al., 1992; Tkachuk et al., 1992; Domer et al., 1993) (herein designated the *MLL* gene), which encodes a 432 kDa protein with several regions of structural homology to other proteins, for instance to *Drosophila* trithorax (Mazo et al., 1990; Djabali et al., 1992; Gu et al., 1992; Tkachuk et al., 1992). More than 30 different chromosomal bands have been found in chromosomal translocations with chromosome 11q23, and molecular studies showed rearrangements with the *MLL* gene in all cases (reviewed in Rowley, 1993; Thirman et al., 1993; Bernard and Berger, 1995; Rubnitz et al., 1996b; Gilliland, 1998). Among the most common reciprocal translocations are...
t(4;11)(q21;q23), t(9;11)(p22;q23) and t(11;19)(p13;q23). These fuse MLL with AF4/FEL, AF9/LTG9 and ENL/LTG19 genes, respectively (Gu et al., 1992; Tkachuk et al., 1992; Chen et al., 1993; Iida et al., 1993; Morrissey et al., 1993; Nakamura et al., 1993; Yamamoto et al., 1993). There is a strong correlation between the leukaemia phenotype and each of these specific MLL translocation fusions (Corral et al., 1993; Thirman et al., 1993). The translocation t(9;11)(p22;q23) is mainly associated with AMLs (Iida et al., 1993; Nakamura et al., 1993) while the translocation t(4;11)(q21;q23) is found predominantly in ALLs (Gu et al., 1992; Domer et al., 1993; Iida et al., 1993; Morrissey et al., 1993; Nakamura et al., 1993; Downing et al., 1994). The t(11;19)(p13;q23) translocation on the other hand occurs in both ALL and AML but with higher frequency in ALL (Nakamura et al., 1993; Yamamoto et al., 1993; Rubnitz et al., 1996a). These findings suggest that the incoming fusion partner of MLL helps to specify the cell type of the tumour. Thus, the fusion protein itself may influence tumour type if the chromosomal translocation occurs in a pluripotent precursor cell. It is nonetheless possible that the chromosomal translocation may occur in a committed precursor because the chromosomal region is accessible for the chromosomal translocation event, in which case the protein fusion would not strictly specify tumour type. Several biological models have been established to gain insights into the role of the MLL fusions in tumorigenesis. The use of retroviruses encoding the MLL/HRX–ENL fusion to infect primitive cells showed that myeloid cell proliferation could be observed and that tumours of this lineage emerged (Lavau et al., 1997; Slany et al., 1997). These experiments suggest that a gain-of-function mechanism at least partly explains the role of this fusion protein which is made after the chromosomal translocation t(11;19) in humans. The fusion of the AF9 gene with the Mll gene also resulted in the emergence of AML in mice (Corral et al., 1996).

In the present study we show that an Mll–AF9 gene fusion [made in embryonic stem (ES) cells by homologous recombination] carried in the mouse germline contributes to AML. In both chimeras and heterozygous mice, the majority of mice developed AML and a small percentage developed ALL. The features of this disease reflect those of the disease which develops in humans carrying the MLL–AF9 translocation. Furthermore, a selective proliferation of Gr-1-positive myeloid bone marrow cells was observed in heterozygous animals before the symptoms of leukaemia occurred. The propensity for myeloid tumour formation therefore seems to be a consequence of the advantageous growth of myeloid precursors caused by the Mll–AF9 fusion, thereby providing a pool of cells in which secondary mutations, necessary for overt tumour development, can occur.

**Results**

**Mll–AF9 mice develop acute leukaemia**

An Mll–AF9 fusion gene was created in mouse ES cells, by knock-in homologous recombination (Corral et al., 1996), in which an AF9 cDNA was fused into exon 8 of one allele of the endogenous Mll gene (a diagram of the targeted allele is shown in Figure 1A). These ES cells were injected into C57Bl/6 blastocysts and chimeric mice were produced which developed haematopoietic tumours, mainly AML, after 6 months (Corral et al., 1996). Germ-line transmission of this Mll–AF9 fusion gene was obtained by crossing chimeras with wild-type females. Heterozygotes obtained from these crosses were identified by filter hybridization of somatic DNA using an internal probe from the targeting region (1.5RXT2 probe, Figure 1A). The probe detects a 10 kb germ-line band and a 13 kb band corresponding to the homologous recombination events [1.5RXT2 (Corral et al., 1996)] is indicated. (B) Filter hybridization of tail biopsy DNA. Lanes a–j show hybridization of DNA extracted from a litter of Mll–AF9 mice produced by crossing a male chimera with a wild-type mouse. The DNA was digested with BglII and hybridized with the EcoRI-Hoxl fragment from clone p1.5RXT2. Two heterozygous carrier mice were present. 129 DNA corresponds to a 129 mouse liver and B1-89 represents DNA from the original Mll–AF9 targeted ES clone. Germline and targeted alleles are indicated.

Our previous report on the Mll–AF9 fusion gene expressed in chimeric mice showed that most animals succumbed to acute leukaemia within one year (Corral et al., 1996). Whilst many of these were diagnosed as myeloid, a complete analysis was not performed in all cases. A detailed investigation of the leukaemias in Mll–AF9 heterozygous and chimeric mice has now been undertaken to define more precisely the type of malignancy and to address issues relating to the mode of onset of overt malignancy. Cohorts of Mll–AF9 mice were compared with mice which had been made with an epitope tag fused (this line of mice has been designated Mll–myc; Corral et al., 1996) at the same Mll exon 8 position at...
Fig. 2. Acute leukaemia occurrence in Mll–AF9 mice. The rate of leukaemia occurrence (age in months of detection of disease versus number of mice with disease) in Mll–AF9 chimeric and heterozygous mice. Diagnosis of leukaemia was obtained by histological analysis of bone marrow, spleen, thymus and liver, and in most cases by FACS analysis of cell surface marker expression. AML developed in 21 out of 28 heterozygous Mll–AF9 mice (circles), in 22 out of 24 Mll–AF9 chimeras (squares) and ALL developed in two out of 24 Mll–AF9 chimeras (diamonds). No Mll–myc heterozygous mice developed a malignancy in the 18 month period of this experiment (four tumours were detected in the cohort of 27 Mll–myc mice but these occurred at 28, 29, 32 and 32 months).

which the AF9 sequences were fused. These groups were analysed over a period of 18 months, and during this time both the Mll–AF9 chimeras and heterozygotes began to show signs of distress. No disease was observed in Mll–myc mice within the 18 month experimental period (there were 27 Mll–myc mice in the control group; four developed AML tumours but these occurred after two years suggesting that they were of sporadic origin rather than due to the manipulation of the Mll gene).

Post-mortem examination of Mll–AF9 mice showed consistent evidence of haematological disease. Signs included pale femurs, splenomegaly, heptomegaly and pale kidneys. All the mice analysed were diagnosed as having acute leukaemias and no tumours of other tissues were observed, despite widespread activity of the Mll promotor (Yu et al., 1995; Corral et al., 1996). The development of these acute leukaemias is shown in Figure 2. The disease was classified as predominantly AML with rare examples of ALL, mirroring the spectrum and ratio of acute leukaemias found in humans with the chromosomal translocation t(9;11). The rate of development of AML was overall slightly faster in heterozygous animals compared with the chimeras (Figure 2), the point at which 50% of each cohort had succumbed to AML being 5 and 7 months for heterozygotes and chimeras, respectively. Twenty-two of the 24 chimeras and 21 of the 28 heterozygous Mll–AF9 mice developed AML in the 18 month period. Two Mll–AF9 chimeras developed ALL rather than AML.

Acute myeloid and lymphoblastic leukaemia in Mll–AF9 mice

Almost all Mll–AF9 heterozygous and chimeric mice developed malignancy within one and a half years. Detailed tissue histology was carried out on mice as pathological signs of disease developed, to classify the types of leukaemia present and to establish a diagnosis of disease. This analysis confirmed the presence of acute leukaemias in all the mice with symptoms and showed the occurrence of either myeloid or lymphoid malignancies. Marked infiltration of leukaemic cells was seen in the bone marrow, peripheral blood and liver of Mll–AF9 mice. Figure 3 shows a comparison of the histology of tissues from an Mll–myc mouse (Figure 3A) with those from Mll–AF9 mice. The AML was observed in two different forms, overt myeloid leukaemia and extramedullary leukaemia. Mice exhibiting overt myeloid leukaemia were distinguished by >30% of nucleated cells in the bone marrow being blasts. Heavy infiltration of the peripheral blood with myeloblasts was observed in these mice (either chimeras or heterozygotes). The myeloblasts were characterized by their large, granular appearance and were frequently observed in the liver (Figure 3B) and in the kidney (Figure 3C). The extensive extramedullary infiltration of myeloblasts was found in the liver, with peri-vascular deposits of malignant cells. In the example shown (histology from a chimeric mouse), the bone marrow shows significantly <30% infiltration with myeloblasts. Similarly, few abnormal cells were observed in the peripheral blood in the extramedullary stage of the malignancy.

Detailed analysis of tissue histology in the Mll–AF9 mice (one example is shown in Figure 3E) indicated that two ALLs arose. In these mice the bone marrow samples were hypercellular, with normal haematopoiesis suppressed, and >90% of the mononuclear cells were lymphoblasts with many active mitoses seen. An enlarged thymus was evident in these mice in addition to a pale femur, enlarged, pale spleen and pale kidneys. Extra-medullary haematopoiesis was evident in the liver which showed the characteristic peri-vascular accumulation of leukaemic cells. The circulating blood of the mouse shown is packed with lymphoblasts. In addition, the peripheral blood film indicates that this mouse was also suffering from anaemia. Immunoglobulin gene rearrangement analysis showed that both of the ALLs were of B cell origin. Filter hybridization was carried out with an immunoglobulin heavy chain probe and spleen cell DNA of the two ALL-bearing mice compared with a myeloma cell line (J558) and a T cell line (BW) and kidney DNA from C57Bl/6 and 129 mice (Figure 4A). The probe detects a 6.5 kb EcoRI germ-line band in the kidney DNA samples and the BW (T cell) DNA, but detects two rearranged bands in the myeloma (B cell) DNA representing heavy-chain gene rearrangements. DNA from both of the Mll–AF9 tumours similarly had two distinct rearranged bands together with faint germ-line bands, indicative of clonal B cell tumours in these mice. Rearrangement of T cell receptor β-chain genes was also investigated with Jβ2 and Cβ1 probes, but no rearrangements were found in the tumour DNA from either ALL mouse (data not shown). FACS analysis of bone marrow cells from mouse 1
Mll–AF9 fusion causes myeloproliferation

Fig. 3. Histology of leukaemias arising in Mll–AF9 mice. Histology was carried out on tissues from all mice with symptoms of disease. Tissues were dissected and fixed in 10% formalin. Sections were prepared from wax-embedded specimens and stained with haematoxylin and eosin (H&E). Blood films were stained with May–Grünwald–Giemsa (MGG). Representative examples of histology are shown. (A) Mll–myc mouse 2021 (age 24 months): this mouse had no symptoms of disease and serves as a control. (B) Mll–AF9 heterozygous mouse 4134 (age 6 months) with overt AML. In this example, the bone marrow is extensively infiltrated with myeloblasts and the liver has significant deposits. The peripheral blood has many circulating myeloblasts. (C) Mll–AF9 chimera 4039 (age 7 months) with overt AML similar to heterozygous mouse 4134. (D) Mll–AF9 chimera 4019 (age 12 months) showing signs of extramedullary leukaemia with myeloblast deposits in the liver but normal bone marrow pattern. (E) Mll–AF9 chimera 4005 (age 12 months). This mouse has ALL, with lymphoblasts in the bone marrow and peripheral blood. The liver has significant lymphoblast infiltration.
confirmed that the tumour was comprised of predominantly B220 antigen-expressing B lymphocytes, which are usually found in low numbers in the bone marrow. In contrast, the normal constituents of the bone marrow (Figure 4B) such as Gr-1- or CD4-positive cells are depleted compared with wild-type mouse controls. A similar pattern of surface marker expression was observed with bone marrow for ALL mouse 2 (data not shown). No evidence of mixed lineage tumours was obtained. Glucose phosphate isomerase (GPI) analysis (Papaioannou and Johnson, 1993) was performed on spleen samples from the two mice with ALL to estimate the contribution originating from the CCB-derived ES cells, injected into the C57Bl/6 blastocysts. Both mice had significant CCB ES cell contribution in the spleen (data not shown) suggesting that the lymphoblastic tumours are of ES cell origin.

Myeloproliferation is observed in Mll–AF9 mice prior to leukaemia occurrence

The normal function of Mll appears to be related to the embryonic developmental plan by affecting Hox gene expression profiles (Yu et al., 1995) and it may also have a specific role in some aspects of haematopoiesis (Fidanza et al., 1996; Hess et al., 1997). One of several possible roles for the tumour-specific MLL–AF9 fusion protein is an influence on the molecular interactions which are important for haematopoietic differentiation, which could thus partly explain the predominant association of the chromosomal translocation t(9;11) with myeloid malignancies. A corollary of this is that the lineage from which the majority of tumours arise (i.e. the myeloid lineage) is selectively increased in the heterozygous Mll–AF9 mice. In order to assess this situation, we first examined the surface antigen phenotype of the myeloid tumours. Antibodies binding to surface proteins of myeloid cells (Mac-1 and Gr-1), B-lymphocytes (B220) and T lymphocytes (CD3 and CD4) were used to detect antigen expression on spleen and bone marrow cells. On the whole, tumour cells were found in bone marrow and in spleen, and in mice with AML malignant cells typically expressed Mac-1 and Gr-1 surface markers. Figure 5 shows FACS analysis of the bone marrow and spleen cells from a typical Mll–AF9 heterozygous mouse (mouse number 4299) with AML. The infiltration of Gr-1-positive tumour cells into the spleen population is very marked with the concomitant loss of B220+ B cells and CD4+ T cells, compared with splenocytes from a wild-type mouse. In the bone marrow there was also a striking difference between AML-bearing mice and wild-type mice. In the latter, there are usually ~50% Gr-1+ cells exhibiting a narrow fluorescence profile corresponding to mature myeloid cells, the fluorescence profile of the Mll–AF9 mouse had a broad appearance consistent with there being mainly immature myeloid cells (Figure 5). A small population of B220+ bone marrow cells found in the wild-type mouse was absent from the Mll–AF9 mouse.

The examination of the Gr-1+/Mac-1+ population in young heterozygous Mll–AF9 mice was used as a basis for determining whether a pre-leukaemic effect on myeloid cell differentiation resulted from the presence of the fusion gene. FACS analysis was carried out on young Mll–AF9 mice using Gr-1 and Mac-1 (myeloid markers), Ter119 (erythrocyte marker), B220 (B cell marker), CD4 (T cell marker), Thy 1.2 (T-lymphocytes, monocytes, B-lymphocytes), Sca-1 (multipotent haematopoietic stem cells, mature myeloid cells), CD44 (leukocytes, erythrocytes, epithelia) and c-Kit (haematopoietic progenitor cells and mast cells). Only effects on the myeloid markers were detectable. Bone marrow from Mll–AF9 heterozygous mice of either 6 days or 5 weeks of age were analysed and compared with either wild-type or Mll–myc mice of similar age (Figure 6). While there was a modest increase in Mac-1/Gr-1 double-positive cells in the Mll–AF9 bone marrow compared with the two controls at 6 days of age...
Mll–AF9 fusion causes myeloproliferation

**Fig. 5.** Surface phenotype of AMLs in Mll–AF9 mice. Mll–AF9 mice with signs of disease were sacrificed and single cell suspensions were made from the spleen and bone marrow. Cells were stained with fluorescent antibodies recognizing the myeloid marker, Gr-1, the T cell markers, CD3 and CD4, and the B cell marker, B220. Twelve out of 24 Mll–AF9 chimeras and 15 out of 28 Mll–AF9 heterozygotes were analysed. In the representative example shown, the Mll–AF9 heterozygous mouse 4299 developed AML (age 4 months) and FACS analysis was performed and compared with a wild-type mouse of comparable age. Graphs represent cell number (y-axis) versus fluorescence intensity (x-axis).

(35% compared with 21 and 15% for wild-type and Mll–myc mice, respectively), a much larger population (~85%) was observed in the Mll–AF9 mice at 5 weeks compared with either the wild-type (48%) or the Mll–myc (54%) mice. The Gr-1+ cells in the Mll–AF9 heterozygotes seem to represent a true increase in myeloid lineage cells, rather than overtly leukaemic cells. This is indicated by the narrow fluorescence profile which is similar to those of the control mice (wild type or Mll–myc; Figure 6), as compared with the broad profile found with AML bone marrow.

These data indicate that an early proliferation of cells in the myeloid lineage, driven by the Mll–AF9 fusion protein, occurs prior to the development of malignancy. The increase in proportion of Mac-1/Gr-1 double-positive cells in Mll–AF9 heterozygous mice at 6 days of age compared with wild-type or heterozygous Mll–myc mice was verified using cells obtained from 6 day pups from litters obtained by mating heterozygous Mll–myc or heterozygous Mll–AF9 mice. The bone marrow cells were analysed by FACS and the percentage of Mac-1/Gr-1 double-positive cells determined (Figure 7A). In these litters, there were four Mll–AF9 heterozygous mice, three of which had high Mac-1/Gr-1 double-positive cell counts compared with the +/− Mll–myc or +/+ litter mates. Thus, a high Mac-1/Gr-1 double-positive count is predictive of Mll–AF9 heterozygosity soon after birth. As the number of Mac-1/Gr-1 bone marrow cells naturally increases as mice mature, we examined groups of littermates at between 5 and 12 weeks of age in which environmental disturbances should be less significant. In this analysis, we examined bone marrow cells from seven Mll–AF9 +/− mice, eight wild-type +/− mice (Figure 7B) and 14 Mll–myc +/− mice (Figure 7C). Of the seven Mll–AF9 heterozygous mice analysed, the proportion of Mac-1/Gr-1 double-positive cells in the bone marrow ranged from 71–93%, with an average of 82%. These figures are consistently higher than those we observed for wild-type mice (Figure 7B), in which the average number of Mac-1/Gr-1 double-positive cells is 52%; one mouse had 77% double-positive bone marrow cells but this was exceptional. Furthermore, the 14 Mll–myc heterozygous mice had an average of 50% Mac-1/Gr-1 double-positive cells, ranging from 43–60%. The standard errors from these cohorts of mice were calculated at 3, 4 and 1.2% for Mll–AF9, +/− wild-type and Mll–myc mice, respectively, indicating a significant proliferative advantage for the myeloid cell compartment in the bone marrow of the Mll–AF9 mice. Only the
Fig. 6. Young Mll–AF9 mice exhibit increased myeloproliferation. Mll–AF9 heterozygous mice were analysed at 6 days or 5 weeks of age to determine the status of haematopoietic differentiation, prior to the development of overt leukaemia. Mice were selected from litters of Mll–AF9, Mll–myc and wild-type mice at 6 days or 5 weeks of age and single cell suspensions were made from the bone marrow. Cells were stained with an anti-Mac-1 antibody coupled with phycoerythrin (PE) together with an anti-Gr-1 antibody coupled with fluorescein isothiocyanate (FITC) to detect the myeloid population of cells. (A) Populations of bone marrow cells expressing both Mac-1 and Gr-1 markers are depicted. Percentage values are shown for the upper right-hand quadrant of Gr-1/Mac-1 double-positive cells. (B) Fluorescence profiles of the same bone marrow cell populations for anti-Gr-1 antibody binding. The peaks of fluorescence intensity for wild-type and Mll–AF9 mouse bone marrow cells are similar indicating these are not transformed myeloid cells (compared with the AML bone marrow shown in Figure 5) at the stage of analysis, but rather there is an increase in myeloid cell compartment size.

The presence of the Mll–AF9 gene fusion was capable of eliciting this change, and not the truncation of the Mll gene by addition of the myc epitope. Thus, one function of the Mll–AF9 fusion gene appears to be to confer a proliferative advantage for cells of the myeloid lineage, prior to the onset of AML.
Discussion

In vivo fusion of Mll and AF9 in mice provides a model for human cancer caused by chromosome 11q23 translocations

In vivo models of cancer development occurring after chromosomal translocations are needed for a comprehensive understanding of the biological consequences of these aberrant chromosomes and as a means to test possible therapies. The propensity of the MLL gene to form fusion genes after chromosomal translocations is important because >15% of all acute leukaemias in man have MLL gene fusions and because there is an association of particular MLL fusions with particular subtypes of acute leukaemia. For instance, MLL–AF4/FEL fusions are typically found in lymphoblastic tumours, whereas MLL–AF9 fusions are typically found in myeloid tumours (Djabali et al., 1992; Gu et al., 1992; Domer et al., 1993; Iida et al., 1993; Morrissey et al., 1993; Nakamura et al., 1993). This implies a specific role for the chimeric protein in determination of the type of leukaemia which develops. Nonetheless, an alternative possibility is based purely on mechanism and accessibility of genes in chromosomes. Thus the ability of MLL and AF9 or MLL and AF4 genomic regions to take part in an inter-chromosomal translocation may be greater in committed myeloid or lymphoid cells, respectively. The alternative models can be assessed using animal models of disease in which gene fusion is either controlled by specific interchromosomal events (Smith et al., 1995; van Deursen et al., 1995) or by knock-in of the fusion gene partner (Thomas and Capecchi, 1987; Corral et al., 1996). We have developed and analysed a model for MLL–AF9-induced leukaemia using homologous recombination to knock-in the AF9 into the endogenous mouse Mll gene (Corral et al., 1996). In this model system, we find that the fusion causes tumours in both chimeric mice and mice heterozygous for the Mll–AF9 fusion gene. The majority of tumours observed in these mice were myeloid as has been reported in humans with chromosomal translocation t(9;11). Two acute lymphoblastic tumours also arise reflecting the relative occurrence of AML:ALL in humans. In the knock-in mice, the ALL cases occurred with a longer latency than the acute myeloid tumours.

The pathological features of the haematopoietic malignancies in the Mll–AF9 mice were very similar to those of human MLL–AF9 associated tumours, providing a striking counterpart between the pathology of the murine and human leukaemias associated with the t(9;11). In man, the most common French-American-British (FAB) subtypes found in this context are AML, M5 (monoblastic/monocytic), M4 (myelomonocytic), and occasionally M1 or M2 (Bain, 1993). Comparable morphologies were evident in the mice used in the investigation. The pattern of organ infiltration described in the mice has close similarities to the clinical findings in man. Thus, enlargement of the superficial lymph nodes, liver and spleen is common in human AML, with 90% of cases showing infiltration of the liver and spleen at autopsy. Extramedullary infiltration of leukaemic myeloblasts is most common in monocytic or myelomonocytic leukaemia. Although usually diffuse, disease may occur as tumorous deposits (granulocytic sarcoma). This may be the initial manifesta-
function to specify the lineage of the tumours, which explains why there is such a strong association between these fusions and lymphoblastic tumours (Gu et al., 1992; Domer et al., 1993; Morrissey et al., 1993; Nakamura et al., 1993; Yamamoto et al., 1993; Downing et al., 1994; Rubnitz et al., 1996a). In the case of MLL–ENL fusions, retroviral transduction experiments with mouse bone marrow cells have clearly shown that a gain-of-function mechanism is operational (Lavau et al., 1997; Slany et al., 1997) and that myeloid differentiation and myeloid transformation are paramount. Therefore, the spectrum of mutations in other genes, which must occur in cells with chromosomal translocations involving the MLL gene, may well differ in the context of tumour types with the different MLL fusions. The nature of these mutations, the pattern of perturbation of gene expression and the biological consequences of AF4 fusion with MLL are now our major objectives.

Materials and methods

Construction of the targeting vectors and analysis of targeted ES cells

The plasmid constructs for gene targeting have been described (Corral et al., 1996). MLL–AF9 comprises a knock-in of the AF9 short form (Iida et al., 1993; Nakamura et al., 1993) into exon 8 of the mouse Mll gene whilst Mll–myc comprises a knock-in of a short epitope tag into the same location in exon 8. CCB ES cells were transfected with the MLL–AF9 targeting vector DNA as described previously (Warren et al., 1994). Homologous recombination was assessed by filter hybridization of RbII digested DNA using the EcoRI–Xhol fragment from clone p1.5XRT2 as a probe. The presence of a single insertion site was confirmed with a neonucin probe. Targeted ES cell clones were injected into C57Bl/6 blastocysts, which were transferred into recipient females. Chimeric mice thereby generated were estimated by coat colour. Heterozygous mice were obtained for both Mll–AF9 and Mll-myc fusion genes by crossing chimeric males with wild-type MF1 females.

Mouse pathology

Leukaemia development in mice was characterized by indolent habit, hunched gait and ruffled hair. On developing sickness, mice were culled and a post-mortem carried out. Tissues were taken for analysis and were fixed in 10% formalin and embedded in paraffin wax. Blocks were sectioned and slides were processed and stained with haematoxylin and eosin (H&E) by routine techniques. Blood films were stained with May–Giemsa–Grünewald (MGG) stain. Slides were photographed using Kodak Tungsten 160 film. Glucose phosphate isomerase (GPI) isozyme analysis was performed on spleen samples as described previously (Papaiouannou and Johnson, 1993).

Immunoglobulin gene rearrangement assays

DNA was prepared from various tissues using standard procedures. The DNA was digested with HindIII, and fractionated on 0.8% agarose gels, transferred to nylon membranes (Southern, 1975) and hybridized with randomly labelled probe (Feinberg and Vogelstein, 1983). An immunoglobulin heavy chain enhancer probe originating from the intron between JH and Cγ gene segments (Neuberger and Williams, 1986) was used. For T cell rearrangement, DNA was digested with EcoRI or HindIII and filter hybridizations carried out with probes for either Jβ2 or Cγ1 (Malissen et al., 1984).

Flow-cytometric analysis of cell surface marker expression

Single-cell suspensions were prepared from mouse thymus, spleen and bone marrow. Cells (100 μl; 5 × 10^6/ml) were incubated at 4°C for 20 min in phosphate-buffered saline (PBS) with 5% fetal calf serum (FCS) with the following antibodies (PharMingen, San Diego, CA): anti-Gr-1 and anti-Mac-1 (granulocytes), anti-B220 (B cells), anti-CD3 and anti-CD4 (T cells), anti-Ter119 (erythrocytes), anti-i–Kit (haematopoietic progenitor cells), anti-Thy 1.2 (T lymphocytes, monocytes, B lymphocytes), anti-CD44 (leukocytes, erythrocytes, epithelia) and anti-Sc-a1 (multipotent haematopoietic stem cells). Antibodies were conjugated with either fluorescein isothiocyanate (FITC), or phycoerythrin (PE).
Isotype-matched fluoresceinated antibodies were used as controls for non-specific immunofluorescence. After antibody labelling, cells were washed once with PBS containing 5% FCS and resuspended in 1 ml of PBS/5% FCS. Flow cytometric analysis was performed using a FACSCALIBUR (Becton Dickinson, San Jose, CA). Data analysis was performed with Cell Quest (Becton Dickinson). Each phenotype analysis was generated by analysis of 10,000 cells.

Acknowledgements

This work was supported by the MRC and partly by a grant from the Kay Kendall Leukemia Research Fund. A.I.W. is the recipient of an MRC Clinician Scientist Fellowship held within the University of Cambridge, Department of Haematology, Cambridge, UK. C.L.D. is the recipient of an MRC Studentship. I.L. was supported by the National Foundation for Cancer Research.

References


MII–AF9 fusion causes myeloproliferation


Received March 24, 1999; revised and accepted April 27, 1999