DISSERTATION

IDENTIFICATION OF PREMALIGNANT TARGET CELLS USING A MOUSE MODEL OF RADIATION-INDUCED ACUTE MYELOID LEUKEMIA

Submitted by

Xiangfei Liu

Graduate Degree Program in Cellular and Molecular Biology

In Partial Fulfillment of the Requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Spring 2010

RC643
.L49
2010

COLORADO STATE UNIVERSITY

10 DECEMBER, 2009

WE HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER OUR SUPERVISION BY XIANGFEI LIU ENTITLED "IDENTIFICATION OF THE PREMALIGNANT TARGER CELL USING A MOUSE MODEL OF RADIATION-INDUCED ACUTE MYELOID LEUKEMIA" BE ACCEPTED AS FULFILLING IN PART REQUIRMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

COMMITTEE ON GRADUATE WORK

MICHAEL M. WEIL

F. ANDREW RAY

JAMES BAMBURG

ANNE C. AVERY

Advisor: ROBERT L. ULLRICH

Department Director: PAUL J. LAYBOURN

COLORADO STATE UNIV. LIBRARIES

ABSTRACT OF DISSERTATION

IDENTIFICATION OF PREMALIGNANT TARGET CELL USING A MOUSE MODEL OF RADIATION-INDUCED ACUTE MYELOID LEUKEMIA

Acute myeloid leukemia (AML) is believed to arise from the clonal expansion of a malignantly transformed blast cell. Whether spontaneous AML arises from a hematopoietic stem cell (HSC) or a more restricted progenitor of myeloid lineage is an area of active investigation. Much less is known about which cell types give rise to radiation-induced or radiotherapy-related AML. We are exploring this question using a mouse model of radiation-induced AML. Several mouse strains including CBA/CaJ are susceptible to radiation-induced AML whereas other strains are resistant. In murine radiation-induced AML the *PU.1* (*Sfpi1*) gene behaves as a classic tumor suppressor gene. In most murine myeloid leukemias, there is a deletion in chromosome 2 encompassing the *PU.1* gene. The second 'hit' is generally a point mutation in the remaining *PU.1* allele. We used the *PU.1* deletion as an early marker for potential radiation-induced AML, and performed immunophenotyping combined with fluorescent in situ hybridization (immunoFISH) to study the persistence of this AML initiating lesion in myeloid and lymphoid lineages. Bone marrow cells were harvested from CBA/CaJ mice irradiated with 3 Gy of ^{137}Cs γ-rays 1, 3 and 6 months post–radiation and assayed by immunoFISH for *PU.1* deletions and cell differentiation markers. We demonstrated that the frequency of *PU.1* deletions were similar in different cell types 1 month after irradiation but increased in the myeloid lineage and decreased in the lymphoid lineage at

3 and 6 months post-irradiation. *PU.1* deletions were only found in myeloid but not in lymphoid lineages in mice with radiation-induced AML. These results indicate that radiation-induced AML is likely to originate from the more restricted progenitor of the myeloid lineage.

Immunostaining the mouse bone marrow cells with a panel of cell differentiation markers, we were able to isolate phenotypically defined hematopoietic stem cells and progenitor subpopulations. In future experiments, studying cytogenetic events in those particular bone marrow subpopulations will help achieve a better understanding on pathogenesis of radiation-induced AML.

Identifying a small fraction of bone marrow cells bearing point mutations in the remaining *PU.1* gene remained as a technical challenge to our study of radiation-induced AML. We assessed a novel in situ hybridization approach that employs padlock probes as a potential tool to detect a *PU.1* point mutation and found it would only be useful if modifications were made to increase the fidelity of this technology.

Xiangfei Liu
Graduate Degree Program in Cellular and Molecular Biology
Colorado State University
Fort Collins, CO 80523
Spring 2010

Acknowledgements

First I would like to thank my family. My wife and my mom thank you for the constant support for me to pursue a PhD degree in this great country.

I would like express my gratitude to my advisor Dr. Robert Ullrich for giving me the opportunity of pursuing graduate studies at Colorado State University. I am also very grateful for your support and positive attitude throughout the entire project. You have always been the role model to me in the aspects of both individual personality and professional achievement.

To Dr. Michael Weil, I would like to thank you for your brilliant scientific mind and professional attitude making my PhD study and research work to be possible.

To F Andrew Ray, you have been helping me with all things no matter big and small. Your endless help was invaluable and unforgettable to me.

My acknowledgments are also to Dr. James Bamburg and Dr. Anne Avery, my other two committee members. Dr. James Bamburg has always been there giving me advices on data analysis and dissertation writing, his advices were as thorough as down to every small detail. Dr. Anne Avery has taught me with every detail how to do experiments using flow cytometry, her generosity and enthusiasm on scientific research have been affecting me throughout the whole process.

Dr. Yuanlin Peng provided me with the DNA probe for this project and taught me how to master the technique of Fluorescent *In Situ* Hybridization.

Special thanks would be given to Lori Williams, her warm heart and lovely smiles make my study in Cell and Molecular Biology program a wonderful life experience.

I would also like to thank Mary Pridgen, Julia Asmus, and Christine Battaglia. Without your help, I would not be able to get the supply in time, either for daily life or for lab work.

Finally I would like to thank my lab mates and office mates. I will always remember those invaluable time we have been working, studying, and having fun together.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Chapter 1

Introduction

1.1 Mammal Hematopoiesis

1.1.1 Human Hematopoiesis

Hematopoiesis is the formation of the various types of blood cell. In an embryonic mammal, this process may occur in the yolk sac, spleen, liver or lymph nodes, whereas in an adult mammal, this process occurs almost exclusively in the bone marrow. Approximately 10^{11}–10^{12} new blood cells are produced daily in a 70-kg man in order to maintain steady state levels in the peripheral circulation. Furthermore the hematopoietic system is capable of responding to a variety of stresses, i.e. hypoxic environment, blood loss, and infections, by increasing the cell counts of certain lineages when needed.

As one of the most active and important processes in the body, hematopoiesis falls into four-tiers of cell types with hierarchical relationships: stem cells, committed progenitor cells, differentiated and maturing cell lineages, and fully-functional blood cells. (A diagram illustrating the hierarchical relationships among major cell types involved in hematopoiesis is shown in Figure 1.1) All mature blood cells in peripheral circulation have a finite life, with the majority of cells being terminally differentiated and unable to replicate. However, a small number of hematopoietic stem cells (HSC) that reside in the bone marrow are capable of self renewal and extensive proliferation. These cells also have been shown in many studies to be able to differentiate into multiple lineages of blood cells, as well as to reconstitute the hematopoietic system of recipient animals [1]. When the HSCs proliferate, at least some of them remain as HSCs, so the pool of stem cells remains steady; while others may give rise to progenitor cells that are committed to certain lineages such as myeloid and lymphoid lineages. The numbers and characteristics of the HSCs and committed progenitors are still the subject of active

investigation and debate. It has been estimated that HSCs represent about 1:10,000 of cells giving rise to myeloid and lymphoid progenitor cells which comprise 2 to 5 per 1000 bone marrow cells [1]. The myeloid progenitor cells then undergo further differentiation to form erythrocytes, megakaryocytes, monocytes/macrophages, and granulocytes. Lymphoid progenitor cells will give rise to T-cells, B-cells and natural killer (NK) cells.

Hematopoietic differentiation and proliferation is a highly regulated process. More than 25 growth factors, cytokines, and other regulators are involved, acting directly on one or more of the major lineages of blood cells or interacting to affect cell proliferations [2]. Various models have been proposed for the regulation of hematopoiesis in previous studies. Some suggested the differentiation and proliferation of hematopoiesis are driven by external influence such as growth factors and stroma [3, 4], whereas others hypothesized a 'stochastic' model in which this process might be regulated intrinsically in the absence of outside factors [5]. One recent model of hematopoietic differentiation proposed that regardless of whether the environmental or stochastic models are involved, transcription factors are the final common pathways that determine differentiation and proliferation [6]. According to this model, a large number of lineage-specific transcription factors, such as *PU.1*, GATA1, and AML1, are expressed at low levels in HSCs or early progenitor cells. The self replication and lineage commitment of hematopoietic cells are driven by alternative expression and specific combination of these transcription factors as a result of either environmental or 'stochastic' influences.

1.1.2 Murine Hematopoieis

Hematopoiesis in mice is considered to be similar to that of humans. In general, all blood cells from in mice are derived from a small population of HSCs that give rise to myeloid and lymphoid progenitor cells. These progenitor cells then undergo further differentiation to eventually form mature and full-functioned blood cells including T and B lymphocytes, erythrocytes, megakaryocyte, monocytes/macrophages, and granulocytes. Mice have been used for many years as animal models of hematopoiesis and leukemogenesis due to their high similarity to most aspects of hematopoiesis in human [1]. However, a number of differences between hematopoieisis in humans and in mice have been noticed. Compared with humans, mice have smaller and more numerous red cells with shorter half-lives, lower neutrophil counts and higher lymphocyte counts, shorter platelet life span, and more numerous platelets. In addition, more circulating polychromatophils and reticulocytes are found in mouse peripheral blood and high percentage of lymphocytes in bone marrow. With regard to pathological characteristics of nucleated blood cells, a large proportion of mouse neutrophile of mice exhibit a ring shaped nucleus rather than the multilobulated type seen in humans. Furthermore, neutrophils in mice have smaller granules which are difficult to stain. In general, hematopoiesis in adult humans occurs exclusively in bone medullary spaces, with occasionally extramedullary hematopoiesis under the conditions of extreme demand. In contrast, small amounts of hematopoiesis always exist in mouse spleen and even in liver tissue. The responses of hematopoiesis of humans and mice to external or internal stresses also vary, i.e. unlike in the human, lymphocyte counts of mice are commonly increased in inflammatory conditions.

As described above, in spite of their similarity, significant differences exist between human and mouse hematopoiesis. One should keep in mind that although murine models have been widely accepted as the preferable animal model of leukemogenesis, these differences in blood cell composition, number, life span, or microenvironment and organization of the bone marrow compartment are likely to influence the characteristics and pathogenesis of hematopoietic disorders seen in human and mouse systems .

1.2 Radiation-Induced Myeloid Leukemias

1.2.1 Classifiction of Leukemias

The most common neoplastic diseases of the hematopoietic system are the leukemias. Leukemia can be defined as an uncontrolled neoplastic proliferation or expansion of blood cell precursors in bone marrow [7]. Unlike solid tumors, the tumor cells do not form distinct tumor masses but proliferate diffusely in the bone marrow, blood, and in the infiltrated tissues such as liver and spleen. Leukemias may be divided into four main groups on the basis of whether the clinical course of the disease is acute or chronic, and on whether the major cell types involved are myeloid or lymphoid. The two main types of chronic leukemias are chronic lymphocytic leukemia (CLL) and chronic myeloid leukemia (CML). The two main types of acute leukemias are acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML). Within these general classifications, each type of leukemia can be divided into several subtypes involving different molecular mechanisms and etiologies, which represent the heterogeneous nature of leukemic diseases. An outline of major types of human leukemias with their subtypes is shown in Table 1.1. For the most common of these human leukemias, tumors affecting

the same cell type and exhibiting similar characteristics have been identified in mice, facilitating research into the underlying pathogenesis and potential therapeutic methods of these diseases [8]. The myelodysplastic syndromes (MDS) are characterized by ineffective hematopoiesis that affects the myeloid cell lineage, resulting in a variable degree of anemia, granulocytopenia, and/or thrombocytopenia. These syndromes are generally considered as preleukemic state of AML because a major proportion of patients develop AMLs as the diseases progress.

Leukemias resulting from exposure to radiation are a distinct group of leukemias because they appear to have some characteristics different from those typically seen in individuals without a history of radiation exposure [9]. Such differences in characteristics include rapid onset of diseases, poor response to therapy, and specific chromosomal aberration, which may indicate variation in molecular mechanisms and etiologies. For many years, leukemias induced by radiation have been studied extensively in humans as well as in animal models. The information collected from these studies is critical for the assessments of risk resulting from exposure. In addition, this information is also valuable for the search of mechanisms leading to the development of leukemia in general.

1.2.2 Radiation-Induced Leukemia

Background

Since the discovery of X rays by Roentgen in 1895, ionizing radiation has been shown to affect a wide range of tissues and organs. Ionizing radiation usually interacts with tissue through the generation of heat. The hazards depend on the ability to penetrate the human body and the absorption characteristics of different tissues. The hematopoietc

cells of bone marrow are among the most sensitive cells in the body to the damaging effects of ionizing radiation (IR). An acute whole-body dose of 2 to 3 Sievert (Sv.; 1 Sv = 100 rem) can cause extensive killing of lymphocytes and their precursors, resulting in severe lymphopenia and immunosuppression within 48 hours. Aplasia, granulocytopenia, and thrombocytopenia may also occur at variable degree due to radiation damage of the hematopoietic cells. Regeneration of the hematopoietic cells within the bone marrow varies depending on the dose, type of radiation, and extent of exposure. It takes around 1 to 2 years for full or partial recoveries of normal bone marrow function, but some studies have shown that in some cases it could take as long as 13 years.

Hematopoietic Neoplasia Induced by Radiation

A large number of studies have provided evidence that chronic exposure to lower levels or high exposures over a short time period is related to development of a variety of cancers. Skin carcinomas have been found with higher incidence in X-ray workers; leukemias, breast cancer, and thyroid cancer in radiologists; osteogenic sarcomas in radium dial painters; and lung cancer in miners. Among these radiation-related cancers, leukemia has the strongest association with radiation exposure. Previous studies have shown various types of leukemia can be induced by ionizing radiation, both in human cases and animal models. The actual risk of these cancers following radiation exposure is affected by multiple factors. These risk factors include the type of radiation, the dose, the part of the body exposed, and the extent of cell killing and DNA repair. Some physiological profile of exposed individual such as age, sex, and genetic background also can influence the risk of cancers. In order to induce malignancy in hematopoietic system,

in which cells are distributed diffusely, a significant proportion of tissue must be irradiated; whereas in solid tumors induced by radiation, such as thyroid cancers, only relatively small areas of body need to be exposed. The extent of cell killing has been shown to affect the risk of leukemia in some atomic-bomb survivors, in individuals who received high doses of primarily gamma radiation, the extensive killing of hematopoietic progenitors reduced the risks of leukemia. This observation has been introduced into the new strategy of radiation therapy to reduce the risk of leukemia by applying higher doses on smaller area of human body and shifting the dose-response curve to the region of higher dose.

Studies of Japanese atomic-bomb survivors have provided the most extensive information on radiation-induced leukemias and lymphomas. These survivors have suffered exposure to both gamma, and to a lesser extent, neutron radiation. The doses of radiation received by bombing survivors cannot be estimated precisely, which may affect the result of risk assessments. In addition, the registry of leukemia cases actually started 5 years after the bombing leading to little information available for the latency period. However, a lot of information is available concerning the types of neoplasia as well as responses to radiation. The latest update of these studies was concluded in 1998, which included 93,696 survivors and 2,778,000 person-years of study. This update reclassifies most of cases in leukemia registry using new criteria known as the French-American-British (FAB) classification, which offers more precise identification of different types of leukemia. According to the updated results, acute myeloid leukemia (AML), acute lymphocytic leukemia (ALL), and chronic myeloid leukemia (CML) comprise more than 80 percent of the leukemia acquired after exposure; whereas no increased risk of adult T-

cell leukemia (ALT), chronic lymphocytic leukemia (CLL), or Hodgkin's lymphoma have been detected in this population. A summary of the hematopoietic neoplasia seen in the atomic-bomb survivors is shown in Figure 1.2, which illustrates types of leukemia and their corresponding percentage to the whole disease population.

Acute Myeloid Leukemia (AML) induced by radiation

As described above, AML is among the most important contributors to the types of leukemia identified in atomic-bomb survivors. A similar conclusion has also been drawn from studies on patients following therapeutic use of radiation. Evidences from previous studies indicated dose-responses curves vary among subtypes of AML. Acute myelogenous leukemia (M2) was the FAB subtype that has the strongest association with gamma-ray exposure. Promyelocytic leukemia (M3) and erythroleukemia (M6) were the only two subtypes falling in high dose region in dose-response curve, whereas the remaining FAB subtypes of AML appeared to be represented at low and intermediate dose. Recent studies conducted on atomic-bomb survivors have indicated that the incidence of AML exhibited a nonlinear fashion over a period of time and was influenced both by gender and age. Highest incidence appeared to represent during the period of 5 to 10 years after exposure, with rapid decline right after. Men tended to have higher incidence of radiation-induced AML than women, and younger individuals were more likely to develop AML after exposure. The latency period of AML may be influenced by the intensity of radiation exposure, given the observation that bombing survivors located within 1500 meters of the hypocenter had much shorter latency periods than those located at greater distance.

1.3 The Pathogenesis of Human Radiation-Induced Leukemia

As seen with other cancers, leukemogenesis is a multistep process involving a series of genetic and possibly epigenetic alterations in the transformation of a normal cell into a malignant cell. Because of the heterogeneous nature of the hematopoietic neoplastic diseases, a large number of genes are likely to be involved. As many as 200 separate genes may be involved in the origin of all leukemias [10]. Some of these genetic alterations can be detected only at the molecular level. However, a large number of these genetic changes can be found directly through the use of cytogenetic and molecular cytogenetic techniques. Nonrandom chromosomal alterations are detected in the neoplastic cells of a majority of patients with leukemias, and identification of genes involved in these alterations has provided valuable insight into leukemogenesis in humans.

Radiation-Induced Chromosomal Alterations

When ionizing radiation passes through biological tissue it forms highly structured tracks as a consequence of energy deposition. The biological consequences of exposure to ionizing radiation include chromosome aberration, gene mutation, cell transformation and cell death. It has been widely accepted most of these changes occur during the cell cycles immediately following radiation exposure, however, that radiation-induced cytotoxicity may be delayed for up to six generation of cell replication. Elevated frequencies of chromosomal aberrations have been seen immediately in lymphocytes and bone marrow of individuals exposed to high levels of radiation. These chromosome

aberrations include both stable and unstable alteration, such as acentric fragments, micro nuclei, dicentric chromosomes, inversions, and translocations [11, 12]. Unstable alterations such as acentric fragments, micro nuclei, and dicentric chromosomes decrease with time, whereas the frequency of stable alterations, primarily translocations and inversions, remains relatively stable for years and may be detected 30 to 40 years later [12, 13]. Studies of chromosomal alterations in peripheral blood cells of highly exposed atomic-bomb survivors conducted many years after bombing have shown that the breakpoints in chromosomes of hemtopoietic cells in these individuals did not occur randomly throughout the genome [13]. Of particular note is that a number of these nonrandom breakpoints (e.g., 5q31, 7q32, 11q23, 21q2) lie within or immediately adjacent to regions that are altered in therapy-related and *de novo* leukemias and are believed to indicate the location of genes involved in leukemogenesis (Pedersen-Bjergaard and Rowley, 1994).

Mechanism of Leukemogenesis

As described above, many nonrandom chromosomal abnormalities observed in radiation-induced leukemia are believed to indicate the location of genes that are involved in the mechanisms leading to leukemogenesis. The chromosomal abnormalities commonly seen in leukemic cells include translocation, deletion and insertions. Chromosomal translocation is the rearrangement of parts between nonhomologous chromosomes and very often a fusion gene/fusion protein may be created as a result of this cytogenetic event. The most well-known translocation in leukemia probably is the translocation of chromosome 9q and 22q that gives rise to the 'Philadelphia' chromosome

and is typically present in chronic myeloid leukemia. Other common chromosomal translocations include translocation of chromosomes 1 and 19 in pre-B acute lymphoblastic leukemia, translocation of chromosomes 4 and 11 in null acute lymphoid leukemia, translocation of chromosomes 8 and 21 in M2 form of AML, and translocation of chromosome 15 and 17 in M3 form of AML. It has been shown that chromosomal translocation resulting in tumor specific fusion proteins are a major feature of the acute leukemia. Interestingly, most of the fusion proteins involved are transcription factors, indicating that aberrant transcriptional regulation play an important role in leukemogenesis. The involvement of aberrant transcription factor activity in human AML was first observed from common somatically acquired chromosomal translocations that result in oncogenic fusion products such as RUNX1–ETO (t(8;21)), CBFβ–MYH11 (core-binding factor-β–myosin heavy chain 11; inv16), fusion proteins involving MLL (mixed lineage leukaemia; t11q23), and PML–RARα (promyelocytic leukaemia–retinoic acid receptor-α; t(15;17)). In addition, some recent studies have shown small recurring mutation in specific chromosome regions may also affect the functions of transcription factors that play critical role in developing leukemia.

Translocations are not the only non-random chromosomal changes encountered in leukemias. Deletions of segments or whole chromosomes, including chromosome 5q, 7q, also occur; and sometimes duplications such as trisomy 8, 21 exist in a significant proportion of leukemic cells. Although the roles of these chromosomal deletion and duplication in cancer development remain unclear, numerous studies have been undertaken in an effort to elucidate their precise contribution to the transformation of normal cells into fully developed leukemic cells. An inspiring study by Shannon and

colleagues suggested that deletion of distal region of 5q is likely to affect the early growth response 1 gene (EGR1), a DNA-binding zinc finger protein than function as a transcriptional regulator. In another interesting study, the presence of ras mutations in the bone marrow of some patients with monosomy 7 was reported by Luna-Fineman and coauthors to indicate that aberrant ras-mediated signaling and alteration of chromosome 7 may cooperate in leukemogenesis, possibly by deregulating different biochemical pathways involved in myeloid growth and differentiation [14].

As seen with other cancers, leukemogenesis is a multistep process involving a series of genetic and possibly epigenetic alterations in the transformation of a normal cell into a malignant cell. The multiple steps of leukemogenesis can be described in terms of initiation, promotion and progression. Initiation is the key event in leukemia, since it is the first genetic event, starts a process which eventually leads to malignant transformation. An initiated bone marrow cell must possess some form of growth advantage to survive and develop into leukemia. This may involve genetic events, leading to delayed apoptosis or increased proliferative capacity, which is referred to as promotion. Promotion themselves have low oncogenic potential but can enhance the yield of neoplasms among the already initiated cells. The enhanced survivability of initiated cells is consolidated by progression, which consists in secondary genetic changed which modify proliferation and cell death.

1.4 Mouse Models for the Study of Radiation-Induced Leukemia

Mouse models have been used for many years as the preferable models for understanding hematopoiesis, for identifying effects of carcinogenic agents, and for

identifying critical genes involved in leukemogenesis. Mice hematopoietic systems have been extensively studied and are considered to be similar to those of humans. In addition, most of the common human hematopoietic cancers have been found to have corresponding tumors in mice that exhibit similar characteristics and affect the same lineages. (Pattengale, 1994; Perkins, 1989).

Certain strains of mice such as RFM, SLJ/J, or CBA/H are believed to be susceptible to radiation-induced leukemia, because significant incidence of myeloid leukemia have been seen at doses from 1 to 5 Gy. Whereas other strains such as C57 mice are considered to be resistant to radiation-induced leukemia since no increases of myeloid leukemia occurs in these strains following exposure. It remains unclear whether the differences in incidence are due to genetic background, hormonal status, or rearing environment. In our study, CBA mice is particularly used as the mouse models of radiation-induced AML because (a) they have a low spontaneous leukemia incidence about 0.1 to 1 %, (b) they develop AML at significantly high incidences after exposure to radiation, (c) and they have cytogenetic, molecular, and histopathological characteristics that are comparable to those seen in human acute leukemia. Although the majority of leukemias induced in CBA mice are originated from myeloid lineage, a few exceptions such as erythroblastic and megakaryoblastic leukemias have been reported. Peak incidences of AML induced in CBA strain can reach to 20 to 25%, which was seen at doses of about 3 Gy. Male CBA mice tend to have high incidences than female. In addition, no evidence has been shown that murine retroviruses or other indirect mechanisms were involved in the induction of AML following radiation exposure.

Chromosomal alterations in mouse model

For many years, it has been known that mouse chromosome 2 has consistently suffered one copy of deletion in most cases of myeloid leukemia induced by radiation. For example, in a study by Bouffler et al. using CBA/H mice, carried out over 24 months, a specific deletion at region D-E of chromosome 2 has been identified. During the 24 months period, a clone of bone marrow cells containing such deletions affecting mouse chromosome 2 could be seen in mice at various times after radiation exposure. This fraction of cells with stable chromosome 2 lesions progressively increased from 8% of analyzed cells at 3 months to 38% at 24 months, suggesting an expansion of cells containing chromosome 2 abnormality. All nine mice with myeloid leukemia exhibited high percentage, up to 100%, of deletion at region D-E of chromosome 2. Based on these results, the authors concluded that region D-E are particularly prone to expression of genomic instability after exposure to radiation and may play an important role in leukemogenesis. A similar study conducted by Hayata have evaluated the chromosomes of 52 myeloid leukemia occurring in 5 different strains of male and female mice, 2 of which developed spontaneously and 50 of which developed following radiation exposure. The chromosomal segment lying between the D and E was also commonly missing in 49 cases of myeloid leukemia. In addition, an alteration affecting chromosome 6 was observed in 16 cases and loss of the Y chromosome occurred in 7 cases of male mice.

Genetic changes in mouse model

Chromosome mapping around the nonrandom breakpoints seen after radiation exposure has provided valuable knowledge about the genes involved in radiation-induced

myeloid leukemogenesis in the mouse. To date, only a few genes associated with differentiation, development, and growth regulation of hematopoiesis have been cloned and mapped to mouse chromosome 2. For example, a homeobox gene *Hox 4.1* has been mapped to the D region of mouse chromosome 2, and a potential role for its involvement in myeloid transformation has been suggested (Rithidech et al., 1995). In addition, Silver and colleagues have reported a deregulation of *IL-1* in 3 murine AMLs involving a rearrangement of mouse chromosome 2 [15]. No such effect was seen in the closely linked *IL-1 alpha* gene, the *beta* 2 *microglobulin* gene, or the *c-abl* oncogene located on chromosome 2 (Silver et al., 1988). Based on the genetic homology of D and E regions on mouse chromosome 2 with the 2q24-32 and 11p11-13 regions of human chromosomes, the possible involvement of the *WT1* tumor suppressor gene located at 11p13 has also been proposed (Rithidech et al., 1995). In addition, some homology between the distal portion of mouse chromosome 2 and the long arm of human chromosome 20 suggests a possible relationship to the 20q13 deletion seen occasionally in de novo and therapy-related human leukemias (Pedersen-Bjergaard and Rowley, 1994;). More recently, Adams and colleagues utilized loss of heterozygosity (LOH) analysis and constructed genome contig to further refine the common deletion region on chromosome 2 in mouse radiation-induced AML. They reported that the common deletion region, which is located about 100 Mbp from the centromere, encompassed a critical transcription factor *PU.1* gene. In addition, mutations affecting the DNA bonding domain of *PU.1* have been frequently found in the remaining allele, strongly suggesting *PU.1* is a tumor suppressor gene for mouse radiation-induced AML.

1.5 The Involvement of PU.1 in Hematopoiesis and Leukemogenesis

The hematopoietic transcription factor PU.1, which is required for hematopoietic differentiation of stem cells, was originally identified as an oncogene. In erythroid progenitors, the integration of spleen focus-forming virus (SFFV) into the PU.1 locus causes its overexpression, which blocks their terminal differentiation into erythrocytes and ultimately leads to the development of erythroleukemia. However, in myeloid lineages, PU.1 promotes granulocytic and monocytic differentiation, and graded reduction in its expression blocks their differentiation or maturation and thereby causes myelogenous leukemia. Thus, in addition to normal hematopoietic regulation, PU.1 plays a critical role in leukemogenesis.

Hematopoiesis

PU.1 is a member of the *ets* transcription factor family. The *ets* region refers to the characteristic 85 residue winged helix-turn-helix domains that bind to DNA sequences with a GGA core [16]. There are more than 20 distinct mammalian *ets* factors, and they are different in their transcriptional activation domains and other functional regions [17].

PU.1 was first described in Friend virus induced erythroleukemia, and its overexpression blocks erythroid differentiation [18]. In cell lines, PU.1 is expressed by myeloid cells and B lymphocytes, but not by T cell lines [19]. Binding sites for PU.1 are various in many cell differentiation promoters. Target genes of interests are the characteristic antigens, CD11b and CD18 [20] and the receptors for the cytokines, M-CSF, GM-CSF, and G-CSF [21]. In addition, PU.1 binds and regulates its own promoter

[22]. Recognition that PU.1 is expressed by myeloid cells and that it binds to numerous myeloid promoters suggested an important role in myeloid differentiation. Its importance in myeloid cells was confirmed when PU.1 was disrupted in mice by two different groups. Their experimental strategies differed, and they observed somewhat different results. However, both groups found that loss of PU.1 disrupted development of both myeloid cells and B lymphocytes; they differed in the observed effects on T cell development [23].

PU.1 expression increases during myeloid differentiation and it may be required for terminal maturation of myeloid cells [24]. Treatment of monocytes with GM-CSF induces macrophage differentiation and causes accumulation of PU.1. Interestingly, transduction of alveolar monocytes with a PU.1-expressing retrovirus was sufficient to drive macrophage differentiation in the absence of GM-CSF [25]. A similar effect is seen with maturation of granulocytes. Expression of PU.1 increases as immature myeloid cells differentiate into mature granulocytes [26]. Thus, studies of PU.1 expression and its were similar in different cell types 1 month after irradiation but increased in the myeloid lineage and decreased in the lymphoid lineage at 3 and 6 months post-irradiation. *PU.1* deletions were only found in myeloid but not in lymphoid lineages in mice with choice between myeloid cells and B cells. They reintroduced PU.1 along with green fluorescent protein (GFP) into PU.1 null cells and rescued development of both lineages. were similar in different cell types 1 month after irradiation but increased in the myeloid lineage and decreased in the lymphoid lineage at 3 and 6 months post-irradiation. *PU.1* deletions were only found in myeloid but not in lymphoid lineages in mice with development [27].

The second 'hit' is generally a point mutation in the remaining *PU.1* allele. We used the *PU.1* deletion as an early marker for potential radiation-induced AML, and performed immunophenotyping combined with fluorescent *in situ* hybridization (immunoFISH) to study the persistence of this AML initiating lesion in myeloid and lymphoid lineages. and cooperate to activate gene transcription [28]. In contrast, PU.1 and the erythroid transcription factor, GATA-1, mutually antagonize their respective transcriptional activities. PU.1 can bind to the DNA binding domain of GATA-1 and block its ability to bind to DNA. Conversely, GATA-1 binds to PU.1 and displaces c-Jun, thereby reducing expression of target genes [29]. Because PU.1 and GATA-1 were similar in different cell types 1 month after irradiation but increased in the myeloid lineage and decreased in the lymphoid lineage at 3 and 6 months post-irradiation. *PU.1* deletions were only found in myeloid but not in lymphoid lineages in mice with GATA-1 expression and caused a switch in lineage fate toward myeloid differentiation [30]. These results indicate that PU.1 and GATA-1 not only activate their distinct lineage specific target genes, but also repress the target genes of the other lineage via protein–protein interactions.

Leukemogenesis

A screening of 126 AML patients led to the identification of 9 patients with mutations in the coding region of PU.1[31]. In most of the patients, mutations were in either the most primitive FAB subtype, myelomonocytic or monocytic, or erythroleukemic. Mutations were reported in different domains of the PU.1 gene; five deletions affecting the DNA binding domain, and five point mutations in the DNA

binding domain, the PEST domain, the transactivation domain. Transactivation of M-CSF receptor promoter was absent in the seven PU.1 mutants that affected the DNA-were similar in different cell types 1 month after irradiation but increased in the myeloid lineage and decreased in the lymphoid lineage at 3 and 6 months post-irradiation. *PU.1* deletions were only found in myeloid but not in lymphoid lineages in mice with suggesting that PU.1 mutations might be involved only in distinct subgroup of AML patients. Furthermore, this implies that in these leukemias the fusion proteins generated as a result of translocation, might disrupt PU.1 function.

Rosenbauer et al. have demonstrated that the graded reduction of PU.1 below a critical level induces an aggressive form of AML in mice, revealing dosage sensitivity for this transcription factor during leukemic progression[32]. They substituted the upstream were similar in different cell types 1 month after irradiation but increased in the myeloid lineage and decreased in the lymphoid lineage at 3 and 6 months post-irradiation. *PU.1* deletions were only found in myeloid but not in lymphoid lineages in mice with defectively differentiated myeloid progenitors. This block in differentiation was suggested to coincide with a preleukemic state. After a short latency (3-8 months), most of the mice died or became moribund after developing a neoplastic disease resembling human AML. were similar in different cell types 1 month after irradiation but increased in the myeloid lineage and decreased in the lymphoid lineage at 3 and 6 months post-irradiation. *PU.1* deletions were only found in myeloid but not in lymphoid lineages in mice with due to expansion of myeloblastic cells (30-75% blasts and promyelocytes in the bone marrow). The restoration of PU.1 expression in PU.1 knockdown mutant cells led to their differentiation into mature macrophages and neutrophils in the presence of IL-

3, IL6, and SCF. Its restoration could also rescue the response of preleukemic PU.1 knockdown progenitor cells to GM- and M-CSF. Thus reintroduction of PU.1 was sufficient to restore normal myeloid differentiation of leukemic PU.1 knockdown cells. These data indicate that downregulated expression of PU.1 is directly responsible for AML in PU.1 knockdown mice and suggest that introduction of PU.1 into blasts of individuals with AML could restore myeloid differentiation.

It has been suggested that PU.1 acts a potent suppressor of myeloid leukemia by promoting myeloid differentiation and that the combination of gene deletion and specific point mutations that impair its ability to bind to DNA are particularly leukemmogenic [33]. In 87% of the γ-radiation-induced tumors with one PU.1 allele deleted, the remaining allele was mutated. The missense mutations were confined to the Ets DNA-binding domain, and most of them were found to ablate its DNA binding ability. It has been shown in this paper that reintroduction of wild-type PU.1 into the leukemia cells impairs their clonal expansion and promote lineage differentiation and concomitant apoptosis.

1.6 The Origin of Malignant Cell in Radiation-induced Myeloid Leukaemia

Generally, diagnosis of acute myelogenous leukemia is based on the presence of excess blast cells in the blood or bone marrow [34]. Histochemical, immunological, and cytogenetic studies are often used to provide confirmatory evidence for the diagnosis. In spite of the observation that AML is a disease of blast cells, it remains controversial whether genetic changes underlying clonal expansion occur in a primitive pluripotent hematopoietic stem cell or whether transformation occurs later in progenitor cells

leukemias) [34]. In an effort to explore this question, variable techniques, including indices of clonality such as X-linked polymorphism and alternative allele expression, Ig gene and T cell receptor rearrangement, and, more recently, in situ hybridization, have been used by different investigators to identify progeny arising from immature cells carrying distinctive molecular or genetic markers [35-38]. However, attempts to elucidate this question have yields conflicting results. For example, the studies by Fialkow and colleagues, analyzing X-linked polymorphism in female leukemia patients, indicated that chronic myelogenous leukemia, polycythemia vera, and certain other myeloproliferative disorders originate in stem cells capable of developing into myeloid, erythroid, and lymphoid lineages [35, 39, 40]. This is perhaps most evident with CML in which, following the blast crisis, many leukemic cells show lymphoid characteristics and surface antigens and are positive for lymphocyte-specific gene expression such as expression of terminal deoxynucleotidyltransferase [39]. With respect to acute myeloid leukemias, Fialkow and colleagues have also provided evidence that two different types of leukemia exist, one in which the transforming event occurs in the primitive pluripotential compartment and another in which the transforming event occurs in a more mature progenitor compartment [39]. Similar conclusions have been drawn from the studies by Pallavicini and colleagues using FISH and immunofluorescent sorting of hematopoietic subpopulations [34]. Their studies have demonstrated that high frequencies of aberrant (aneuploid) cells were present in the primitive stem-cell compartment (CD34+) of patients with AML, even in cases in which the leukemic cells did not express the CD34 antigen (CD34-). Aberrations were also seen in lymphoid and erythroid cells in cases where the predominant leukemic cells lacked lymphoid or erythroid differentiation

markers. Based on an analysis of aberrant-cell frequency and compartment size, the authors proposed that these cytogenetically abnormal primitive stem cells represent preleukemic cells requiring the expression of aberrant genes or additional transforming events (i.e., clonal expansion, decreased apoptosis, etc.) for the leukemic phenotype to be expressed. Similar results were also seen by Haase and coinvestigators indicated that malignant transformation could occur at the level of the early hematopoietic stem cell [CD34+/CD38-] [41]. However, results from other studies have argued that the lineage-restricted myeloid progenitor cells are more commonly involved in AML. Recently, several studies have provided additional evidences in support of the involvement of more restricted progenitor cells in myeloid lineage [36, 37, 42]. For example, Kibbelaar et al. used panning, FISH, and immunophenotypic discrimination of myeloid and lymphoid cells in MDS patients to determine that granulocytes and monocytes contained trisomy 8 or monosomy 7, but T and B cells did not carry cytogenetic abnormalities found in the leukemic clone. Similar results were reported by Anastasi et al. using FISH with a chromosome 8 enumerator probe to measure trisomy 8 in MDS patients with chromosome 8 aneusomy. Van Lom et al. reported FISH analysis of AML patients showing that lymphocytes do not carry trisomy X. whereas neutrophils, eosinophils, and monocytes were trisomic. Based on these recent studies, in general, aberrant cells involved in AML can be considered as occurring in progenitor cells restricted to myeloid and erythroid lineages, with occasional involvement of the megakaryocyte lineage (see [34] for discussion). A small number of molecular studies have suggested that lymphoid progenitor cells are also clonal in AML; however, if so, they apparently have a lesser

tendency than myeloid or monocytic cells to undergo cytogenetic transformation and to contribute to the aggressiveness of the disease.

Most of the above studies were performed on patients with de novo leukemias. Much less is known about the origin of alterations and transforming events in leukemic cells resulting from exposure to chemicals and radiation. The induction of CML, ALL, and ANLL by ionizing radiation clearly indicates that the pluripotent stem cell is the target in radiation-induced leukemias [43]. Most treatmentrelated leukemias show multiple lineage involvement, consistent with an origin at the multipotent myeloid progenitor cell (CFU-GEMM) [44]. The critical event possibly occurs in the more primitive pluripotential stem cell but, as part of the leukemic process, the cells become lineage-restricted and differentiate within the myeloid lineage [44].

Involvement of bone-marrow–derived stem or progenitor cells also has been implicated in leukemogenesis and lymphomagenesis in mouse models. Due to the myeloid lineage involvement, critical transforming event in radiation-induced myeloid leukemogenesis almost certainly involve bone-marrow myeloid progenitor or pluripotent stem cells. However, the stage at which critical events occur has not been established. Studies of radiation-induced myeloid leukemias have indicated that they were monoclonal in origin [45]. In some mice, the leukemias appeared to originate in a cell that had been committed to a specific myeloid lineage whereas in other mice, the target cell appeared to be capable of differentiating into both myelomonocytic and erythroid cells[45].

1.7 Specific Aims

It has been suggested transcription factor PU.1 acts as a tumor suppressor in mouse radiation-induced AML. The frequency of PU.1 single copy deletion was more than 90% in γ-radiation-induced AML, and in 87% of the tumors with one PU.1 allele deleted, the remaining allele contained a point mutation. The most common point mutation is the transition of 703C to T in the Ets domain of PU.1, impairing its DNA binding capacity. Since PU.1 is a transcription factor critical for proper differentiation of myeloid and B cell lineages, it is reasonable to postulate that PU.1 malfunction disrupting the differentiation and maturation of certain lineages, especially myeloid lineage, is the mechanism underling radiation leukemogenesis. Therefore, characterizing PU.1 deletion and mutation in different cell types including stem cell, myeloid and lymphoid compartment in a time course following γ irradiation, as well as in mice with γ radiation-induced AML , will provide us clues to a better understanding of the process of radiation-induced leukemogenesis.

In addition, although the target cells of spontaneous AML have been actively investigated, their identity is still not clear: it has been proposed that leukemia may be initiated by transforming events that take place in HSCs; however, some evidences show leukemia may also arise from more restricted progenitors within myeloid lineage. Even less information is known about the target cells of radiation-induced AML. To study PU.1 deletion and mutation in different hematopoietic cell types of irradiated mice will also help resolve this question.

The purpose of this research is to study PU.1 deletion and mutation in the process of radiation-induced leukemogenesis using a mouse model. Such studies have three specific aims: 1. Develop proper methods for the identification of PU.1 deletion and

mutation in irradiated mouse model; 2. Characterize PU.1 deletion in different hematopoietic cell types in a time course following γ irradiation, as well as in leukemic mice; 3. Characterize PU.1 mutation in different hematopoietic cell types in a time course following γ irradiation, as well as in leukemic mice.

References

1. Orlic, D. and D.M. Bodine, *What Defines a Pluripotent Hematopoietic Stem-Cell (Phsc) - Will the Real Phsc Please Stand Up.* Blood, 1994. **84**(12): p. 3991-3994.
2. Mihich, E. and D. Metcalf, *6th Annual Pezcoller-Symposium - Normal and Malignant Hematopoiesis - New Advances.* Cancer Research, 1995. **55**(15): p. 3462-3466.
3. Metcalf, D., *Hematopoietic Regulators: Redundancy or Subtlety?* Blood, 1993. **82**(12): p. 3515-23.
4. Roberts, R., et al., *Heparan-Sulfate Bound Growth-Factors - a Mechanism for Stromal Cell-Mediated Hematopoiesis.* Nature, 1988. **332**(6162): p. 376-378.
5. Fairbairn, L.J., et al., *Suppression of Apoptosis Allows Differentiation and Development of a Multipotent Hematopoietic-Cell Line in the Absence of Added Growth-Factors.* Cell, 1993. **74**(5): p. 823-832.
6. Tenen, D.G., *Disruption of Differentiation in Human Cancer: Aml Shows the Way.* Nature Reviews Cancer, 2003. **3**(2): p. 89-101.
7. Sawyers, C.L., C.T. Denny, and O.N. Witte, *Leukemia and the Disruption of Normal Hematopoiesis.* Cell, 1991. **64**(2): p. 337-50.
8. Pattengale, P.K. and C.R. Taylor, *Experimental Models of Lymphoproliferative Disease. The Mouse as a Model for Human Non-Hodgkin's Lymphomas and Related Leukemias.* Am J Pathol, 1983. **113**(2): p. 237-65.

9. Philip, P. and J. Pedersen-Bjergaard, *Cytogenetic, Clinical, and Cytologic Characteristics of Radiotherapy-Related Leukemias.* Cancer Genet Cytogenet, 1988. **31**(2): p. 227-36.

10. Greaves, M.F., *Request for Leukaemic Samples from Twins.* Leukemia, 1995. **9**(2): p. 362-3.

11. Bender, M.A., et al., *Chromosomal Aberration and Sister-Chromatid Exchange Frequencies in Peripheral Blood Lymphocytes of a Large Human Population Sample.* Mutat Res, 1988. **204**(3): p. 421-33.

12. Gray, J.W., et al., *Structural Chromosome Analysis by Whole Chromosome Painting for Assessment of Radiation-Induced Genetic Damage.* J Radiat Res (Tokyo), 1992. **33 Suppl**: p. 80-6.

13. Tanaka, K., et al., *Nonrandom Distribution of Chromosome Breaks in Lymphocytes of Atomic Bomb Survivors.* J Radiat Res (Tokyo), 1983. **24**(4): p. 291-304.

14. Luna-Fineman, S., K.M. Shannon, and B.J. Lange, *Childhood Monosomy 7: Epidemiology, Biology, and Mechanistic Implications.* Blood, 1995. **85**(8): p. 1985-99.

15. Silver, A., et al., *Molecular Mapping of Chromosome 2 Deletions in Murine Radiation-Induced Aml Localizes a Putative Tumor Suppressor Gene to a 1.0 Cm Region Homologous to Human Chromosome Segment 11p11-12.* Genes Chromosomes Cancer, 1999. **24**(2): p. 95-104.

16. Donaldson, L.W., et al., *Solution Structure of the Ets Domain from Murine Ets-1: A Winged Helix-Turn-Helix DNA Binding Motif.* EMBO J, 1996. **15**(1): p. 125-34.

17. Wasylyk, B., S.L. Hahn, and A. Giovane, *The Ets Family of Transcription Factors.* Eur J Biochem, 1993. **211**(1-2): p. 7-18.

18. Paul, R., et al., *The Sfpi-1 Proviral Integration Site of Friend Erythroleukemia Encodes the Ets-Related Transcription Factor PU.1.* J Virol, 1991. **65**(1): p. 464-7.

19. Klemsz, M.J., et al., *The Macrophage and B Cell-Specific Transcription Factor PU.1 Is Related to the Ets Oncogene.* Cell, 1990. **61**(1): p. 113-24.

20. Rosmarin, A.G., et al., *Cd18 (Beta 2 Leukocyte Integrin) Promoter Requires PU.1 Transcription Factor for Myeloid Activity.* Proc Natl Acad Sci U S A, 1995. **92**(3): p. 801-5.

21. Reddy, M.A., et al., *Opposing Actions of C-Ets/PU.1 and C-Myb Protooncogene Products in Regulating the Macrophage-Specific Promoters of the Human and Mouse Colony-Stimulating Factor-1 Receptor (C-Fms) Genes.* J Exp Med, 1994. **180**(6): p. 2309-19.

22. Chen, H., et al., *PU.1 (Spi-1) Autoregulates Its Expression in Myeloid Cells.* Oncogene, 1995. **11**(8): p. 1549-60.

23. Scott, E.W., et al., *Requirement of Transcription Factor PU.1 in the Development of Multiple Hematopoietic Lineages.* Science, 1994. **265**(5178): p. 1573-7.

24. Cheng, T., et al., *Temporal Mapping of Gene Expression Levels During the Differentiation of Individual Primary Hematopoietic Cells.* Proc Natl Acad Sci U S A, 1996. **93**(23): p. 13158-63.

25. Shibata, Y., et al., *Gm-Csf Regulates Alveolar Macrophage Differentiation and Innate Immunity in the Lung through PU.1.* Immunity, 2001. **15**(4): p. 557-67.

26. Bjerregaard, M.D., et al., *The in Vivo Profile of Transcription Factors During Neutrophil Differentiation in Human Bone Marrow.* Blood, 2003. **101**(11): p. 4322-32.

27. DeKoter, R.P. and H. Singh, *Regulation of B Lymphocyte and Macrophage Development by Graded Expression of PU.1.* Science, 2000. **288**(5470): p. 1439-41.

28. Behre, G., et al., *C-Jun Is a Jnk-Independent Coactivator of the PU.1 Transcription Factor.* J Biol Chem, 1999. **274**(8): p. 4939-46.

29. Nerlov, C., et al., *Gata-1 Interacts with the Myeloid PU.1 Transcription Factor and Represses PU.1-Dependent Transcription.* Blood, 2000. **95**(8): p. 2543-51.

30. Rekhtman, N., et al., *Direct Interaction of Hematopoietic Transcription Factors PU.1 and Gata-1: Functional Antagonism in Erythroid Cells.* Genes Dev, 1999. **13**(11): p. 1398-411.

31. Dohner, K., et al., *Mutation Analysis of the Transcription Factor PU.1 in Younger Adults (16 to 60 Years) with Acute Myeloid Leukemia: A Study of the Aml Study Group Ulm (Amlsg Ulm).* Blood, 2003. **102**(10): p. 3850; author reply 3850-1.

32. Rosenbauer, F., et al., *Acute Myeloid Leukemia Induced by Graded Reduction of a Lineage-Specific Transcription Factor, PU.1.* Nat Genet, 2004. **36**(6): p. 624-30.

33. Cook, W.D., et al., *PU.1 Is a Suppressor of Myeloid Leukemia, Inactivated in Mice by Gene Deletion and Mutation of Its DNA Binding Domain.* Blood, 2004. **104**(12): p. 3437-44.

34. Mehrotra, B., et al., *Cytogenetically Aberrant Cells in the Stem Cell Compartment (Cd34+Lin-) in Acute Myeloid Leukemia.* Blood, 1995. **86**(3): p. 1139-47.

35. Fialkow, P.J., et al., *Clonal Development, Stem-Cell Differentiation, and Clinical Remissions in Acute Nonlymphocytic Leukemia.* N Engl J Med, 1987. **317**(8): p. 468-73.

36. Saitoh, K., et al., *Fluorescence in Situ Hybridization of Progenitor Cells Obtained by Fluorescence-Activated Cell Sorting for the Detection of Cells Affected by Chromosome Abnormality Trisomy 8 in Patients with Myelodysplastic Syndromes.* Blood, 1998. **92**(8): p. 2886-92.

37. Kibbelaar, R.E., et al., *Combined Immunophenotyping and DNA in Situ Hybridization to Study Lineage Involvement in Patients with Myelodysplastic Syndromes.* Blood, 1992. **79**(7): p. 1823-8.

38. Kibbelaar, R.E., et al., *Detection of Trisomy 8 in Hematological Disorders by in Situ Hybridization.* Cytogenet Cell Genet, 1991. **56**(3-4): p. 132-6.

39. Fialkow, P.J., *Stem Cell Origin of Human Myeloid Blood Cell Neoplasms.* Verh Dtsch Ges Pathol, 1990. **74**: p. 43-7.

40. Raskind, W.H., et al., *Evidence for a Multistep Pathogenesis of a Myelodysplastic Syndrome.* Blood, 1984. **63**(6): p. 1318-23.

41. Haase, D., et al., *Evidence for Malignant Transformation in Acute Myeloid Leukemia at the Level of Early Hematopoietic Stem Cells by Cytogenetic Analysis of Cd34+ Subpopulations.* Blood, 1995. **86**(8): p. 2906-12.

42. Anastasi, J., et al., *Cytogenetic Clonality in Myelodysplastic Syndromes Studied with Fluorescence in Situ Hybridization: Lineage, Response to Growth Factor Therapy, and Clone Expansion.* Blood, 1993. **81**(6): p. 1580-5.

43. Preston, D.L., et al., *Cancer Incidence in Atomic Bomb Survivors. Part Iii. Leukemia, Lymphoma and Multiple Myeloma, 1950-1987.* Radiat Res, 1994. **137**(2 Suppl): p. S68-97.

44. Keinanen, M., et al., *Clonal Chromosomal Abnormalities Showing Multiple-Cell-Lineage Involvement in Acute Myeloid Leukemia.* N Engl J Med, 1988. **318**(18): p. 1153-8.

45. Kotler, M., et al., *Radiation Leukemogenesis - Quantitative Relationship between Pre-Leukemic Cells in the Thymus and Lymphoma Induction.* International Journal of Cancer, 1994. **56**(5): p. 761-765.

Figures

Figure 1.1 Hierachical relationships between major cell types involved in hematopoiesis. for simplicity, a number of steps in maturation pathways have been omitted. (modified from Wright 1995)

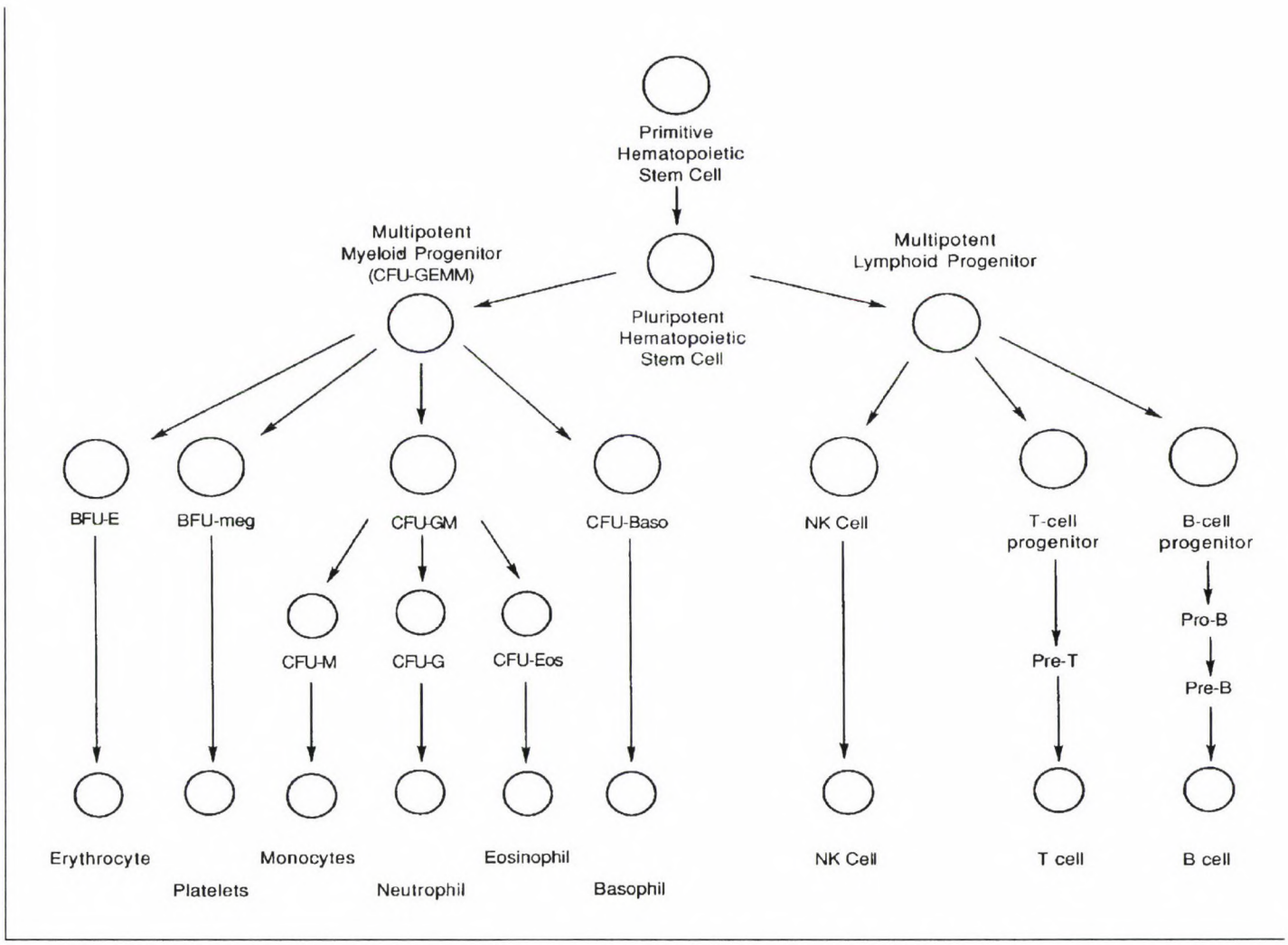


Figure 1. 2 Types of lymphohematopoietic neoplasia in Nagasaki survivors of the atomic-bomb blast (MDS: myelodysplasia syndrome, ANLL: acute nonlymphoid leukemia, ALL: acute lymphoid leukemia, ATL: adult T-cell leukemia, CML: chronic myeloid leukemia, CLL: chronic lymphoid leukemia, MM: multiple myeloma). Types of cancer for which evidence is sufficient for association with radiation are labeled with an S. Data are from Matsuo et al., 1988.

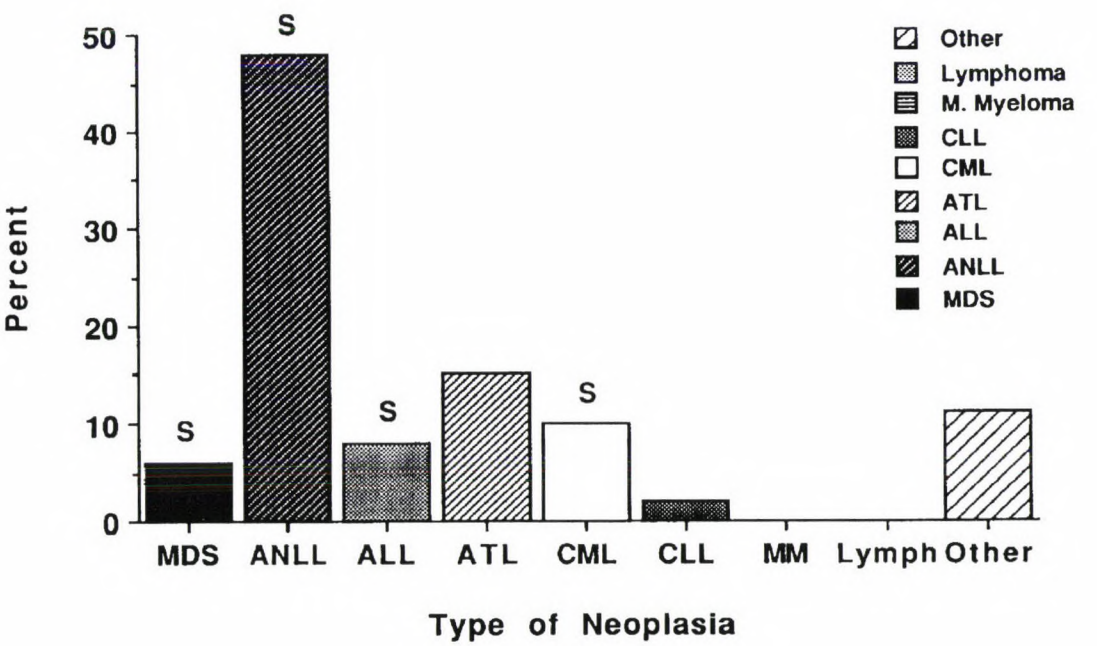


Table 1.1. Classification of major lymphohematopoietic neoplastic diseases in humans

I. Nonlympholytic leukemia	
Chronic myelogenous leukemia	CML
Myelodysplastic syndromes	MDS
Acute myeloid leukemia	ANLL/AML
Myeloblastic leukemia with minimal differentiation	M0
Myeloblastic leukemia without maturation	M1
Myeloblastic leukemia with maturation	M2
Promyelocytic leukemia	M3
Myelomonocytic leukemia	M4
Monocytic leukemia	M5
Erythroleukemia	M6
Megakaryoblastic leukemia	M7
Malignant histiocytosis	
II. Lympholytic leukemia	
Acute lymphoblastic leukemia	ALL, L1,L2
B-cell lineage	b-ALL
T-cell lineage	t-ALL
Prolymphocytic leukemia	
Chronic lymphocytic leukemia	CLL
B-cell lineage	b-CLL
T-cell lineage	t-CLL
Hairy cell leukemia	

Modified from Sullivan (1993).

Chapter 2

Clonal cytogenetic abnormalities (*PU.1* hemizygous deletion) in radiation-induced mouse AML development studied with combined immunophenotyping and *in situ* hybridization.

Abstract

Acute myeloid leukemia (AML) is believed to arise from the clonal expansion of a malignant transformed blast cell. Whether spontaneous AML arises from a hematopoietic stem cell (HSC) or a more restricted progenitor of myeloid lineage is an area of active investigation. Much less is known about which cell types give rise to radiation-induced or radiotherapy-related AML. We are exploring this question using a mouse model of radiation-induced AML. Several mouse strains including CBA/CaJ are susceptible to radiation-induced AML whereas other strains are resistant. In murine radiation-induced AML the *PU.1* (*Sfpi1*) gene behaves as a classic tumor suppressor gene. In most murine myeloid leukemias, there is a deletion in chromosome 2 encompassing the *PU.1* gene. The second 'hit' is generally a point mutation in the remaining *PU.1* allele. We used the *PU.1* deletion as an early marker for potential radiation-induced AML, and performed immunophenotyping combined with fluorescent *in situ* hybridization (immunoFISH) to study the persistence of this AML initiating lesion in myeloid and lymphoid lineages. Bone marrow cells were harvested from CBA/CaJ mice irradiated with 3 Gy of ^{137}Cs γ-rays 1, 3 and 6 months post –radiation and assayed by immunoFISH for *PU.1* deletions and cell differentiation markers. We demonstrated that the frequency of *PU.1* deletions were similar in different cell types 1 month after irradiation but increased in the myeloid lineage and decreased in the lymphoid lineage at 3 and 6 months post-irradiation. *PU.1* deletions were only found in myeloid but not in lymphoid lineages in mice with radiation-induced AML. These results indicate that radiation-induced AML is likely to originate from the more restricted progenitor of the myeloid lineage.

Introduction

Acute myeloid leukemia (AML) is believed to arise from the clonal expansion of a malignant transformed blast cell [1, 2]. It is still not clear about the origin of this clonal disease, some studies suggested the cytogenetic change and transformation of the cells leading to clonal expansion and leukemogenesis occur in multipotent stem cell capable of both myeloid and lymphoid differentiation[3, 4], whereas several recent studies suggested the involvement of a more restricted progenitor of myeloid lineage[5, 6]. The conflicting results from those reports could be due to different techniques employed, and variation in patient resources. To date, most of those studies are conducted on spontaneous AML without history of chemical or radiation exposure. Much less information is available for the identification of target cells in the radiation-induced or radiotherapy-related AML, and radiation-induced AML is known to exhibit characteristic differences from the spontaneous disease, suggesting the disease may arise by different mechanisms [7].

CBA/CaJ mice have found wide acceptance as a robust animal model and have provided valuable insight into the leukemogenesis in human and animal [8]. Mouse radiation-induced AML arises in CBA/CaJ mice after exposure to high or low LET radiation and there is very low incidence of spontaneous leukemia. Cytogenetic, molecular, and histopathological characteristics of radiation-induced AML in CBA/CAJ mice are comparable to those seen in human AML. In contrast, C57BL/6 mice are resistant to radiation-induced AML. The difference in susceptibility between these two strains could be attributed to genetic background, cell composition, number, proliferation rates, or organization of the stem-cell compartmen cellular composition in mouse hematopoietic tissues.

Some nonrandom cytogenetic abnormalities, ie, -5, 5q-, -7, 7q-, +8, 20q-, have been found in human spontaneous AML and have been used as markers to identify the target cell of this disease. [3]. Unfortunately, none of those genetic markers are specific for a significant proportion of AML. In addition, those aberrations have been reported to appear late in the development of neoplastic cells and are therefore not adequate to be used as a genetic marker to identify the origin of AML. In the CBA/CaJ model of radiation-induced AML, more than 90% affected mice acquire deletion on one copy of chromosome 2 [9], which encompasses the coding region of *PU.1* gene[10]. The PU.1 protein is known as one of the Ets transcription factors family members that play important roles in regulating stepwise differentiation of HSC. In the studies of *PU.1* knock-out mice, the *PU.1* gene has been associated with the differentiation of myeloid cells and B cells [11]. One recent study has shown inactivation of *PU.1* in adult mice led to myeloid leukemia [12]. All these studies indicate that deletion or mutation of the *PU.1* gene is a common and specific genetic lesion associated with mouse radiation-induced AML. Thus, measurement of *PU.1* deletion in early transformed pro-leukemia cells can be considered as a reliable method to study the cellular origin of radiation-induced AML.

In this study, *PU.1* DNA probe was used as the specific marker for the identification of premalignant cells in radiation-induced AML. *In situ* hybridization with *PU.1* probe was combined with immunophenotyping to identify cytogenetically aberrant cells in phenotypically defined hematopoietic cell. The analysis has enabled us to identify the clonal lineages involved in the developing of radiation-induced AML. Our results show that the frequency of *PU.1* deletions were similar in different cell types 1 month after irradiation but increased in the myeloid lineage and decreased in the lymphoid

lineage at 3 and 6 months post-irradiation. *PU.1* deletions were only found in myeloid but not in lymphoid lineages in radiation-induced AML mice. These results indicate that radiation-induced AML is likely to originate from the more restricted progenitor of the myeloid lineage.

Materials and methods

Mouse irradiations:

8-12 week-old male CBA/CaJ and C57BL/6 mice obtained from Jackson Laboratory were used in this study. The mice were handled according to the procedures approved by the Animal Care and Use Committee at Colorado State University (Fort Collins, CO). Groups of CBA/CAJ, and C57BL/6 mice were exposed to a single acute dose of 3.0 Gy gamma rays from a ^{137}Cs irradiator. Animal health was monitored daily. AML was identified by using the criteria that described by Major et al. 1979. Briefly, murine AML was distinguished by a replacement of normal bone marrow by abnormal myeloid cells and a marked splenomegaly and hepatomegaly. The diagnosis of myeloid leukaemia was routinely confirmed by pathological examination of blood, bone marrow, and spleen sample for morphology and phenotype.

Bone Marrow Collection

Bone marrow was collected from both CBA/CAJ and C57BL/6 mice, including control non-irradiated, 1 month post-irradiation, 3 months post-irradiation, 6 months post-irradiation and AML group (4 mice a group). Of note is that the AML group did not include C57BL/6 mice because of extremely low incidence of radiation-induced AML. Bone marrow cells from sacrificed mice were flushed from each femur, tibia and radius with PBS. The bone marrow PBS suspension was centrifuged at 1000rpm for 8 min at 4 C. The cell pellet was then resuspended using ACK lysis buffer, incubated for 5 min at 20 ^{o}C, and then diluted in PBS to 10^{7} cells/ml for further analysis.

Immunophenotyping

Bone marrow cells were attached to microscope slides using a cytospin instrument. One slide was used for evaluation of immunophenotyping, two were used for subsequent FISH procedures, and the remaining slides were frozen and stored. For immunophenotyping analysis, cytospinned slides were washed with ice cold PBS and fixed in PBS containing 4% paraformaldehyde for 15 min. After three further washes with PBS for 10 min, cells were treated with 0.2% Triton X-100 solution in PBS for 5 min. Before immunocytochemical detection, cells were blocked with 10% goat serum solution for 1 hour at room temperature or overnight at 4 ^{o}C to reduce subsequent non-specific antibody binding. For the identification of different lineages in bone marrow or spleen cells, a panel of antibodies was used: anti-mouse CD34, which recognizes an antigen present on hematopoietic progenitors; anti-mouse CD3 (mature T cells), anti-mouse CD19 (mature B cells), anti-mouse Gr-1 (granulocytes/monocytes), and anti-mouse TER119 (erythroid cells). Isotype antibodies were used as a control. All the primary antibodies were monoclonal, purchased from Ebioscience (San Diego, CA); and were diluted 1: 100. Slides with the cell monolayers were incubated in the diluted primary antiserum for 1 hour at 37 ^{o}C. Cells were then washed with PBS 3 times for 10 min. each, followed by incubation with secondary goat-anti-rat antibodies conjugated with FITC or Texas red for another 1 hour at 37^{o}C. Slides were mounted in a solution of 1.5ug/ml DAPI containing slow-fade (Molecular Probes, Carlsbad, CA) following 4 washes with PBS for 10 min each.

BAC DNA Probe

BAC clone 253H22 containing the murine *PU.1* gene was used to make FISH probes for measurement of *PU.1* loss. This probe was labeled with Alexa fluor 594-5-dUTP or FITC by nick translation and was provided by Dr. Yuanlin Peng.

Combined immunophenotyping and FISH study (ImmunoFISH)

For the combined immunophenotyping and FISH procedures, the slides were fixed for 15 min in methanol/acetic acid (3:1) after immunophenotyping; subsequently, the slides were washed and fixed for 10 min in 1% PBS-formalin, washed again, and dehydrated. FISH was performed on the slides as described by Pinkel et al. with the following modifications. Briefly, air-dried cells were treated with freshly prepared Carnoy's fixative for 5 minutes, allowed to air dry, and then baked at 65°C for 15 minutes. Cellular DNA was denatured at 72°C for 2.5 minutes in 70% formamide (Omnisolv; EM Science, Gibbston, NJ), saline sodium citrate (SSC; 0.30 mol/L NaCl, 0.03 mol/L sodium citrate, pH 7.0). After dehydration in ethanol series and air-drying at 42"C, *PU.1* DNA probe (1 to 2 uL) was denatured in hybridization mixture [fomamide, 50%; dextran sulfate (Sigma. St Louis. MO) 10%; 2x SSC] at 72°C for 5 minutes and immediately applied to cells on slides that were hybridized overnight in a humidified chamber at 37°C. Slides were washed three times at 45°C in 50% formamide and once each in 2X SSC and 0.2 X SSC. Chromosomal DNA was counterstained with diamino-2-phenyl-indole dihydrochloride (DAPI) in antifade solution.

Microscopical evaluation and scoring of *PU.1* deletion

The slides were evaluated using a Zeiss fluorescence microscope equipped with following filter sets: DAPI, FITC, and Texas red. *PU.1* deletions were scored in both immune-positive and immunonegative cells. For each cell group, at least 50 nuclei were evaluated. Cells were considered to have a *PU.1* hemizygous deletion if the cell showed one hybridization signal. Bone marrow cells from disease-free non-irradiated mice served as controls, and the mean percentage of false-positive nuclei (one signal) was determined at a level of 5.1% (cutoff).

Statistical analysis

The data are presented as the mean number of *PU.1* deletion frequency ± SD. Significant differences between samples were determined by one-tail t test. The criterion for statistical significance is a P value of 0.05.

Results

1. *PU.1* deletion detected in different lineages of bone marrow cells one month following γ irradiation.

One month following γ irradiation, *PU.1* single copy deletion was detected in all cell types including lymphocytes (T and B cells), granulocyte/monocyte, erythroid cells, and CD34 positive progenitors (Figure 2.1). For CBA/CAJ mice, all the cell types showed a similar frequency of *PU.1* deletion. The percentage of cells with PU.1 deletion is 19.4% in T cell, 20.3% in B cells, 21.4% in granulocytes/monocytes, 20.6% in erythroid cells and 19.4% in CD34 positive progenitor cells (Figure 2.2A), those values are significantly higher than the cutoff value (5.1%) which was determined by disease-free non-irradiated mice served as controls ($P<0.05$). However, no significant difference of PU.1 deletion has been found among different cell types ($P>0.05$). In C57BL/6 mice, a lower frequency of PU.1 loss was detected, which is 6.5% in T cells, 7.2% in B cells, 7.5% in granulocytes/monocytes, 7.1% in erythroid cells, and 6.5% in CD34 positive progenitor cells (Figure 2.2A). The percentages in C57BL/6 mice is not significantly higher than the cutoff value (5.1%). Again, no significant difference was observed among different cell types in C57BL/6 mice ($P>0.05$).

The results above showed that radiation tended to induce higher levels of PU.1 deletion in CBA/CAJ mice than in C57BL/6, which is not surprising given the fact that CBA/CAJ strain is more susceptible to radiation exposure. Another observation is that radiation exposure induced similar levels of PU.1 deletion among different types of bone marrow cells, such as lymphocytes, granulocytes/monocytes, erythroid cells, and CD34

positive progenitors, within the same strain of mouse, either CBA/CAJ or C57BL/6. It seems that radiation did not preferentially cause PU.1 deletions in any particular type of bone marrow cells at this early time point. However, it is possible that certain cell types may gain expansion or proliferation advantages over others once they have suffered a deletion in PU.1 gene. Therefore, we also investigated mice housed longer than 1 month after radiation exposure.

2. Expansion of myeloid cells with *PU.1* deletion at 3 and 6 months following γ irradiation

Indeed, in CBA/CAJ mice that have been kept for 3 or 6 months after radiation exposure, increasing levels of PU.1 deletion were observed in granulocytes/monocytes, erythroid cells, and CD34 positive cells. It seems that increasing percentage of PU.1 deletion in granulocytes/monocytes, erythroid cells, and CD34 positive cells is an indication of clonal expansions within myeloid lineage. In contrast, the frequency of PU.1 deletion in lymphocytes (either T or B cells) was decreasing at the same period (Figure 2.3A and 3B). The expansion of myeloid cells and shrinkage of lymphoid cells that suffered a PU.1 deletion are further demonstrated with a line chart (Figure 2.3C). The line chart showed that there was no significant difference in *PU.1* deletion frequency between myeloid cells and lymphoid cells at one month following radiation exposure. However, after 3 months and 6 months following radiation exposure, myeloid cells appeared to exhibit significantly higher level of *PU.1* deletion than that seen in lymphoid cells as a result of expansion in myeloid cells and shrinkage in lymphoid cells ($P<0.05$). Based on those observations, we may predict that later at certain time point after radiation

exposure, *PU.1* single copy deletion will be removed from lymphoid cell population. In contrast, myeloid cells maintain a relatively high level of *PU.1* deletion and these cells may represent cell populations that are more susceptible to additional mutation ('second hit') which eventually lead to the development of leukemia. It has also been noticed that in C57BL/6 mice, the *PU.1* deletion levels maintained at a low level and showed no significant variation over the same six months period (Figure 2.3A and 3B).

3. Clonal myeloid *PU.1* deletion in CBA/CAJ strain with radiation-induced AML.

Although having a low life time spontaneous leukemia incidence (0.1 to 1%), CBA/CAJ mice have been shown to develop AML in a relatively high frequency (25 to 30%) following exposure to ionized radiation. In contrast, C57BL/6 mice are considered to be resistant to radiation-induced AML and incidence of acquiring radiation-induced AML is very low. In this study, three cases of CBA/CaJ mice with radiation-induced AML were obtained for the immunoFISH assay with PU.1 probe and cell differentiation markers. Interestingly, in those three AML mice, *PU.1* deletions were detected at high frequencies in myeloid cells and CD34 positive progenitor cells, ranging from 70.8% to 95.1%, but none of AML mice showed *PU.1* deletion in the cells bearing B- or T- cell antigen (Figure 2.4).

Discussion

The susceptibility to radiation-induced AML in CBA/CAJ mice and resistance in C57BL/6 is considered to be complex polygenic traits. Compared to C57BL/6 mice, bone marrow cells from the CBA/CAJ strain are more susceptible to radiation-induced chromosome 2 aberrations [13, 14]. In a recent study by Peng et al. [15], a significantly higher frequency of *PU.1* deletions was found in the bone marrow cells from the CBA/CAJ mice than cells from the C57BL/6 mice at the one month time point after radiation exposure. The underlining mechanism of susceptibility to *PU.1* deletion in CBA/CAJ mice following radiation is not clear. It could be due to genetic background, hormonal status, or bone marrow microenvironment. Similarly in the present study, bone marrow cells from CBA/CAJ mice exhibit a higher percentage of *PU.1* deletion than those from C57BL/6 mice at one month following γ radiation. In addition, the present study shows there is no difference of *PU.1* deletion frequency among bone marrow cell lineages within the same strain, although differences can exist between the two different stains. Further investigation on underlying mechanisms involved in different responses to radiological exposure between CBA/CAJ and C57BL/6 mice will provide valuable insight into radiation-induce leukemogensis.

To explore the clonal expansion of cell populations suffering *PU.1*, which possibly is part of the process of radiation-induced leukemia, we examined *PU.1* deletion frequency within different lineages at different time points, i.e. 1, 3 and 6 month following radiation exposure. Our results show that in CBA/CaJ mice, *PU.1* deletion frequency is similar in different cell types 1 month after γ irradiation, but increased in myeloid compartment over a six month period whereas decreased in lymphoid

compartment, which imply *PU.1* loss has different impact on the development of myeloid cells and lymphoid cells. Indeed, in some previous studies, *PU.1* has been shown to be a key regulator of hematopoietic system, playing different roles in regulating the proliferation and differentiation of lymphoid and myeloid lineages [16, 17]. The observation that *PU.1* deletion frequency was increasing in myeloid lineages over time is an indication of expansion of cytogenetic abnormal cells within myeloid lineage. This myeloid expansion probably is one of the critical steps in the developing of AML, given the fact that the nature of AML is an uncontrolled proliferation or expansion of immature myeloid cells [18]. Such an observation is also in agreement with the scenario seen in the studies of *PU.1*+/- mice. In those studies, reduced *PU.1* expression has been shown to promoted myeloid progenitor expansion [19, 20]. However, one fact needed to be mentioned here is that radiation-induced *PU.1* deletion in CBA/CAJ mice is not the same genetic abnormality as the haploinsufficient phenotype in genetically engineered *PU.1*+/- mice; the former event may involves other genes immediately adjacent to the location of *PU.1* and could be much more complex. In addition, at 3 months or 6 months post-irradiation, although we found expansion of myeloid cells with a *PU.1* deletion in all CBA/CAJ mice, none of them demonstrated AML symptoms, strongly suggesting radiation-induced AML arises from multiple genetic events and *PU.1* deletion is one of the early events leading to leukemia. Cook et al. suggested a point mutation on Ets domain of the residual *PU.1* allele might be "the second hit" leading to leukemia [10]. Decreased *PU.1* frequency in lymphoid compartment at 3 months and 6 months post-irradiation suggested *PU.1* deletion was not able to persist in lymphoid cells. The specific mechanism responsible for the disappearance of radiation-induced *PU.1* deletion in

lymphoid compartment over time is not clear. A possible mechanism could be that lymphocytes with a *PU.1* deletion are maintained by long term HSCs with self-renewal potential, and those long term HSCs with *PU.1* deletion favor myeloid but not lymphoid development. Another possibility is that lymphoid cells with *PU.1* deletion following radiation could be more prone to apoptosis.

AML has been known as oligoclonal disease, malignant cells are derived from one or several transformed cells[21]. Whether the HSC or more restricted progenitor of myeloid lineage is the origin of the disease is still not clear. Much less information is available about radiation-induced AML. In the present study, we demonstrated *PU.1* deletion in granulocyte/monocytes and CD34 positive progenitor cells, but not in the lymphocytes in radiation-induced AML mice. Our results are similar to some previous studies conducted on human myelodysplastic syndrome [5, 6], which measured trisomy 8 with chromosome 8 specific repetitive DNA probes in myeloid and lymphoid cell fraction from patients who had myelodysplastic syndrome. In those studies, none of the lymphocytes had been identified with trisomy 8, whereas cells from myeloid cells and progenitor cells contained trisomy 8. Similar to those study, our results suggest the radiation-induced AMLs arise from more restricted myeloid progenitors due to the fact that plenty of cytogenetically abnormal cell exist in myeloid compartment but not in the lymphoid compartment. However, although cytogenetically abnormal cells exclusively exist in myeloid compartment, we may not necessarily exclude the possibility that these cells are originated from a HSC. A recent study by Cozzio and coworkers [22] reported both HSC and common myeloid progenitor (CMP), but not common lymphoid progenitor (CLP), could be transformed in vitro by a leukemogenic MLL fusion gene, and

subsequently induced AML in lethally irradiated recipients. Interestingly in their study, all transformed cell lines, including HSC and CMP, exhibited a similar immunophenotype with granulocyte/macrophage potential. If so, in radiation-induced AML the early critical event such as *PU.1* deletion could possibly occur in either HSC or CMP, but lately the transformed cell become lineage-restricted and differentiate within the myeloid lineage. Unlike spontaneous AML, the target cells of the malignant cell in radiation-induced AML is less studied and understood. The present study provided new evidence to the current understanding on the target cells of radiation-induced AML.

In summary, the study of *PU.1* deletion in different lineages of hematopoietic cells has permitted the identification of the origin of premalignantly transformed hematopoietic cells in mouse radiation-induced AML. We observed dynamic changes in *PU.1* deletion frequency over a six-month period following radiation exposure, with increases in myeloid cells and decreases in lymphocytes. The increased frequency of *PU.1* deletion in myeloid cells over a six-month period indicated expansion of myeloid lineage with deletion, which can be a critical early event in the initiation and development of AML. *PU.1* deletion was observed only in myeloid lineages but not in lymphoid lineages in mouse radiation-induced AML, suggesting the origin and expansion of neoplastic cells from a restricted myeloid progenitor. Further studies will be needed to characterize *PU.1* deletion in hematopoietic stem cells (HSCs) and lineage restricted progenitors in mouse radiation-induced AML, which will provide more profound information for us to understand the effect of *PU.1* deletion in the process of radiation leukemogensis, as well as for the determination on the target cells of radiation-induced AML.

References

1. Raskind, W.H., et al., *Evidence for a multistep pathogenesis of a myelodysplastic syndrome.* Blood, 1984. **63**(6): p. 1318-23.
2. Jacobson, R.J., et al., *A clonal complete remission in a patient with acute nonlymphocytic leukemia originating in a multipotent stem cell.* N Engl J Med, 1984. **310**(23): p. 1513-7.
3. Mehrotra, B., et al., *Cytogenetically aberrant cells in the stem cell compartment (CD34+lin-) in acute myeloid leukemia.* Blood, 1995. **86**(3): p. 1139-47.
4. Greaves, M.F., *Stem cell origins of leukaemia and curability.* Br J Cancer, 1993. **67**(3): p. 413-23.
5. Kibbelaar, R.E., et al., *Combined immunophenotyping and DNA in situ hybridization to study lineage involvement in patients with myelodysplastic syndromes.* Blood, 1992. **79**(7): p. 1823-8.
6. Anastasi, J., et al., *Cytogenetic clonality in myelodysplastic syndromes studied with fluorescence in situ hybridization: lineage, response to growth factor therapy, and clone expansion.* Blood, 1993. **81**(6): p. 1580-5.
7. Rowley, J.D., *Chromosome abnormalities in leukemia and lymphoma.* Ann Clin Lab Sci, 1983. **13**(2): p. 87-94.
8. Rithidech, K.N., E.P. Cronkite, and V.P. Bond, *Advantages of the CBA mouse in leukemogenesis research.* Blood Cells Mol Dis, 1999. **25**(1): p. 38-45.
9. Silver, A., et al., *Molecular mapping of chromosome 2 deletions in murine radiation-induced AML localizes a putative tumor suppressor gene to a 1.0 cM*

region homologous to human chromosome segment 11p11-12. Genes Chromosomes Cancer, 1999. **24**(2): p. 95-104.

10. Cook, W.D., et al., *PU.1 is a suppressor of myeloid leukemia, inactivated in mice by gene deletion and mutation of its DNA binding domain.* Blood, 2004. **104**(12): p. 3437-44.
11. Scott, E.W., et al., *Requirement of transcription factor PU.1 in the development of multiple hematopoietic lineages.* Science, 1994. **265**(5178): p. 1573-7.
12. Metcalf, D., et al., *Inactivation of PU.1 in adult mice leads to the development of myeloid leukemia.* Proc Natl Acad Sci U S A, 2006. **103**(5): p. 1486-91.
13. Watson, G.E., et al., *Genetic factors influencing alpha-particle-induced chromosomal instability.* Int J Radiat Biol, 1997. **71**(5): p. 497-503.
14. Gowans, I.D., et al., *Genotype-dependent induction of transmissible chromosomal instability by gamma-radiation and the benzene metabolite hydroquinone.* Cancer Res, 2005. **65**(9): p. 3527-30.
15. Peng, Y., et al., *Radiation leukemogenesis in mice: loss of PU.1 on chromosome 2 in CBA and C57BL/6 mice after irradiation with 1 GeV/nucleon 56Fe ions, X rays or gamma Rays. Part II. Theoretical considerations based on microdosimetry and the initial induction of chromosome aberrations.* Radiat Res, 2009. **171**(4): p. 484-93.
16. DeKoter, R.P. and H. Singh, *Regulation of B lymphocyte and macrophage development by graded expression of PU.1.* Science, 2000. **288**(5470): p. 1439-41.

17. DeKoter, R.P., J.C. Walsh, and H. Singh, *PU.1 regulates both cytokine-dependent proliferation and differentiation of granulocyte/macrophage progenitors.* Embo J, 1998. **17**(15): p. 4456-68.

18. Sawyers, C.L., C.T. Denny, and O.N. Witte, *Leukemia and the disruption of normal hematopoiesis.* Cell, 1991. **64**(2): p. 337-50.

19. Walter, M.J., et al., *Reduced PU.1 expression causes myeloid progenitor expansion and increased leukemia penetrance in mice expressing PML-RARalpha.* Proc Natl Acad Sci U S A, 2005. **102**(35): p. 12513-8.

20. Dahl, R., et al., *Regulation of macrophage and neutrophil cell fates by the PU.1:C/EBPalpha ratio and granulocyte colony-stimulating factor.* Nat Immunol, 2003. **4**(10): p. 1029-36.

21. Fialkow, P.J., et al., *Clonal development, stem-cell differentiation, and clinical remissions in acute nonlymphocytic leukemia.* N Engl J Med, 1987. **317**(8): p. 468-73.

22. Cozzio, A., et al., *Similar MLL-associated leukemias arising from self-renewing stem cells and short-lived myeloid progenitors.* Genes Dev, 2003. **17**(24): p. 3029-35.

Figures

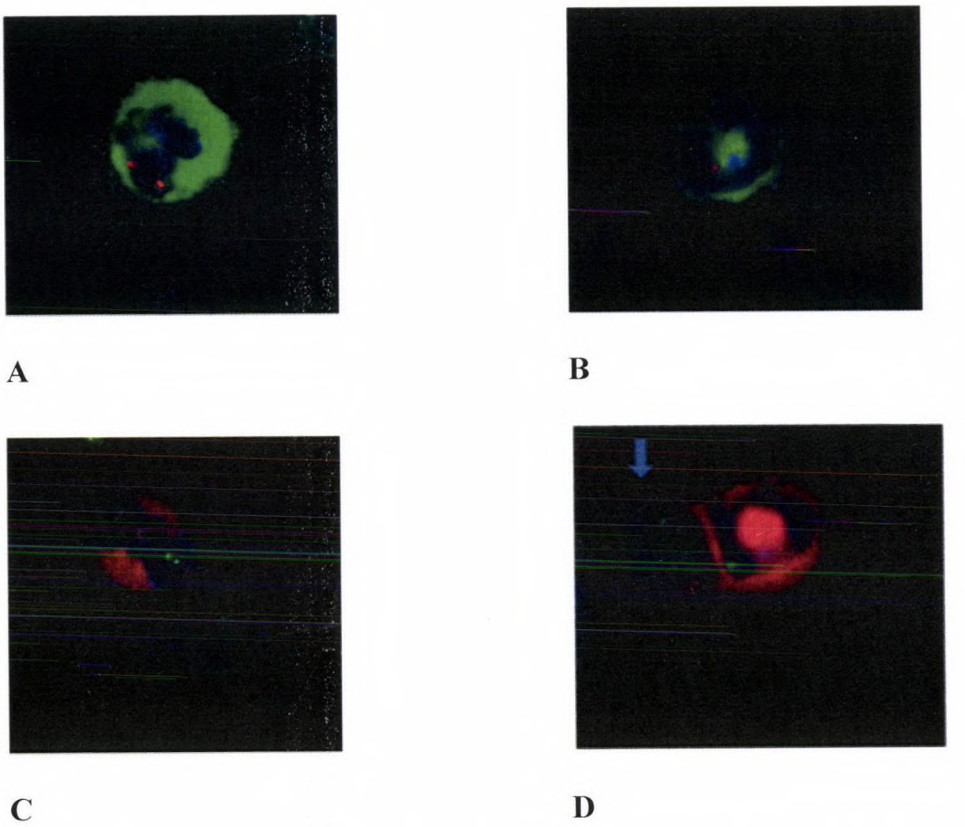


Figure 2.1. Examples of combined immunophenotyping and FISH analysis of bone marrow smears in irradiated CBA/CAJ mice using *PU.1* probe. Cells shown in (A) and (B) was immunophenotyped with CD3 antibody (T cell), FISH probe for *PU.1* was used. The cell nuclei were counter stained with DAPI (blue). The CD3 Positive cells were stained with green fluorescence (FITC) and FISH signals were stained with red fluorescence (Alexa fluor 594-5-dUTP). The T cell in in (B) has only one red signal, and was scored as a *PU.1* deletion. Cells shown in (C) and (D) were immunophenotyped with Gr-1 (granulocyte/monocyte), FISH probe for *PU.1*. The Gr-1 Positive cells were stained with red fluorescence (Texas red) and FISH signals were stained with green fluorescence (FITC). The cell nuclei were counter stained with DAPI (blue). The

granulocyte/monocyte in (D) with a single signal was scored as a *PU.1* deletion. Of note is that in (D): next to the Gr-1 positive cell, a Gr-1 negative cell also showed a *PU.1* deletion.

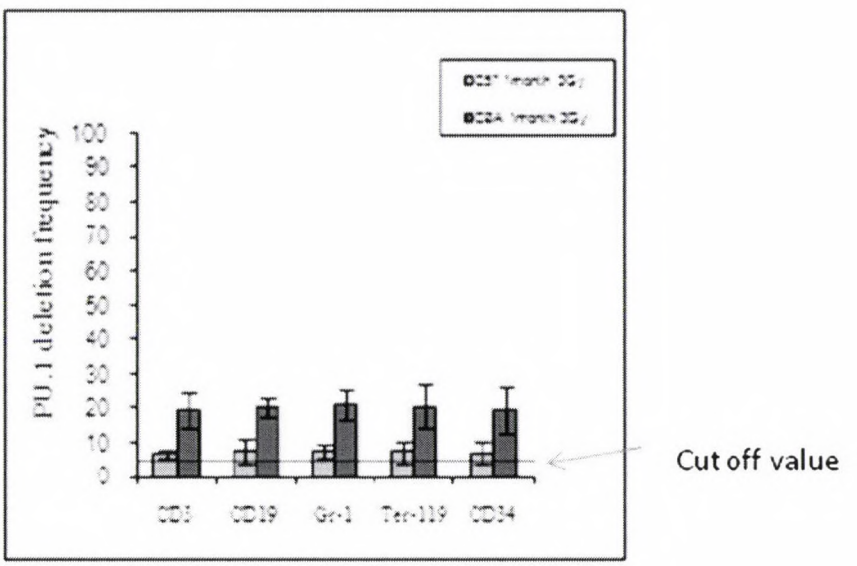
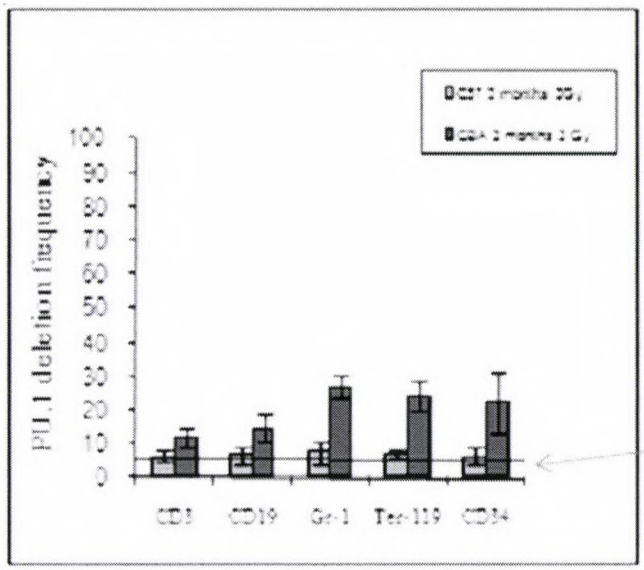
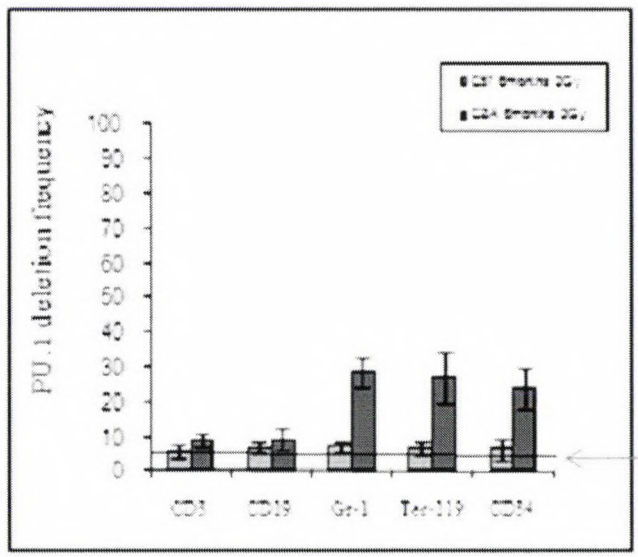


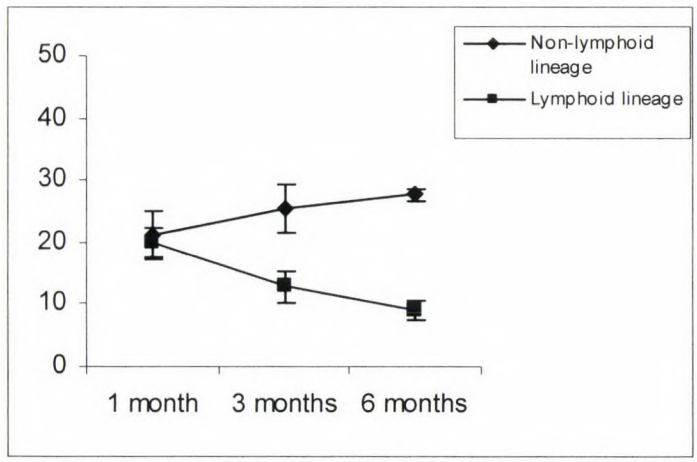
Figure 2.2. *PU.1* deletion frequency in different cell types one month after3 Gy γ radiations. Each point is the average of samples from three individual mice with standard error. No significant differences were found among different cell types within the same strain of mouse, CBA/CAJ or C57BL/6 ($P>0.05$). Significant difference exists between CBA/CAJ and C57BL/6 strain in every cell type ($P<0.05$). Gr-1, granulocyte/monocyte; Ter-119, erythroid cells; CD34, progenitors; CD3, T lymphocytes; CD19, B lymphocytes.


PU.1 deletion frequency
100
90
80
70
60
50
40
30
20
10
0
CD3
CD19
Gr-1
Ter-119
CD34
Cut off value

A

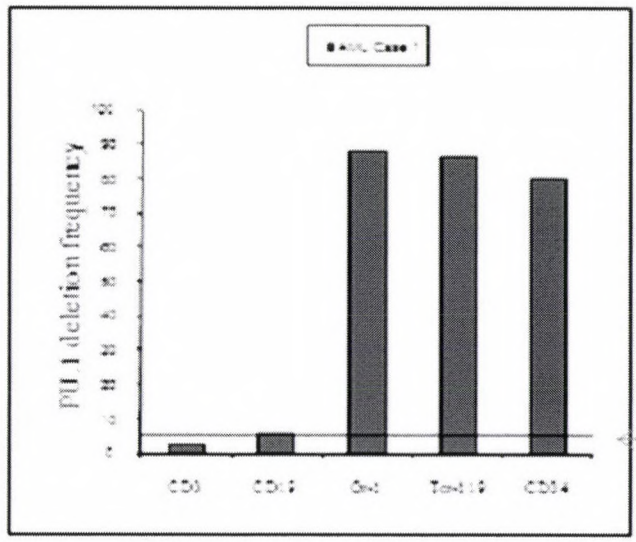

PU.1 deletion frequency
100
90
80
70
60
50
40
30
20
10
0
CD3
CD19
Gr-1
Ter-119
CD34
Cut off value

B

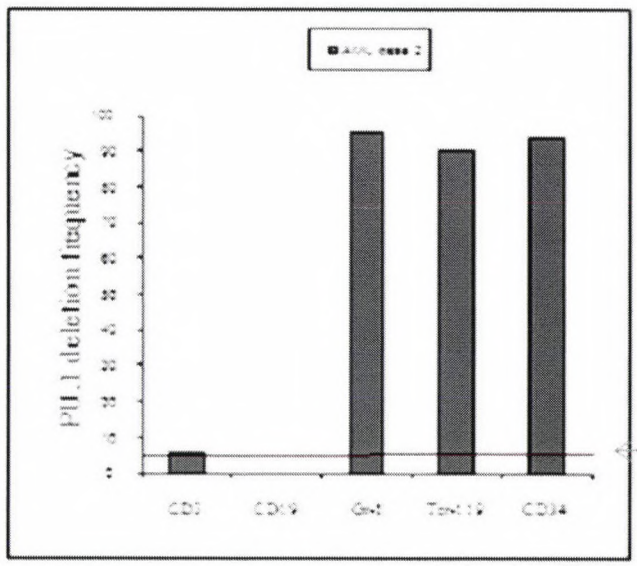


C

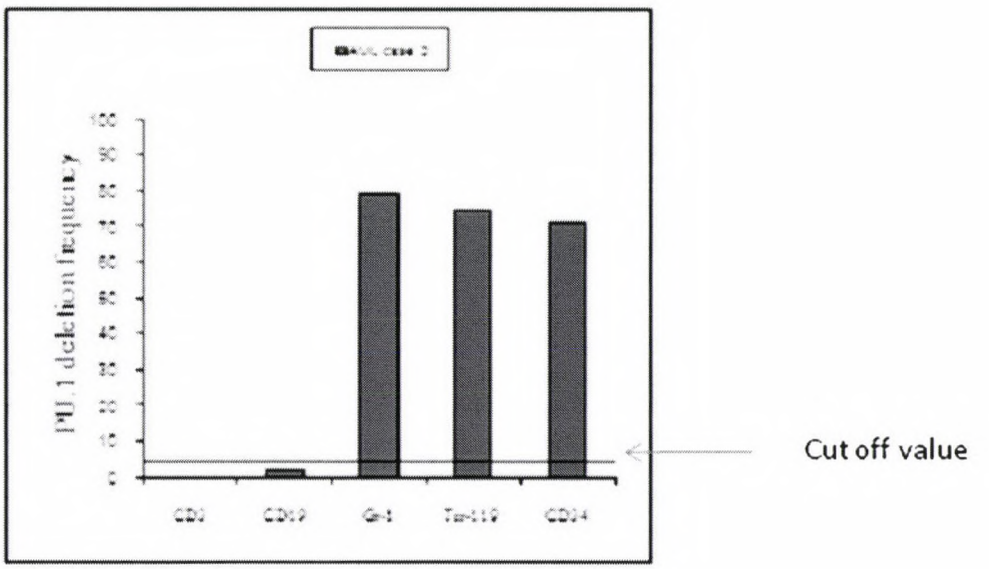
Figure 2.3. *PU.1* deletion in different cell types at 3 and 6 months after 3 Gy γ radiations. (A) and (B) showed *PU.1* deletion in different cell types at 3 and 6 months after 3 Gy γ radiations, respectively. Compared with 1 month after radiation (Figure 2.2), for CBA/CAJ mice, *PU.1* deletion frequency increased in granulocyte/monocyte (Gr-1), erythroid cells (Ter-119), and CD34 positive progenitors, but decreased in T (CD3) and B (CD19) lymphocytes. For C57BL/6 mice, *PU.1* deletion maintained at a low level in every cell types without obvious changes. (C) The plotted line chart showed the trends that frequency of *PU.1* deletion fluctuated over 6 months in CBA/CAJ mice. Different trends were observed between myeloid cells (granulocyte/monocyte and erythroid cell) and lymphoid cells (T and B cells), increased in myeloid cells but decreased in lymphoid cells.



A



B



C

Figure 2.4. Analysis of *PU.1* deletion in 3 cases of CBA/CAJ mice with radiation-induce AML. In all 3 cases, high frequency of *PU.1* deletion was in granulocyte/monocyte (Gr-1), erythroid cells (Ter-119) and CD34 positive progenitors, ranging from 70.8% to 95.1%, whereas no *PU.1* deletion was found in T (CD3) or B (CD19) lymphocytes.

Chapter 3

Characterizing *PU.1* deletion in flow-sorted subpopulation of bone marrow cells in γ irradiated CBA/CaJ and C57BL/6 mice

Abstract

Dysfunction of the transcription factor *PU.1* has been strongly associated with radiation-induced leukemogenesis. Deletion of *PU.1* gene in one copy of chromosome 2 has been observed in more than 90% of mouse radiation-induced acute myeloid leukemia. Given *PU.1* deletions are common and early events in the development of myeloid leukemia, experiments were designed to characterize the deletions of *PU.1* in different cell types of bone marrow harvested from irradiated mice at various post-irradiation time points. The results demonstrate that the frequency of *PU.1* deletions is similar in different cell types 1 month after irradiation, and then increased in the myeloid lineage and decreased in the lymphoid lineage at 3 and 6 months post-irradiation. These results support the multistep theory of leukemogenesis, as well as implicate the origin of radiation-induced acute myeloid leukemia as the more restricted myeloid progenitors.

Introduction

Acute myeloid leukemia (AML) is characterized by uncontrolled proliferation or expansion of myeloid cells with differentiation and maturation defects. AML is widely accepted as a clonal disease, which means AML arise from the clonal expansion of a malignantly transformed cell [1, 2]. Several techniques have been used to study the clonality: eg, use of constitutionally defined characteristics in heterozygous female patients, such as glucose-6-phosphate dehydrogenase (G6PD) isoenzymes, and restriction length polymorphisms of hypoxanthine phosphoribosyl transferase (HPRT) and phosphoglycerate kinase (PGK) [1]. Another approach uses tumor-specific markers: eg, genetic alterations detectable with banding analysis, or mutated, duplicated, or deleted sequences detectable with Southern blot or polymerase chain reaction (PCR) techniques [3]. An alternative strategy to study the clonality of AML is to use immunophenotype and genotype measurements to detect cells carrying characteristic cytogenetic abnormalities in phenotypically defined lineage compartments [3, 4]. Flow cytometric analysis and sorting using lineage specific markers has been widely used to identify and isolate phenotypically defined lineages both in research and clinical work. Fluorescent *in situ* hybridization (FISH) techniques have been shown to be useful tools to investigate nonrandom cytogenetic abnormalities in AML, ie, -5, 5q-, -7, 7q-, +8, 20q-[5]. Combining these two technologies has been shown efficient by several researchers to study the clonal origin of AML [4, 6].

Therapy-related or radiation-induced AMLs appear to have characteristics different from those seen in spontaneous AMLs. Karyotype comparison between cases with spontaneous leukemia (AML) and patients previously treated with chemotherapeutic or

radiological agents reveal that the latter groups had a significant higher frequency of leukemic cells with abnormal karyotypes, primarily loss and deletion of certain chromosomes[7]. In addition, immunological studies revealed that leukemic cells of 80% of AML patients with history of chemical or radiological exposure were positive for the CD34, whereas only 20% of leukemic cells of nonexposed patients were positive for this marker, suggesting more frequent involvement of the more primitive hematopoietic cells [8]. Furthermore, exposed patients showed a much lower frequency of remission following conventional chemotherapy [8]. These differences suggest a variation in origin and etiology of therapy-related or radiation-induced AMLs from those of spontaneous AMLs. However, compared with spontaneous AMLs, much less studies have been done to investigate the clonal origin of radiation-induced AMLs due to the limited number of patients. Introducing animal models may help in this regard.

Mouse models have proven to be useful models for understanding underlying mechanism, and for identifying critical genes involved in leukemogenesis. In certain strains of mice such as RFM, SLJ/J, or CBA/CaJ, significant increases of AML have been seen after acute doses of irradiation. In those radiation-induced mouse AMLs, high frequency (more than 90%) of chromosome 2 deletions is observed and the commonly deleted region containing the *PU.1* gene has been characterized [9, 10].

In the present study, two strains of mice, CBA/CaJ and C57BL/6, were irradiated by an acute dose of γ radiation. Flow cytometric analysis/sorting was combined with FISH to quantify potentially transformed cells in sorted subsets of bone marrow cells from these irradiated mice: lymphocyte, granulocyte/monocyte, and erythroid cell were labeled with antibodies recognizing differentiation antigens present on the membranes;

and *PU.1* DNA probes were used as the specific marker for the identification of potentially transformed cells. The idea was to identify and study the specific bone marrow cell types that undergo *PU.1* deletion over time and may give rise to radiation-induced AML. Our results show that the frequency of *PU.1* deletions were similar in different cell types 1 month after irradiation but increased in the myeloid lineage and decreased in the lymphoid lineage at 3 and 6 months post-irradiation. These results indicate that radiation-induced AML is likely to originate from cells restricted within myeloid lineage.

Materials and Methods

Mouse strains and irradiation.

8-12 week-old male CBA/CaJ and C57BL/6 mice obtained from Jackson Laboratory were used in this study. The mice were housed in the Laboratory Animal Resource facility at Colorado State University (Fort Collins, CO). Animal handling and care procedures were approved by the Animal Care and Use Committee at CSU. Cohorts of CBA/CAJ, and C57BL/6 mice were exposed to a single acute dose of 3.0 Gy gamma rays from a ^{137}Cs irradiator. Animal health was monitored daily.

Bone Marrow Collection

Bone marrow was collected from both CBA/CaJ and C57BL/6 mice, including control non-irradiated, 1 month post-irradiation, 3 months post-irradiation, 6 months post-irradiation and AML group (4 mice per group). Bone marrow cells were flushed from each femur, tibia and radius with PBS. The bone marrow PBS suspension was centrifuged at 1000rpm for 8 min at 4 ^{o}C. The cell pellet was then mixed well with ACK lysis buffer (NH_4Cl 8g/l, $KHCO_3$ 1g/l, and $EDTANa_2 \cdot 2H_2O$ 3.7g/l), incubated for 5 min at 20 ^{o}C, resuspended in PBS at 10^8 cells/ml for further flow cytometric analysis/sorting.

Cell phenotyping and separation.

After incubated with anti-mouse CD16/32 (blocks Fc binding) for 15 minutes, marrow cells was incubated at 20 ^{o}C for 30 min with the following antibodies: anti-CD3 (mature T cells), anti-CD19 (mature B cells), anti-Gr-1 (granulocyte/monocyte), and anti-Ter119 (erythroid precusor). Antibodies were conjugated with Allophycocyanin- cy7

(APC-cy7), fluorescein isothiocyanate (FITC), or Allophycocyanin (APC). After antibody labeling, cells were washed twice with phosphate-buffered saline, pH 7.4 (PBS). Flow cytometric analysis and sorting of subpopulations were performed using a MoFlo high speed cell sorter. Forward light scatter, perpendicular light scatter, and fluorescence signals were measured for each cell and saved in list mode data files using Summitview software .

Marrow cells not incubated with antibodies were used as negative controls. Cells gated on the basis of immunofluorescence intensity were sorted into 15ml tubes with PBS plus 2% FBS. The sorted cells were centrifuged at 1000rpm for 8 minutes at 4 ^{o}C. The cell concentration was then placed into an incubation mixture (9 ml 0.075KCl, 200ul colcemid), mixed and incubated for 30 min at 37 ^{o}C. After incubation, 5 ml fresh Carnoy's fixative (3:1 methanol : acetic acid) was added, mixed and centrifuged at 1000 rpm for 5 min. Cells were washed with fresh fixative two more times. The supernatant was decanted, a small amount of fixative was added, and cells were dropped onto clean, wet microscope slides and allowed to dry.

Fluorescence *in situ* hybridization (FISH)

The RP23-263H8 BAC clone containing the murine *PU.1* gene was used to make FISH probe for measurement of *PU.1* loss. The BAC probe provided by Dr. Yuanlin Peng was 197kbp in size and labeled with Alexa fluor 594-5-dUTP or FITC by nick translation. FISH was performed as described by Peng et al [11]. with the following modifications. Briefly, air-dried cells were treated with freshly prepared Carnoy's fixative for 5 minutes, allowed to air dry, and then baked at 65°C for 15 min. Cellular DNA was

denatured at 72°C for 2.5 min in 70% formamide (Omnisolv; EM Science, Gibbston, NJ), saline sodium citrate (SSC; 0.30 mol/L NaCl, 0.03 molL sodium citrate, pH 7.0). After dehydration in ethanol series and air-drying at 42 °C, DNA probe (1 uL) was denatured in hybridization mixture [fomamide, 50%; dextran sulfate (Sigma. St Louis. MO). 10%; 2x SSC] at 72°C for 5 min and immediately applied to cells on slides that were hybridized overnight in a humidified chamber at 37°C. Slides were washed three times at 45°C in 50% formamide 2X SSC; and once each in 2X SSC and 0.2 X SSC. Chromosomal DNA was counterstained with diamino-2-phenyl-indole dihydrochloride (DAPI) in antifade solution.

Microscope analysis

The slides were evaluated using a Zeiss fluorescence microscope equipped with following filter sets: DAPI and Texas red. The frequencies of cells showing hybridization signals and the number of signals per cell were determined. Cells with disrupted nuclear membranes were excluded from the analysis. For each cell group, at least 50 nuclei were evaluated. Hybridization signals were considered to represent two copies of *PU.1* if the fluorescent hybridization signals were separate and nonoverlapping. Cells were considered to be *PU.1* hemizygous deletion if the cell showed one hybridization signal corresponding to the control slide. Bone marrow cells from disease-free non-irradiated mice (4 mice) served as controls, and the mean percentage of false-positive nuclei (one signal) was determined at a level of 3.8% (cutoff).

Statistical methods

The data are presented as the mean number of *PU.1* deletion frequency ± SD. Significant differences between lineages were determined by one-tail t test. The criterion for statistical significance is a P value of 0.05.

Results

Flow sorting of bone marrow of irradiated mice

In this study, 12 CBA/CAJ and 12 C57BL/6 age-matched mice (12 weeks) were irradiated with a single dose of 3.0 Gy of γ rays. 6 CBA/CAJ and 6 C57BL/6 were used as the control without irradiation. Groups of randomly selected mice (4 treated CBA/CAJ or C57BL/6 mice and 2 non-treated CBA/CAJ or C57BL/6 mice) were killed at 1 month, 3 months, and 6 months after irradiation. At each sacrifice time, bone marrow from each mouse was harvested and analyzed immediately. Hematopoietic subpopulations were identified on the basis of fluorescence intensity. Cells blocked with isotype antibody were used to define the immunofluorescence intensity above which cells were considered labeled specifically. Flow analysis of the sorted subpopulation confirmed that the purity exceeded 98%. The proportion of erythroid cells with Ter-119 positive were estimated to be the frequency of cells in R2 window (Figure 3.1A.). The lymphocytes bearing CD3 (T cell) and CD19 (B cell) antigens were defined and sorted as the cells in R4 window (Figure 3.1B.). Two distinct groups of cells with positive Gr-1 expression can be seen on the chart of flow cytometric analysis, one is cell with high Gr-1 expression and the other is cell with lower Gr-1 expression. Thus, we decided to isolate and analyze these two groups of cells separately. The high Gr-1 group was considered to be mature granulocyte/monocyte and low Gr-1 was considered to be granulocyte/monocyte in a more primitive stage.

The immunophenotyping profiles of bone marrow

The immunophenotyping profiles of CBA/CAJ and C57BL/6 at different time points following radiation exposure are shown in Table 3.1. In C57BL/6 mice, the percentage of Gr-1 positive cells in bone marrow increased from 26.8% to 36.3% over a six months period following radiation, and the percentage of CD3 and CD19 positive cells in bone marrow of C57BL/6 mice decreased from 21.8% to 7.4% during the same period. In CBA/CAJ mice, the Gr-1 positive cells increased at 3 months but decreased at 6 months following irradiation; and the CD3 and CD19 positive lymphocytes appeared to decrease at 3 months but increase at 6 months following irradiation. In both strains, the erythroid cells showed significant decrease over time, implying erythropoietic function was commonly affected by radiation exposure and anemia possibly existed in those irradiated mice.

FISH on sorted subpopulations

Cells subpopulations with *PU.1* deletion were quantified using fluorescence microscopy after hybridization of sorted cells with *PU.1* probes (Figure 3.2). Background *PU.1* deletion levels in the normal and nonirradiated control mice were determined to define the levels above which cells were considered with *PU.1* deletion. Bone marrow cells from disease-free non-irradiated mice served as controls, and the mean percentage of false-positive nuclei (one signal) was determined at a level of 3.8% (cutoff). Figure 3.3 showed frequency of *PU.1* deletion in different hematopoietic cell types of CBA/CAJ or C57BL/6 mice at 1 month, 3 months, and 6 months after radiation exposure. One month following γ irradiation, *PU.1* deletions were detected in all cell types including lymphocytes (T and B cells), granulocyte/monocyte, erythroid cells (Figure 3.3A).

CBA/CAJ showed similar frequency of *PU.1* deletion among different cell types. The same is true for C57BL/6. However, when CBA/CaJ and C57BL/6 were compared, there was a clear difference between the two strains, higher in CBA/CAJ and lower in C57BL/6 ($P<0.05$) (Figure 3.3A). The results above were consistent with those of the former study utilizing immunoFISH assay to identify *PU.1* deletion in different cell types.

After 3 and 6 months, there were differences between cell types of CBA/CAJ mice in respect of *PU.1* deletion frequency (Figure 3.3B and C). The cells with positive Gr-1 surface marker, both high and low expression groups, appeared to have increased *PU.1* deletion. In contrast, decreases in *PU.1* deletion were observed in cells bearing T- and B-antigen. Those results were also similar to the finding in our former study using immunoFISH technique. One exception was that the increase of *PU.1* deletion in Ter-119 positive cells was not as obvious as that observed in the former study, reflecting a variation of results by using different techniques and antibodies from different companies. On the other hand, the *PU.1* deletion level in C57BL/6 mice maintained at a low level and showed no significant variation over time (Figure 3.3).

Figure 3.3D showed the trends of *PU.1* deletion frequency changing in different cell types over a six month period after radiation. Although no significant difference in the *PU.1* deletion frequency was found in different cell types at 1 month following irradiation. Because of the increase in granulocyte/monocytes and decrease in lymphocytes with *PU.1* deletion, granulocyte/monocytes possessed significant higher *PU.1* deletion frequency than that of lymphcytes at 3 months and 6 months following irradiation ($P<0.05$).

Discussion:

The involvement of a deletion at region D-E of mouse chromosome 2 (del2 (D-E)) in radiation-induced murine myeloid leukemia has been documented for more than a decade[12]. Recently, genome mapping has narrowed this common deletion region to a 1.0 cM length chromosome fragment, franking the *PU.1* gene sequence, a hematopoietic transcription factor regulating development and differentiation of multiple lineages in hematopoietic tissues [13]. Not only does PU.1 function as a master regulator of proper differentiation of both myeloid and lymphoid lineages [14-16], it also plays a critical role in murine leukemogenesis[17]. Recent views of the genetic mechanism of leukemia are that mutated or dysregulated transcription factors (i.e. *PU.1*) disrupt the differentiation of hematopoietic cells and lead to uncontrolled proliferation of immature leukemic cells. This concept has been functionally confirmed in mouse models [17]. Mice with an engineered deletion of the URE of *PU.1* had decrease *PU.1* expression and this led to an aggressive form of AML[18]. Similarly, mice with γ irradiation-induced myeloid leukemia regularly acquired both a deletion in one copy of *PU.1* and a point mutation in the ETS domain, which impaired DNA binding, of the remaining *PU.1* allele[10]. Consequently, these findings prompted us to design a study to investigate *PU.1* deletion status during the transformation of normal hematopoietic cells to the leukemic phenotype.

A mouse model was used in this study. Two strains of mice, one (CBA/CaJ) is sensitive to radiation-induced AML and the other (C57BL/6) is resistant, were irradiated with an acute dose of 3 Gy γ rays. We combined flow cytometric sorting and fluorescence *in situ* hybridization to quantify *PU.1* deletion status in different types of bone marrow cells at a time course after irradiation exposure. One month after radiation

exposure, significant higher *PU.1* deletion frequency was observed in CBA/CAJ mice than in C57BL/6 mice, consistent with the results reported by Peng et al. (in press). However, at the time point of one month after radiation, it appeared that radiation induced deletions occurred with equal frequencies in all cell types examined in both strains. The *PU.1* deletion level in C57BL/6 mice maintained at a low level and showed no significant variation over time (Figure 3.3A and 3B). This is not the case in CBA/CaJ mice; however, at 3 and 6 month after radiation exposure, cells with *PU.1* deletions were increased in granulocyte/monocyte; and decreased T- and B- lymphocyte (Figure 3.3A and 3B). The results were consistent with our previous study, which used immunoFISH to identify *PU.1* deletion in different lineages of bone marrow cells. A minor difference was observed in erythroid cells: the increasing of *PU.1* deletion at 3 and 6 months was not as obvious as in the previous study, possibly due to antibodies ordered from different sources, or the different methodologies employed. Gr-1 positive cells were discriminated into two groups in this study: The high expression group, which was considered to be mature granulocyte/monocyte; and low expression group, which was considered to be granulocyte/monocyte in a more primitive stage. Interestingly, the low Gr-1 expression group appeared to have higher frequency of *PU.1* deletion than high Gr-1 expression group, although the difference is not significant. This result indicated immature granulocyte/monocyte may be the cell type primarily affected by *PU.1* deletion. A similar observation has been report by several studies that *PU.1* haploinsufficiency promoted myeloid progenitor expansion and increased compartment sizes of granulocyte/monocyte [19].

The results of the above experiments demonstrated that PU.1 deletion had different impact on the myeloid and lymphoid lineages, which fit in the prevailing concept that PU.1 plays different roles in the regulation of development of multiple lineages. This study also reinforces the thesis that the development of AML is a multiple-step process, which arises from cumulative effects of several genetic events. This inference was based on the finding that none of the mice examined developed AML symptoms, even though the expansion of abnormal myeloid cells had occurred in the bone marrow. We hypothesize that those expanded myeloid cells are more likely to acquire additional genetic aberrations, such as point mutation in the remaining *PU.1* copy or dysfunction of other transcription factors mutually interacting with *PU.1*, which eventually lead to myeloid leukemia.

Our results suggest the originally transformed target cells likely exist in myeloid compartment, since the abnormal lymphocytes with *PU.1* deletion were removed from bone marrow over time and unlikely to undergo further transformation into leukemic cells. The mechanism responsible for the disappearance of lymphocyte bearing *PU.1* deletion is not clear, although it could possibly be due to apoptosis.

In summary, we characterized *PU.1* deletions in post-irradiated mice in a time course and discovered dynamic change of *PU.1* deletion frequencies in subpopulations of bone marrow cells, increasing in myeloid cells and decreasing in lymphoid cells over a six-month period of time. Our results support *PU.1* deletion is an early event of the multiple-step leukemogenesis, and suggest radiation-induced AML is originated from a restricted myeloid progenitor.

References

1. Fialkow, P.J., et al., *Clonal development, stem-cell differentiation, and clinical remissions in acute nonlymphocytic leukemia.* N Engl J Med, 1987. **317**(8): p. 468-73.
2. Fialkow, P.J., *Stem cell origin of human myeloid blood cell neoplasms.* Verh Dtsch Ges Pathol, 1990. **74**: p. 43-7.
3. Kibbelaar, R.E., et al., *Combined immunophenotyping and DNA in situ hybridization to study lineage involvement in patients with myelodysplastic syndromes.* Blood, 1992. **79**(7): p. 1823-8.
4. Mehrotra, B., et al., *Cytogenetically aberrant cells in the stem cell compartment (CD34+lin-) in acute myeloid leukemia.* Blood, 1995. **86**(3): p. 1139-47.
5. Mitelman, F., et al., *Chromosome pattern, occupation, and clinical features in patients with acute nonlymphocytic leukemia.* Cancer Genet Cytogenet, 1981. **4**(3): p. 197-214.
6. Haase, D., et al., *Evidence for malignant transformation in acute myeloid leukemia at the level of early hematopoietic stem cells by cytogenetic analysis of CD34+ subpopulations.* Blood, 1995. **86**(8): p. 2906-12.
7. Rowley, J.D., *Chromosome abnormalities in leukemia and lymphoma.* Ann Clin Lab Sci, 1983. **13**(2): p. 87-94.
8. Cuneo, A., et al., *Morphologic, immunologic and cytogenetic studies in acute myeloid leukemia following occupational exposure to pesticides and organic solvents.* Leuk Res, 1992. **16**(8): p. 789-96.

9. Alexander, B.J., et al., *Gene deletion explains both in vivo and in vitro generated chromosome 2 aberrations associated with murine myeloid leukemia.* Leukemia, 1995. **9**(12): p. 2009-15.

10. Cook, W.D., et al., *PU.1 is a suppressor of myeloid leukemia, inactivated in mice by gene deletion and mutation of its DNA binding domain.* Blood, 2004. **104**(12): p. 3437-44.

11. Peng, Y., et al., *Radiation leukemogenesis in mice: loss of PU.1 on chromosome 2 in CBA and C57BL/6 mice after irradiation with 1 GeV/nucleon 56Fe ions, X rays or gamma Rays. Part II. Theoretical considerations based on microdosimetry and the initial induction of chromosome aberrations.* Radiat Res, 2009. **171**(4): p. 484-93.

12. Azumi, J.I. and L. Sachs, *Chromosome mapping of the genes that control differentiation and malignancy in myeloid leukemic cells.* Proc Natl Acad Sci U S A, 1977. **74**(1): p. 253-7.

13. Silver, A., et al., *Molecular mapping of chromosome 2 deletions in murine radiation-induced AML localizes a putative tumor suppressor gene to a 1.0 cM region homologous to human chromosome segment 11p11-12.* Genes Chromosomes Cancer, 1999. **24**(2): p. 95-104.

14. Anderson, K.L., et al., *Neutrophils deficient in PU.1 do not terminally differentiate or become functionally competent.* Blood, 1998. **92**(5): p. 1576-85.

15. DeKoter, R.P. and H. Singh, *Regulation of B lymphocyte and macrophage development by graded expression of PU.1.* Science, 2000. **288**(5470): p. 1439-41.

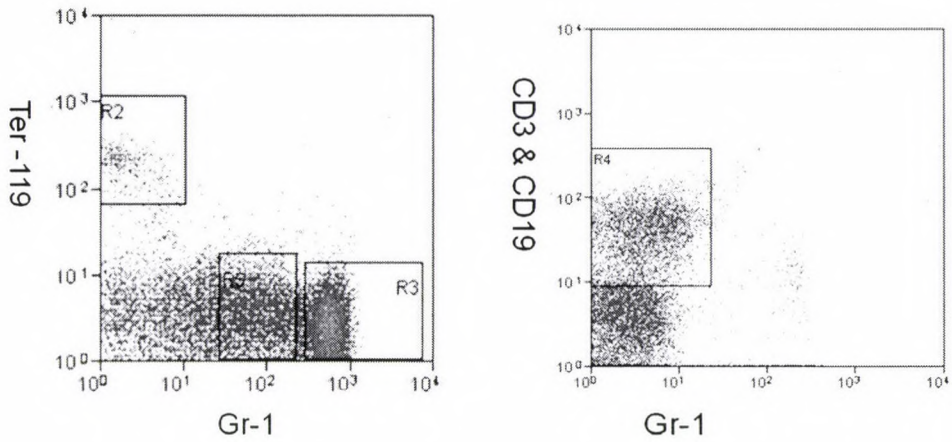
16. DeKoter, R.P., J.C. Walsh, and H. Singh, *PU.1 regulates both cytokine-dependent proliferation and differentiation of granulocyte/macrophage progenitors.* Embo J, 1998. **17**(15): p. 4456-68.

17. Metcalf, D., et al., *Inactivation of PU.1 in adult mice leads to the development of myeloid leukemia.* Proc Natl Acad Sci U S A, 2006. **103**(5): p. 1486-91.

18. Rosenbauer, F., et al., *Acute myeloid leukemia induced by graded reduction of a lineage-specific transcription factor, PU.1.* Nat Genet, 2004. **36**(6): p. 624-30.

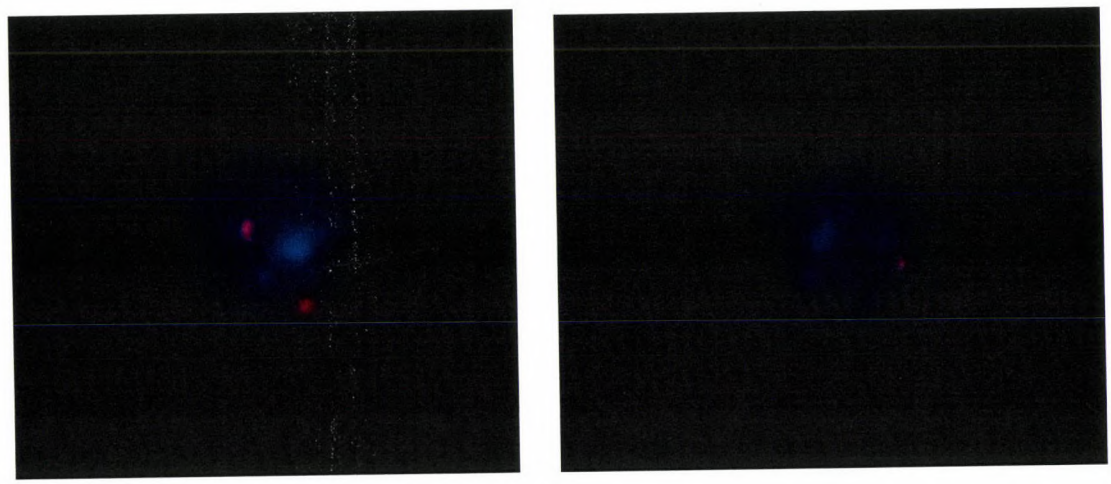
19. Walter, M.J., et al., *Reduced PU.1 expression causes myeloid progenitor expansion and increased leukemia penetrance in mice expressing PML-RARalpha.* Proc Natl Acad Sci U S A, 2005. **102**(35): p. 12513-8.

Figures and Table



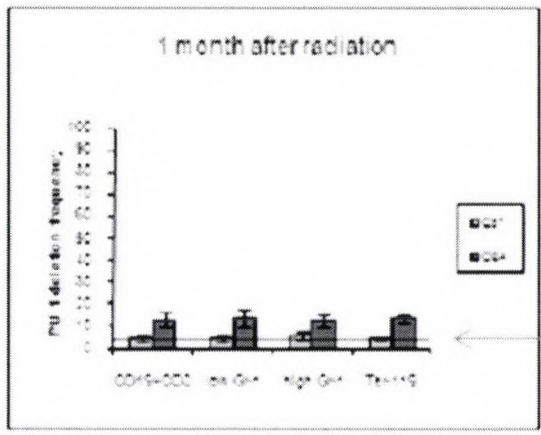
A B

Figure 3.1. Flow cytometric analysis and sorting on subpopulation of bone marrow from irradiated mice. (A) Erythroid cells in R2 window were positive for Ter-119 conjugated with APC, but negative for Gr-1 conjugated with FITC. R3 included mature granulocyte with high Gr-1 expression level. R5 included primitive cells in granulocyte/monocyte compartment with lower expression of Gr-1. (B) R4 included lymphocytes immunostained positively by CD3 and CD19 antibody conjugate with APC-cy7.

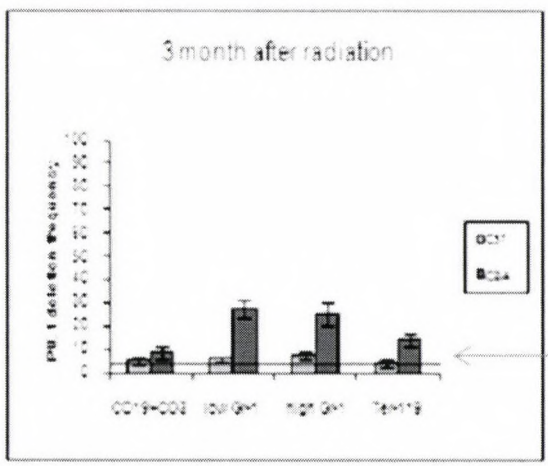


A B

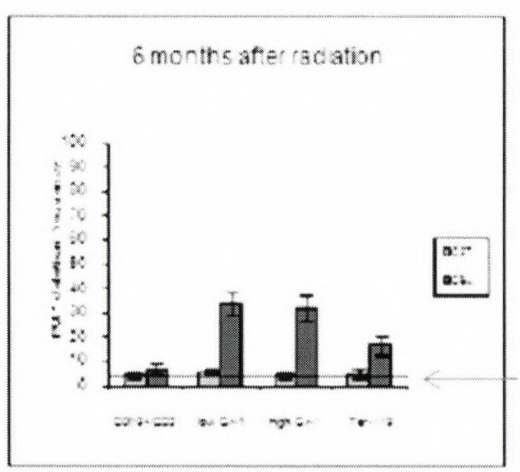
Figure 3.2. Examples of FISH analysis of sorted bone marrow subpopulations. Cells were FISHed with *PU.1* probe labeled with Alexa fluor 594-5-dUTP. Hybridization signals were stained with red fluorescence (Alexa fluor 594-5-dUTP). The cell nucleuses were counter stained with DAPI (blue). The cell in (A) was sorted as a lymphocyte and has two red signals. The cell in (B) was also sorted as a lymphocyte but has only one red signal, which indicated *PU.1* single copy deletion.


1 month after radiation
Cut off value

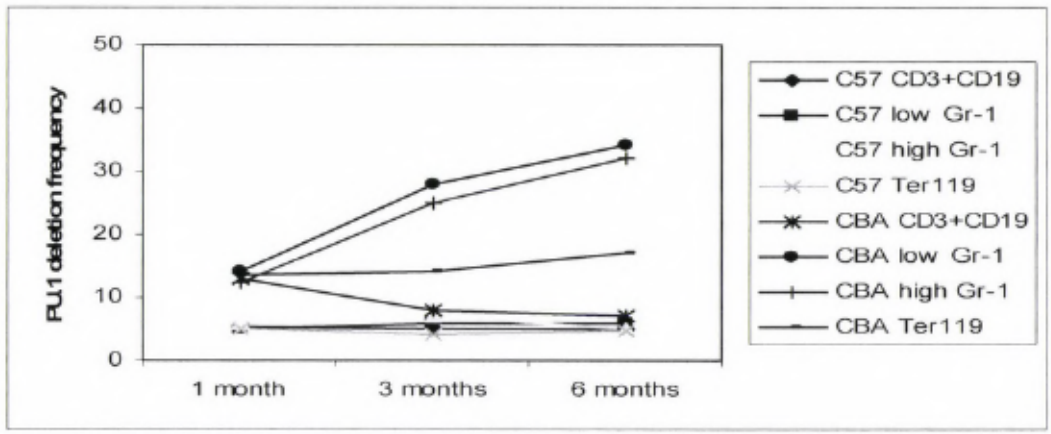
A


3 month after radiation
Cut off value

B


6 months after radiation
Cut off value

C



D

Figure 3.3. *PU.1* deletion frequency in different cells types in a time course of 1, 3 and 6 months after radiation exposure. (A) showed *PU.1* deletion frequency at one month after γ radiation. Each point is the average of 4 mice samples with standard error. No significant differences were found among different cell types in the same strain of mouse, CBA/CAJ or C57BL/6 ($P>0.05$). Significant differences do exist between CBA/CAJ and C57BL/6 strain in every cell type ($P<0.05$) at 1 month. Gr-1, granulocyte/monocyte; Ter-119, erythroid cells; CD3, T cells; CD19, B. (B) and (C) showed *PU.1* deletion frequency at 3 and 6 months after 3 Gy γ radiations, respectively. The *PU.1* deletion frequency increased with time in granulocyte/monocyte (Gr-1), and erythroid cells (Ter-119), but decreased in T (CD3) and B (CD19) lymphocytes in the CBA/CAJ mice only. In C57BL/6 mice, *PU.1* deletions were at a low level in all cell types at every timepoint. (D) The line chart showed the trends that frequency of *PU.1* deletion fluctuated over 6 months. Different trends in CBA/CAJ mice were observed between myeloid cells (granulocyte/monocyte and erythroid cell) and lymphoid cells (T and B cells), increased in myeloid cells but decreased in lymphoid cells.

Table 3.1. The immunophenotyping profiles of bone marrow samples

	1 month after 3 Gy		3 month after 3 Gy		6 month after 3 Gy	
	C57BL/6	CBA/CAJ	C57BL/6	CBA/CAJ	C57BL/6	CBA/CAJ
Lymphocyte (CD3 and CD19)	21.8±1.1%	27.0±4.7%	5.36±0.1%	17.9±3.4%	7.4±0.5%	31.5±0.7%
Low Gr-1	22.9±0.2%	24.4±3.1%	24.2±3.2%	25.4±1.7%	28.8±2.5%	28.6±1.6%
High Gr-1	26.8±2.5%	30.4±4.1%	34.4±1.0%	36.2±3.1%	36.3±1.3%	34.3±1.4%
Ter-119	8.7±0.6%	6.2±0.7%	2.7±0.7%	4.8±0.3%	1.5±0.7%	3.7±0.2%

Each data is the average of 4 mice with standard error. Bone marrow samples from these mice was analyzed separately.

Chapter 4

The originally transformed cells in radiation-induced acute myeloid leukemia: hematopoietic stem cells or restricted progenitors?

Abstract

Leukemia can be viewed as a newly formed, abnormal hematopoietic tissue initiated by a few transformed leukemic cells that undergo an aberrant and poorly regulated process analogous to that of normal hematopoietic stem cells (HSCs). A hallmark of all cancers is the capacity for unlimited self-renewal, which is also a defining characteristic of normal stem cells. Given this shared attribute, it has been proposed that leukemias may be initiated by transforming events that take place in hematopoietic stem cells. Alternatively, leukemias may also arise from more committed progenitors caused by mutations and/or selective expression of genes that enhance their otherwise limited self-renewal capabilities. Identifying the target cells for each type of leukemia is a current challenge and a critical step in understanding their respective biologies and may provide key insights into more effective treatments. Moreover, target cell identification and purification will provide a powerful diagnostic, prognostic, and therapeutic tool in the clinic. We set out to study this issue by characterizing *PU.1* deletion, a genetic event strongly associated with mouse radiation-induced acute myeloid leukemia, in phenotypically defined HSCs and restricted progenitors in irradiated mice and mice with radiation-induced leukemia. Our preliminary data validated the feasibility of our original goal and provided meaningful methods for the future study.

Introduction

Leukemia can be viewed as a clonal disease that malignant hematopoietic tissue is initiated by a few leukemic stem cells (LSCs)[1, 2]. In normal hematopoietic tissues, the formation of blood cells is supported by a few number of clonogenic cells, termed hematopoietic stem cells (HSCs), which are capable of both self-renewal and multilineage differentiation. HSCs have been isolated in both human and mice. HSCs can be divided into a long term subset (LT-HSC), capable of indefinite self-renewal, and short term subset (ST-HSCs) that self-renew for a defined interval. HSCs give rise to nonself-renewal oligolineage progenitors, which in turn give rise to progeny that are more restricted in their differentiation potential, and finally to functionally mature cells [3]. LSCs resemble HSCs in many respects, such as self-renewal, clonal expansion and differentiation. Many pathways associated with leukemogenesis also regulate normal stem cells development. Given all these shared attributes, it has been proposed that leukemias may be initiated by transforming events that take place in hematopoietic stem cells. Alternatively, leukemias may also arise from more committed progenitors caused by mutations and/or selective expression of genes that enhance their otherwise limited self-renewal capabilities. Until recently, however, the isolation and characterization of HSCs and progenitors subsets have not been well characterized and this represented a significant problem in studies investigating hematopoiesis and leukemia.

Using multicolor flow cytometry, the Weissman laboratory in particular succeeded identifying an ordered sequence of phenotypically distinct stem-cell and intermediate-progenitor populations, allowing for the first time the prospective isolation and characterization of these populations [3]. The Weissman model proposes that long-term

HSCs (defined phenotypically as $Lin^{-}IL\text{-}7R\alpha^{-}Sca\text{-}1^{+}c\text{-}kit^{+}FLT3^{-}Thy^{low}CD34^{-}$) are included within a low-frequency bone marrow subpopulation with the unique ability for life-long self-renewal and multilineage differentiation potential. Long-term HSCs give rise to short-term HSCs ($Lin^{-}IL\text{-}7R\alpha^{-}Sca\text{-}1^{+}c\text{-}kit^{+}FLT3^{-}Thy^{low}CD34^{+}$), which retain the ability for multilineage differentiation potential, but have decreased self-renewal potential. The next phenotypically distinct precursor population, the multipotential progenitors MPPs (Lin-IL7R-Sca1+ckit+FLT3lowThy-CD34+) has lost self-renewal potential completely, but maintains the abilities to differentiate into all blood-cell lineages. The first lymphoid progenitor population that is known to arise from MPPs is the common lymphoid progenitors (CLPs; Lin-IL7R+Sca-ckit-). CLPs have lost all myeloid potential, but retain the ability to form all cells of the lymphoid lineage. Analogous to the view that all lymphoid lineages develop form CLPs, the Weissman model proposes that all myeloid (including erythroid) cells arise from common myeloid progenitors (CMPs; Lin-Sca1-ckit+CD34+FcR-). CMPs subsequently develop into more specified progenitors, such as granulocyte/monocyte progenitors (GMPs; Lin-Sca1-ckit+CD34+FcR+), megakaryocyte/erythroid progenitors (MEPs; Lin-Sca1-ckit+CD34-FcR-) and, as has recently been shown, basophil progenitors, as well as shared macrophage and dendritic-cell progenitors.

The deletion of the *PU.1* gene from one allele of chromosome 2 is a genetic abnormality specifically associated with radiation-induced acute myeloid leukemia in mouse [4, 5]. In a study of γ radiation-induced acute myeloid leukemia in mouse, more than 90% of leukemic mice acquired deletion in one copy of *PU.1*, among them 87% appeared to have point mutation in Ets domain of the remaining *PU.1*allele [6]. Based on

these observations, the authors believed that *PU.1* was a tumor suppressor in mouse radiation-induced acute myeloid leukemia, and *PU.1* deletion in one copy of chromosome 2 is an early initiating event caused by the radiation; an additional hit is the point mutations in the remaining *PU.1*gene that may allow expansion of preleukemice cells. Therefore, the deletion event can be used as an early marker for the identification of the potentially transformed cells.

We predicted that characterizing *PU.1* deletions in phenotypically defined HSCs or progenitor subset in irradiated mice would provide valuable information for studying the origin or target cell of radiation-induced leukemia. Here, we explored the methodologies that might be useful for this. Those techniques include multicolor flow cytometric sorting on HSCs and progenitors, as well as *PU.1* fluorescence in situ hybridization (FISH) to sorted cells,

Materials and Methods

Mouse strains

CBA/H, C57BL/6, Balb/c and several other strains of mice were used in this experiment. The mice were housed in the Laboratory Animal Resource facility at Colorado State University (Fort Collins, CO). Animal handling and care procedures were approved by the Animal Care and Use Committee at CSU.

Bone Marrow Collection

According to the Weissman model, HSCs and progenitors subsets are low frequency bone marrow populations, less than 0.1%. We decided to pool bone marrow from 10 mice into one sample, in order to obtain enough cells for further experiments. Mice were sacrificed and bone marrow cells were flushed from each femur, tibia and radius with PBS. The bone marrow PBS suspension was centrifuged at 1000rpm for 8 min at 4 ^{o}C. The cell pellet was then mixed well with ACK lysis buffer (NH_4Cl 8g/l, $KHCO_3$ 1g/l, and $EDTANa_2 \cdot 2H_2O$ 3.7g/l), incubated for 5 min at 20 ^{o}C, resuspended in PBS at 10^8 cells/ml for further flow cytometric analysis/sorting.

Lineage depletion of bone marrow cells

In order to enrich the HSCs and progenitors subpopulations, lineage positive cells in bone marrow were depleted by magnetic separation using Cell Separation Columns (products of Miltenyi Biotech) and strepavidin microbeads. In brief, the cells were first stained with biotinylated primary antibodies specific for the following lineage markers: CD3, CD19, Ter-119, and Gr-1. Subsequently, the cells were magnetically labeled with

straptavidin microbeads. Then the cells suspension was loaded onto a Cell Separation Column which is placed in a strong magnetic field. The magnetically labeled cells were retained in the column while the unlabeled cells run through. The unlabeled cells were collected in PBS buffers for further isolation.

HSCs and progenitors phenotyping and separation.

Following lineage depletion, the remaining cells was incubated at 20 °C for 30 min with the following antibodies: APC conjugated anti-FcγRII/III, FITC conjugated anti-CD34, Pacific blue conjugated anti-Sca1, APC-cy7 conjugated anti-c-kit, and PE-cy7 conjugated anti-IL-7Rα. After antibody labeling, cells were washed twice with phosphate-buffered saline, pH 7.4 (PBS). Flow cytometric analysis and sorting of subpopulations were performed using a MoFlo high speed cell sorter. Forward light scatter, perpendicular light scatter, and fluorescence signals were measured for each cell and saved in list mode data files using Summitview software.

Marrow cells without incubation with antibody were used as negative control. Cells gated on the basis of immunofluorescence intensity were sorted into 15ml tubes with PBS. The sorted cells were centrifuged at 1000rpm for 8 minutes at 4 °C. The cell concentration was then placed into an incubation mixture (9 ml 0.075M KCl), mixed and incubated for 30 min at 37°C. After incubation, 5 ml fresh Carnoy's fixative (3:1 methanol : acetic acid) was added, mixed and centrifuged at 1000 rpm for 5 min. Cells were washed with fresh fixative two more times. The supernatant was decanted, a small amount of fixative was added, and cells were dropped onto clean, wet microscope slides and allowed to dry.

Fluorescence in situ hybridization (FISH)

The RP23-263H8 BAC clone containing the murine *PU.1* gene was used to make FISH probe for measurement of *PU.1* loss. The BAC probe provided by Dr. Yuanlin Peng was 197kbp in size and labeled with Alexa fluor 594-5-dUTP or FITC by nick translation. FISH was performed as described by Peng et al. with the following modifications. Briefly, air-dried cells were treated with freshly prepared Carnoy's fixative for 5 minu, allowed to air dry, and then baked at 65°C for 15 min. Cellular DNA was denatured at 72°C for 2.5 min in 70% formamide (Omnisolv; EM Science, Gibbston, NJ), saline sodium citrate (SSC; 0.30 mol/L NaCl, 0.03 molL sodium citrate, pH 7.0). After dehydration in ethanol series and air-drying at 42°C, DNA probe (1 to 2 uL) was denatured in hybridization mixture [fomamide, 50%; dextran sulfate (Sigma. St Louis. MO). 10%; 2x SSC] at 72°C for 5 min and immediately applied to cells on slides that were hybridized overnight in a humidified chamber at 37°C. Slides were washed three times at 45°C in 50% formamide 2X SSC; and once each in 2X SSC and 0.2 X SSC. Chromosomal DNA was counterstained with diamino-2-phenyl-indole dihydrochloride (DAPI) in antifade solution.

Microscope analysis

The slides were evaluated using a Zeiss fluorescence microscope equipped with following filter sets: DAPI and Texas red. The frequencies of cells showing hybridization signals and the number of signals per cell were determined. Cells with disrupted nuclear membranes were excluded from the analysis. For each cell group, at least 50 nuclei were evaluated. Hybridization signals were considered to represent two copies of *PU.1*if the

fluorescent hybridization signals were separate and nonoverlapping. Cells were considered to be *PU.1* hemizygous deletion if the cell showed one hybridization signal corresponding to the control slide. The hybridization efficiency (i.e, two hybridization signal) of control DNA probes exceeded 96%.

Results

Flow cytometric analysis and sorting on HSCs and progenitor subsets

Using the antibodies proposed by the Weissman laboratory, we were able to isolate several distinct bone marrow subpopulations. HSCs existed exclusively in $Lin^-Sca1^+c\text{-}kit^+$ fraction (Figure 4.1 B). $Lin^-Sca1^-c\text{-}kit^+$ fraction included both lymphoid and myeloid progenitors (Figure 4.1B). The expression of the interleukin-7 receptor a chain (IL-7Ra) marks the common lymphoid progenitors (CLPs) and other downstream lymphoid progenitors, therefore the lymphoid progenitors were found in $Lin^-\ Sca1^-c\text{-}kit^+\ IL\text{-}7Ra^+$ fraction (Figure 4.1C), and the myeloid progenitor in $Lin^-\ Sca1^-c\text{-}kit^+\ IL\text{-}7Ra^-$ fraction (Figure 4.1C). $Lin^-\ Sca1^-c\text{-}kit^+\ IL\text{-}7Ra^-$ fraction could be further divided into three subpopulations according to the expression profiles of the of the Fcγ receptor-II/III (FcγR), an important marker for myeloimonocytic cells and a progenitor marker in fetal liver hematopoiesis, and CD34, which marks a fraction of hematopoietic stem cells and progenitors: the $Fc\gamma R^{hi}CD34^+$, $Fc\gamma R^{lo}CD34^-$, and $Fc\gamma R^{lo}CD34^+$ populations (Figure 4.1D). The $Fc\gamma R^{hi}CD34^+$ cells were shown to form colonies composed only of macrophages and/or granulocytes such as CFU-M, CFU-G, or CFU-GM in response to any of the growth factor combinations, and are therefore termed granulocyte/macrophage lineage-restricted progenitors (GMPs). The $Fc\gamma R^{lo}CD34^-$ cells were believed to give rise to CFU-Meg, BFU-E or CFU-MegE colonies that contained only megakaryocytes and/or erythrocyte, and are thus termed megakaryocyte/erythrocyte lineage-restricted progenitors (MEPs).The $Fc\gamma R^{lo}CD34^+$ populations are upstream of both $Fc\gamma R^{hi}CD34^+$ GMP and $Fc\gamma R^{lo}CD34^-$ MEP cells and termed common myeloid progenitors (CMPs).

The compartment sizes of HSCs and progenitors subsets in our study were estimated to be the following: HSCs 0.01%, CLPs 0.002 %, CMPs 0.01%, GMPs 0.03%, and MEPs 0.01%. The compartment sizes are smaller than those reported by Weissman's laboratory, which can be due to more stringent gating standard we have used in this study. The stringent gating standard should maximize the purity of sorted cell populations and reduce false conclusion in further experiments. Conversely, the stringent gating standard may cause insufficient cell numbers for further cytogenetic examination. One way to solve this problem would be to increase the number of mice sacrificed, which is both uneconomical and laborious. Another way to enrich the sorted cell subpopulation would be to culture sorted cells in the presence of cell growth factors, which is probably a better way since morphological and biological characteristics can be identified in the cultured cells. Analogous to bone marrow stem cells, mammary stem cells have been successfully cultured and studied in our lab using a mammasphere system (Magers et al. in manuscript). However, we have yet not applied the culture method to increase cell number in this study.

PU.1 FISH assay of sorted cell subsets

Examples of *PU.1* FISH applied on sorted HSCs and progenitor subsets are shown in Figure 4.2. The FISHs applied were generally successful and spontaneous *PU.1* loss is scarcely found (less than 0.5%) in sorted HSCs and progenitors. Further experiments will focus on characterizing *PU.1* loss in those hematopoiesis subpopulations in irradiated mice and/or leukemic mice.

Discussion

For most leukemias, as for most cancers, the target cell of transformation is still unknown. Because normal stem cells and leukemic cells share the ability of self-renewal, as well as various developmental pathways, it has been postulated that leukemia arises from HSCs that have become leukemic as the result of accumulated mutations. HSCs persist through a life time thus have a much higher risk to accumulate genetic abnormalities than more mature cells. In addition, HSCs are naturally self-renewing and require fewer mutations during the transformation from normal cells into leukemic phenotype. Alternatively, leukemia can be originated from more restricted progenitors such as CMPs and CLPs when they somehow gained the capacity of self-renewal and extensive proliferation. The onset of AML has been studied extensively, but the origin of the cells is still inconclusive.

Studies by Pallavicini and colleagues using FISH and flow sorting of hematopoietic subpopulation have demonstrated that high frequencies of aberrant (aneuploid) cells were present in the primitive stem-cell compartment (CD34+CD38-) of a patient with AML, aberrations were also seen in lymphoid and erythroid cells in cases where the predominant leukemic cells lacked lymphoid or erythroid differentiation markers [7]. Based on those observations, the authors suggested that certain types of human AML arise from mutations that accumulated in HSCs. However, several other studies have indicated that, in some samples obtained from MDS or AML patients, monosomy 7 and trisomy 8 seen in leukemic cells were only present in myeloid lineage but not in lymphoid lineage, indicating the transformation occur in committed myeloid progenitor [8-10]. Moreover, in APML patient samples, the M3 subtype of AML, it has

been shown that the APML-associated fusion gene PML/retinoic acid receptor α (RARα) , which results from the t(15,17) balanced reciprocal translocation, was present in CD34-CD38+ cell population but not in CD34+CD38- HSC-enriched cell population. Currently, human HSCs and progenitors subsets have not been well characterized and this may represent a significant obstacle for investigating the origin of AML. Using mouse models, Weissman and coworkers in particular succeeded in identifying an ordered sequence of phenotypically distinct stem-cell and intermediate-progenitor populations. This allowed for the first time the prospective isolation and characterization of these populations. It is obvious that the nonambiguous definition of HSCs and progenitors in the mouse model will cast new light on the study of target cells in human AML.

Although the origination of spontaneous AML, which refers specially to those AML patients without a history of exposure to chemical or radiological carcinogens, has been extensively studied, few studies have been reported about chemical or radiation-induced AML, a type of AML that possibly has a different origin and etiology from spontaneous AML. The present study tries to explore this question by characterizing cytogenetic aberration in HSCs and downstream progenitors using a mouse model of radiation-induced AML.

Increasing evidence has shown leukemia originated from and was sustained by a few 'stem-cell-like' tumor cells which acquired self-renewal and extensive proliferation capacity, so termed LSCs. The concept of LSCs emerged in 1970's from several studies showing only a small subset of leukemia cells was capable of extensive proliferation in *vivo* and in *vitro*. However, it is not until the study published by Blair and coworkers that researchers have tried to phenotypically identify and purify LSCs. In their study, LSCs

for human AML were identified prospectively and purified as $Thy^{-}CD34^{+}CD38^{-}$ cells from various patient samples. Although these cells represent a small and variable proportion of the total AML cells, they are the only cells capable of transferring AML from human patient to nonobese diabetic/severe combine immunodefecient (NOD/SCID) mice and were referred as SCID leukemia-initiating cells or SL-IC [11]. LSC identification and prospective isolation would facilitate the study of gene expression profiles of malignant progenitors compared with their normal counterparts by using microarray technology. Purified LSCs will also provide a powerful substrate for preclinical testing of the efficacy of newly developed chemotherapeutic agents, immune-based therapies, and biologic response modifiers. Until recently, the identification and prospective isolation of LSCs mostly depended upon a cell's phenotype and capacity to form colonies in immunosupressive recipient mice. The present study providesvj new method for the identification and purification of LSCs: the combination of both flow cytometric sorting and cytogenetic analysis could potentially be a more precise method than simply depending on flow cytometric sorting for the isolation of LSCs.

References

1. Fialkow, P.J., *Stem cell origin of human myeloid blood cell neoplasms.* Verh Dtsch Ges Pathol, 1990. **74**: p. 43-7.
2. Fialkow, P.J., et al., *Clonal development, stem-cell differentiation, and clinical remissions in acute nonlymphocytic leukemia.* N Engl J Med, 1987. **317**(8): p. 468-73.
3. Akashi, K., et al., *A clonogenic common myeloid progenitor that gives rise to all myeloid lineages.* Nature, 2000. **404**(6774): p. 193-7.
4. Bouffler, S.D., et al., *Chromosome 2 hypersensitivity and clonal development in murine radiation acute myeloid leukaemia.* Int J Radiat Biol, 1997. **72**(2): p. 181-9.
5. Azumi, J.I. and L. Sachs, *Chromosome mapping of the genes that control differentiation and malignancy in myeloid leukemic cells.* Proc Natl Acad Sci U S A, 1977. **74**(1): p. 253-7.
6. Cook, W.D., et al., *PU.1 is a suppressor of myeloid leukemia, inactivated in mice by gene deletion and mutation of its DNA binding domain.* Blood, 2004. **104**(12): p. 3437-44.
7. Mehrotra, B., et al., *Cytogenetically aberrant cells in the stem cell compartment (CD34+lin-) in acute myeloid leukemia.* Blood, 1995. **86**(3): p. 1139-47.
8. Kibbelaar, R.E., et al., *Combined immunophenotyping and DNA in situ hybridization to study lineage involvement in patients with myelodysplastic syndromes.* Blood, 1992. **79**(7): p. 1823-8.

9. Anastasi, J., et al., *Cytogenetic clonality in myelodysplastic syndromes studied with fluorescence in situ hybridization: lineage, response to growth factor therapy, and clone expansion.* Blood, 1993. **81**(6): p. 1580-5.

10. Jacobson, R.J., et al., *A clonal complete remission in a patient with acute nonlymphocytic leukemia originating in a multipotent stem cell.* N Engl J Med, 1984. **310**(23): p. 1513-7.

11. Blair, A., et al., *Lack of expression of Thy-1 (CD90) on acute myeloid leukemia cells with long-term proliferative ability in vitro and in vivo.* Blood, 1997. **89**(9): p. 3104-12.

Figures

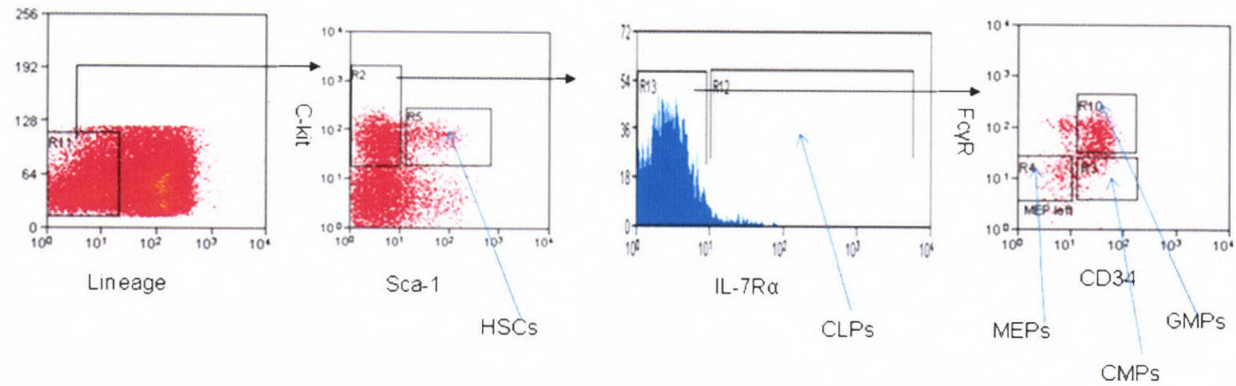


Figure 4.1. Isolation of HSCs, CLPs, CMPs, GMPs, and MEPs in mouse bone marrow.

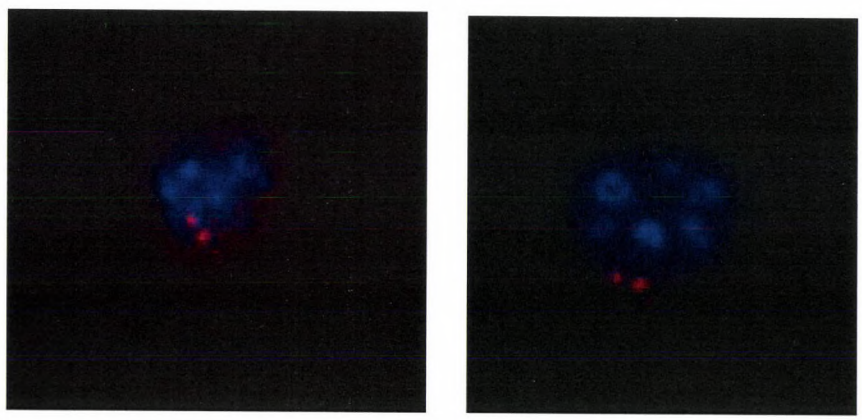


Figure 4.2. Examples of *PU.1* FISH applied on sorted HSCs and CMP. (A) HSC; (B) CMPs.

Chapter 5

A strategy for the measurement of PU.1 point mutation in radiation-induced murine AML: rolling circle amplification of padlock probe

Abstract

In murine radiation-induced AML the *PU.1* (*Sfpi1*) gene behaves as a classic tumor suppressor gene. More than 90% of the cases of murine myeloid leukemia suffer the deletion of *PU.1* gene on one copy of chromosome2. The second 'hit' is generally a point mutation in the remaining *PU.1* allele. Unlike murine leukemia, PU.1 mutations are rarely found in human AML. Point mutations in PU.1 gene are considered to be a low incident event in human AML and in the early development of murine AML. For the detection of point mutation in a small fraction of cells, DNA sequencing, the traditional method to analyze point mutation, is not adequate. A novel rolling circle amplification (RCA) of padlock probe has been reported to be an alternative method for the detection of a single nucleotide change. Here we designed experiments to examine the fidelity and sensitivity of this novel technique. Our results indicated the RCA of a padlock probe is not reliable for the analysis of a single nucleotide change in cells unless further a improvement of this probe is made. The low efficiency of applying padlock probe to in situ cytological target observed in our study also hampers the using of this technology in our project. However, further modification could be made to improve the fidelity and sensitivity of this potential technique.

Introduction

The mouse model of radiation-induced acute myeloid leukemia has been used to localize a putative tumor suppressor gene to a 1.0cM region homologous to the human chromosomal segment 11p11-p12[1]. PU.1 (sfpi1) maps to this chromosomal region and the majority (>90%) of induced AMLs were found to have lost one copy of the gene. In the tumors with PU.1 hemizygous deletion, 87% of the cell lines had point mutations in the DNA-binding Ets domain of the remaining PU.1 allele and the most commonly found point mutations were 703C-T (62%) [2]. In contrast to mouse radiation-induced AML, PU.1 mutations are rarely found in human AML and the frequency with which PU.1 mutations arise in human AML remains controversial. Muller et al. reported 7% (9/126) PU.1 heterozygous mutations in human AML [3]. The 10 mutant alleles identified affected the Ets DNA-binding domain, the Pest domain and the transactivation domain. However, several subsequent studies have failed to identify a single mutation [4, 5]. For all of those studies, point mutations were identified by DNA sequencing, the results of which only represent the majority of the many cell analyzed and could possibly neglect a small number of cells bearing point mutations. Therefore, rather than averaging across many cells or whole tissues and all phases of the cell cycle, it is important to develop assays that allow point mutations to be monitored in individual cells and subcellular compartments with sufficient precision. A novel in situ hybridization with a padlock probe is one of the possible assay formats [6, 7].

The padlock probe was developed by Nilsson M. et al. in 1994 [6], in order to discriminate point mutations within a gene sequence. The padlock probe is actually a linear probe composed of two target complementary segments in 3' and 5' terminal

regions. The two ends of the probe are juxtaposed by hybridization to a target sequence and can be circularized by enzymatic ligation, becoming topologically locked to the target (Figure 5.1). The requirement for simultaneous hybridization of two different probe segments to target molecules and the following act of ligation should permit distinction of a single base mismatch.

The padlock probes can be amplified by RCA, a novel amplification technology based on the rolling circle replication mechanism used by many viruses to rapidly generate multiple copies of their genome. RCA is able to generate long single-stranded DNA molecules containing tandem repeats complementary to the padlock probe sequence. Up to ten thousand copies of the circle can be produced at the site of padlock probe binding on the glass surface and these RCA products can be visualized by hybridization with fluorescently labeled probe. A study by Lizardi et al. showed padlock probes could serve as templates for RCA and could be amplified more than ten thousand times after 20 minutes [7][Figure 5.2].

Application of RCA to in situ targets in fixed or permeabilized cells has not been uniformly successful to date. Whereas recent work has demonstrated that the concept is viable, DNA detection efficiencies of 20-30% lessen the utility of RCA as an assay [7, 8]. Lack of success has been attributed to the background noise and possible blocking of the enzymes by target strands. In an interesting approach, target sequences were enzymatically rendered single stranded having 5' ends near the binding sites for padlock probes and the results showed the efficiency of DNA detection was increased to about 90% following this enzymatic treatment [9].

We set out to apply FISH based on rolling circle amplification of padlock probe in this project to detect a PU.1 point mutation in bone marrow cells of irradiated mice. In order to do this, we first need to examine whether this technique has high fidelity to detect a single base pair difference in point mutated cells, and whether this technique has high sensitivity to find small amount of abnormal cells mixed with normal cells.

Methods and materials

DNA oligonucleotides.

Oligonucleotides containing a phosphate group at the 5′ end were purchased from the Colorado State University Macromolecular Resources facility (Table 5.1)

Table 5.1. Sequences of Oligo

Oligo	Sequence
Artificial Mutant Target	5'-CAT*GGGCGGCATGAACCAGAGGCCCATCCTCAC*CTTC-3'
Artificial Wildtype Target	5'-CAT*GGGCGGCATGAACCGGAGGCCCATCCTCAC*CTTC-3'
Mutant Probe	5'-P-*GGTTCATGCCGCCC*GGATTAACCCTCACTAAAGGGACCCTATAGTGAGTCGTATTA CC*GTGAGGATGGGCCTCT*-3'
Primer	5'-TAATACGACTCACTATAGGG-3'

Oligo was purchased from Macromolecular Resources in CSU. P, 5' phosphate. Target complementary sequences of padlock probes are shown in italics. The single nucleotide mutated is colored red.

Cell lines with point mutation for in situ hybridization experiment.

Two cell lines were used in these experiments. One was a human lymphoblastoid (HLB) line (Coriell Cell Repositories, Camden, NJ), putatively normal with regard to karyotype and gene expression. The other was a Molt-4 lymphoid cell line (American Type Culture Collection, ATCC) derived from a patient with acute lymphoblastoid leukemia. HLB cells were expected to have two normal copies of the Tp53 gene and to be normal with regard to Tp53 expression. Molt-4 cells are reported by ATCC to express no normal Tp53 and to have one normal and one or more abnormal copies of the Tp53 gene, in which there is a G to A transition in codon 248 of exon 7.

Enzymatic treatment to induce single strand DNA target.

Cells were prepared for DNA detection by first incubating in a hypotonic solution (0.075 M KCl) for 30 min at 37°C followed by three fixations in methanol : acetic acid (3:1 vol : vol) and dropped on clean glass microscope slides. Fixed cells on slides were covered with 50 µl of ribonuclease A (500 µg/µml, Roche Biochemicals) under a glass coverslip. Slides were incubated 1 h at 37°C and then rinsed with sterile water. Restriction enzymes were used to cut 20 base pairs either 3' or 5' of the sequence of interest, Tp53 in this case. Either *Afl*III or *Bbs*I (0.1 unit/µl, New England Biolabs) was applied for 12 h at 37°C. Cells were treated with exonuclease III (1.3 units/µl, Life Technologies, Grand Island, NY) in 1X exonuclease III buffer (50mMTris, pH 8.0, 5 mM $MgCl_2$, and1 mM DTT), then incubated 1 h at 37°C and rinsed with sterile water.

FISH based on rolling circle amplification of padlock probe.

Sequences of designed probes were shown in Table 5.1. Simultaneous hybridization and ligation were performed with 0.8 µM of probe, 20 units of Ampligase thermostable DNA ligase (0.43 unit/µl, Epicentre Technologies, Madison, WI), and 1X Ampligase buffer. The 50-µl reaction was placed on the slide, covered with a glass coverslip, and sealed with rubber cement. The slide was heated to 94°C for 10 min to ensure that both probe and target DNA were single stranded and then lowered to 42°C for 1 h to allow hybridization and ligation of the probe. Slides were washed in 2 X SSC at 42°C for 15 min, rinsed in sterile water, and blown dry. The RCA reaction mixture consisted of 4 µM of T7 primer, 200 µM of each dNTP (Roche), either 63 nM digoxigenin-11-dUTP (Roche) or 63 nM biotindUTP (Roche), 2 units of ø29 DNA

polymerase with pyrophosphatase (United States Biochemical), and 1X ø29 DNA polymerase buffer, which was added to the slide, which was then covered with a coverslip, sealed with rubber cement, and heated 12 h at 54°C. Slides were washed in 2 X SSC at 45°C for 5 min, 1 X PBS at 45°C for 5 min, and rinsed in sterile water at room temperature. Anti-digoxigenin–fluorescein antibody (Roche, 200 ng/µl) was incubated on the slide at 37°C for 10 min and washed 2 X 5 min in 1 X PBS at room temperature. Slides were mounted in 4',6-diamidino-2- phenylindole in anti-fade and viewed with an Axiophot fluorescence microscope (Zeiss).

Results and discussion

Rolling circle amplification of padlock probes

The padlock probes described contain two adjacent probe sequences of 20 bases. When hybridized with the target DNA sequence, the probe formed a circle which was ligated by DNA ligase to form a closed padlock probe (Figure 5.3A). Primer complement to the linker segment of the padlock probe then was used to amplify the closed padlock probe (Figure 5.3B). We designed a padlock probe for a 46-nt target sequence in the *T53* gene locus with a point mutation in exon 7. The circularizable probe was first hybridized with an artificial DNA target bearing that certain point mutation, and then was ligated by T4 ligase to form a closed padlock. To catalyze this linear RCA reaction, we used the DNA polymerase of phage ø29 [10], a highly processive enzyme that displays strand-displacing activity in the absence of additional proteins or cofactors. We analyzed the primer extension by gel electrophoresis in a denaturing alkaline agarose gel. Consistent with previous studies, RCA products with more than 4136 nucleotides were generated after a 30 mins reaction if the there was no mismatch between padlock probe and artificial target (Figure 5.4). The observed reaction rate is approximately 53 nt per second, a value consistent with previously published data on the replication of single stranded M13 DNA by ø29 DNA polymerase. Sequence analysis of cloned RCA products revealed that the amplified DNA contained the sequence expected for each different target-dependent, allele-specific, circle-ligation event. In order to assure that the padlock probe is able to discriminate that point mutation from the wild type DNA sequence, the probe was also incubated with artificial wild type DNA target that has a single nucleotide mismatch with the probe, followed by rolling circle amplification with

the same primer. To our surprise, the padlock probe incubated with the wild type target was also amplified by RCA and the amplified DNA product was observed on the agarose gel at the similar size (Figure 5.5). The RCA products were lesser in the wild type panel, suggesting RCA of the padlock probe with one base pair mismatch is less efficient than that of the probe matched perfectly with target. Our results argued that the fidelity of routing RCA amplification of padlock probe was not high enough for identifying point mutations and were not in agreement with what reported by Nilsson et al.

Detection of padlock probes in cytological samples

Although linear RCA of padlock probe was shown efficient in the above experiments, we wanted to test this amplification reaction for the detection of padlock probes *in situ.* In spite of the efficiency of linear RCA in solution, padlock probes represent a serious challenge for amplification in cells. Differential chromatin condensation and probe accessibility within a cellular structure could present obstacles for a rolling-circle reaction. Indeed, attempts to detect the TP53 locus in metaphase chromosomes by RCA of ligated padlock probes were unsuccessful. In the interphase cells, the efficiency of detecting RCA signal was about 20% (Figure 6.6A), which is in agreement with some former studies [7, 8]. However, a technique with an efficiency of 20% is not sensitive enough for the detection of low incidence genetic event, such as point mutation in human AML or early developed murine AML. Besides, nonspecific signals were also found in the negative control cells (Figure 5.6B), similar to nonspecific RCA amplification in solutions (Figure 5.5).

Our study has thrown some suspects on the fidelity and sensitivity of RCA amplification of padlock probe. However, further modifications can be made to improve the fidelity of this technique. For example, an asymmetric padlock probe, which make the hybridization unstable, has been reported to improve the fidelity of padlock probe[11]. To improve the efficiency of padlock probe applied to in situ detection, modifications should be made to decondense or deproteinize the genetic material within cells for the purpose of a better accessibility to both enzymes and probes.

References

1. Silver, A., et al., *Molecular mapping of chromosome 2 deletions in murine radiation-induced AML localizes a putative tumor suppressor gene to a 1.0 cM region homologous to human chromosome segment 11p11-12.* Genes Chromosomes Cancer, 1999. **24**(2): p. 95-104.
2. Cook, W.D., et al., *PU.1 is a suppressor of myeloid leukemia, inactivated in mice by gene deletion and mutation of its DNA binding domain.* Blood, 2004. **104**(12): p. 3437-44.
3. Mueller, B.U., et al., *Heterozygous PU.1 mutations are associated with acute myeloid leukemia.* Blood, 2002. **100**(3): p. 998-1007.
4. Dohner, K., et al., *Mutation analysis of the transcription factor PU.1 in younger adults (16 to 60 years) with acute myeloid leukemia: a study of the AML Study Group Ulm (AMLSG ULM).* Blood, 2003. **102**(10): p. 3850; author reply 3850-1.
5. Ley, T.J., et al., *A pilot study of high-throughput, sequence-based mutational profiling of primary human acute myeloid leukemia cell genomes.* Proc Natl Acad Sci U S A, 2003. **100**(24): p. 14275-80.
6. Nilsson, M., et al., *Padlock probes: circularizing oligonucleotides for localized DNA detection.* Science, 1994. **265**(5181): p. 2085-8.
7. Lizardi, P.M., et al., *Mutation detection and single-molecule counting using isothermal rolling-circle amplification.* Nat Genet, 1998. **19**(3): p. 225-32.
8. Zhong, X.B., et al., *Visualization of oligonucleotide probes and point mutations in interphase nuclei and DNA fibers using rolling circle DNA amplification.* Proc Natl Acad Sci U S A, 2001. **98**(7): p. 3940-5.

9. Christian, A.T., et al., *Detection of DNA point mutations and mRNA expression levels by rolling circle amplification in individual cells.* Proc Natl Acad Sci U S A, 2001. **98**(25): p. 14238-43.

10. Blanco, L. and M. Salas, *Characterization and purification of a phage phi 29-encoded DNA polymerase required for the initiation of replication.* Proc Natl Acad Sci U S A, 1984. **81**(17): p. 5325-9.

11. Szemes, M., et al., *Diagnostic application of padlock probes--multiplex detection of plant pathogens using universal microarrays.* Nucleic Acids Res, 2005. **33**(8): p. e70.

Figures

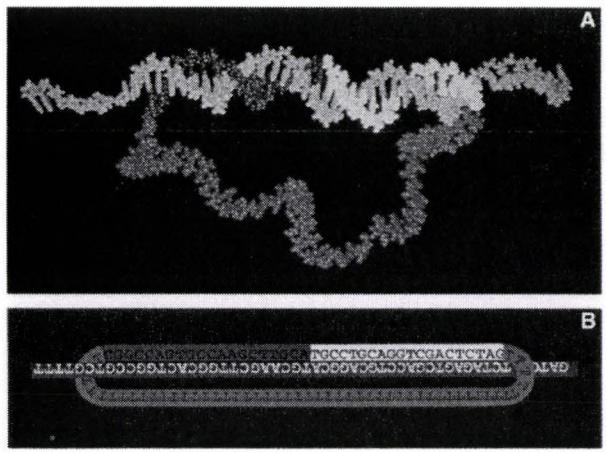


Figure 5.1. structure of a padlock probe interacting with its target sequence. (A) molecular model of the probe-target complex. (B) sequence composition of a probe. At the 5' end of the probe, beginning with a phosphate group, 20 target-complementary nucleotide positions are shown in red. Directly contiguous with these is a linker segment of 50 T residues, shown in green. Finally, the 20 nucleotides at the 3' end of the probe are yellow. The target sequence is shown in blue. (adopted from Nillson et al. 1994)

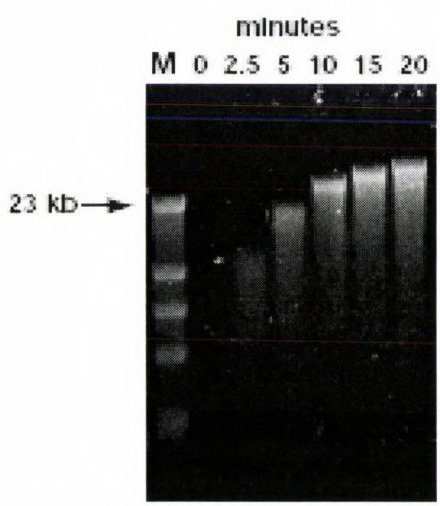
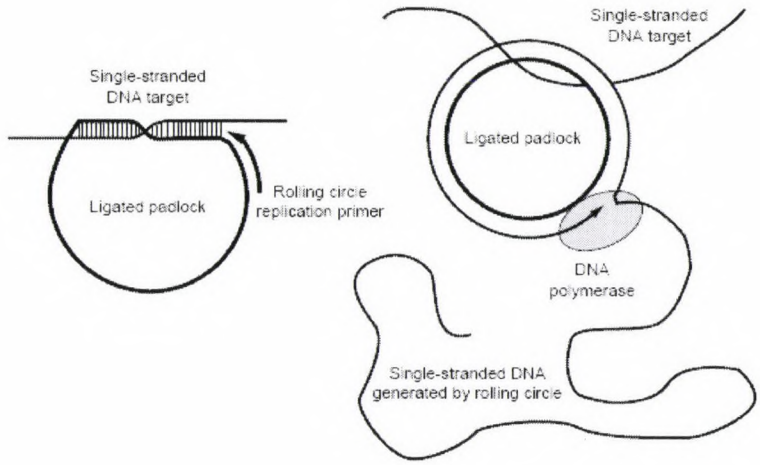


Figure 5.2. Analysis of RCA products. Time course of amplification of circularized probes, catalyzed by ø29 DNA polymerase (Lizardi et al. 1998).



A B

Figure 5.3. Illustration of rolling circle amplification of padlock probes (A) Hybridized and Ligated (padlock) probe, and binding of complementary primer for rolling circle amplification. The primer 3′ end is located five or six bases away from the last paired base in the hybridized probe arm. (B) Rolling-circle amplification of a padlock probe, catalyzed by a strand displacing DNA polymerase.

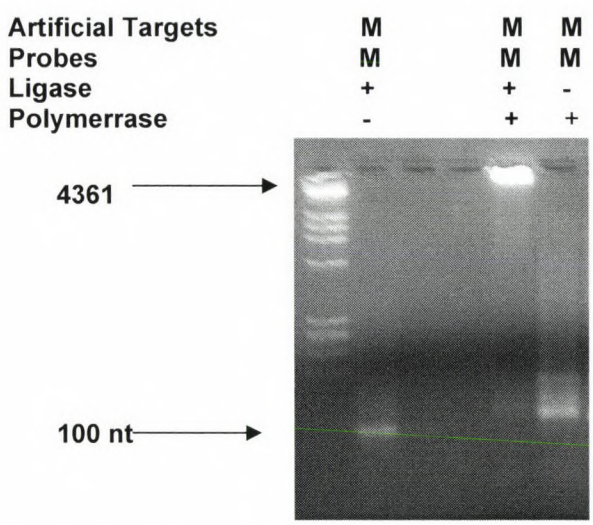


Figure 5.4. RCA reaction after 30 minutes. M, mutant target or probe designed for mutant target.

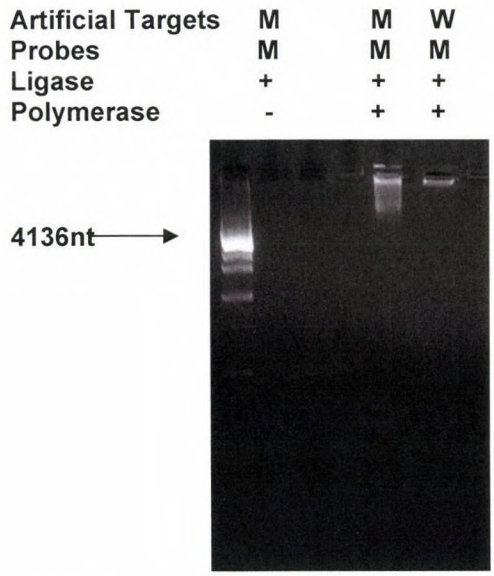
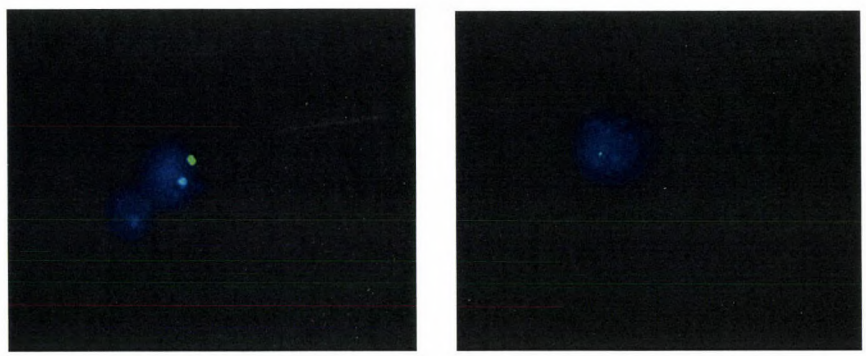


Figure 5.5. RCA reaction after 30 minutes. M, mutant target or probe designed for mutant target; W, wild type target.



A B

Figure 5.6. (A) RCA detected in Molt-4 cells in the presence of digoxigenin-dUTP. (B) RCA signal detected in negative control lymphoblast in the presence of digoxigenin-dUTP

Chapter 6

Discussion

Risk of radiation exposure comes from various resources in modern world, such as patients receiving radiological therapy, and astronauts traveling through outer space. AML is one of the most frequently diagnosed hematopoietic malignancies as a consequence of radiation exposure. In spite of extensive research being carried, the pathogenesis of AML is still not well understood, which hampers successful therapy of this disease. Animal models, especially mouse models, have been utilized to help investigate the pathogenesis of AML and those models did provide valuable insight for AML study. Based on numerous studies on human patients and mouse models, a recent view of AML proposed disruption of transcription-factor function can disrupt normal cellular differentiation and lead to cancer. Transcription factors, for example PU.1, C/EBPα, AML1, and c-jun, have been recognized as key components in the orchestration of myeloid-cell differentiation and maturation [1, 2]. These transcription factors regulate the expression of many myeloid genes, such as those encoding receptors for colony-stimulating factor M-CSF, G-CSF, and GM-CSF, and those encoding granule components such as lactoferrin and neutrophil gelatinase [3], thereby explaining why their dysfunction can be detrimental to the development of the myeloid lineage and will be especially myeloid-leukemogenic.

PU.1 is one of the transcription factors closely linked with both hematopoiesis and myeloid leukemogenesis. Mice with PU.1 knocked out were fatal in embryo due to severe hematopoietic defects in the development of myeloid cells and B cells [4]. Moreover, PU.1 expression levels seem to be a crucial determinant in cell-fate decisions of both myeloid and lymphoid progenitors: in lymphoid lineage, low levels of PU.1 are associated with B-cell development; whereas in the myeloid lineage, low PU.1 levels

seem to support granulocyte production [5].

PU.1 levels are also associated with the development of leukemia. Although haploinsufficient PU.1 expression in PU.1+/- mice did not induce myeloid leukemia, mice with an engineered deletion of the URE of PU.1 had a decreased PU.1 level to about 20% of normal, and this led to an aggressive form of AML [6]. Moreover, deletion of one PU.1 gene copy in combination with downregulation of the other copy by PML–RARα was identified as the mechanism in mouse acute promyelocytic leukaemia (APL) [7]. Interestingly, complete loss of PU.1 was not detected in either model. This suggests that, at least in the mouse models used, low but detectable amounts of PU.1 might favor the malignant potential of leukaemic cells.

PU.1 deletion on one allele of chromosome 2 can be observed early in irradiated mice, long before the mice show symptoms of AML, implying PU.1 abnormality may be crucial in the initiation of radiation-induced AML. In mice with γ radiation-induced AML, the majority of the tumor acquired a deletion in one copy of PU.1, and an additional point mutation in the ets domain of the remaining PU.1 copy, suggesting PU.1 acts like a tumor suppressor in radiation-induced AML[8]. However, the precise role of PU.1 during the initiation, progression, and promotion of radiation-induced leukemogenesis has not been well demonstrated. In this case, characterizing PU.1 abnormality in mice at the time course following irradiation may be helpful for answering this question.

Leukemia can be viewed as a clonal disease that malignant hematopoietic tissue is maintained by clonal expansion of a few cells [9]. For most leukemia, as for most cancers, the target cell of transformation is still unknown. Because normal stem cells and

leukemic cells share the ability of self-renewal, as well as various developmental pathways, it has been postulated that leukemia arises from HSCs that have become leukemic as the result of accumulated mutations. Alternatively, leukemia can originate from more restricted progenitors such as CMPs and CLPs when they somehow gained the capacity of self-renewal and extensive proliferation. AML is one of the leukemias that have been most extensively studied for its' target cell. However, evidence from different researchers has been remarkably controversial. Much less is known about the target cell of radiation-induced AML, a type of AML that possibly has different origins and etiology from *de novo* AML. Characterizing PU.1 abnormality in irradiated mice before they acquire AML may also help identify the target cell.

To answer those questions, we utilized a mouse model and studied the PU.1 abnormalities in the hematopoietic system of irradiated mice, as well as of mice that have acquired radiation-induced AML. In our study, PU.1 deletion was detected by in situ hybridization utilizing a BAC probe flanking the PU.1 gene locus. Short term (one month) following γ radiation exposure, deletion on one copy of PU.1 was found in both the AML susceptible mouse strain CBA and AML resistant strain C57. However, the CBA mice acquired a significant higher frequency of PU.1 single copy deletion than C57 mice. Furthermore, we sought to investigate PU.1 deletion in different cell types, such as T cell, B cell, granulocytes, erythroid cells and progenitors, at different time points following irradiation. Interestingly, although the different cell types had similar frequency of PU.1 deletion at one month post-radiation, the myeloid cells including granulocytes and erythroid cells had increased PU.1 deletion frequency at three and six months after radiation exposure, while the lymphoid cell including T and B cells had

decreased PU.1 deletion at the same period of time. This result indicated there was expansion of myeloid cells bearing the PU.1 deletion in the bone marrow of irradiated mice. Another interesting finding in our study is that in the mice with radiation-induced AML, PU.1 deletion was found at very high frequency in myeloid cells, but was not found in lymphoid cells. Taking those data together, we proposed a mechanistic model for the radiation-induced myeloid leukemogenesis: following the whole body γ radiation exposure, all the cell lineages in mouse hematopoietic system acquired similar frequency of PU.1 deletion. However, abnormal clonal expansion only occurred in the myeloid cells with PU.1 deletion, which put myeloid cells at higher risk of gaining additional transforming mutation, i.e. point mutation in the ets domain of the remaining PU.1 gene. One or several such myeloid cells with PU.1 deletion, most likely progenitors, were transformed into leukemic cells by those cumulative genetic aberrations and underwent uncontrolled proliferation, which led to the aggressive form of leukemia (Figure 6.1). This model agrees with the clonal theory of AML in that AML originates from the clonal expansion of a single or oligo transformed cells. This model suggested that radiation-induced AML arises from a more restricted progenitor in myeloid lineage and it also reinforced the theory that cancer development is a multiple-step process as the result of cumulative genetic events.

Future directions

Similar to PU.1 deletion, PU.1 point mutation may be found in a small fraction of premalignant bone marrow cells following irradiation. Study of those cells will be particularly important to understand the pathogenesis of radiation-induced AML. However, identifying such a small fraction cells with PU.1 point mutation remains as a

challenge since in situ hybridization is not able to distinguish a single base difference, and DNA sequencing is only useful to measure the majority of bone marrow cells. A novel in situ hybridization based on rolling circle amplification of padlock probe has been reported as a potential method capable of distinguishing a single point mutation [10, 11]. Our previous study showed although this technology does not have such high fidelity as reported by other researchers to be able to distinguish a single nucleotide difference. Further modifications need to be made to this technology to increase its fidelity in order to use it in the future study to analyze the point mutation of PU.1 in irradiated mice.

Isolation of phenotypically defined HSCs and progenitor subset is important for the identification of target cells in radiation-induced AML. Using the cell surface markers proposed by Weissman and coworker [12], we were able to isolate distinct HSCs and progenitors subpopulations from mouse bone marrow. In the future study, we will focus on studying cytogenetic aberration such as PU.1 deletion and mutations in those subpopulations of bone marrow cells.

References

1. Behre, G., et al., *c-Jun is a JNK-independent coactivator of the PU.1 transcription factor.* J Biol Chem, 1999. **274**(8): p. 4939-46.
2. Chen, H., et al., *PU.1 (Spi-1) autoregulates its expression in myeloid cells.* Oncogene, 1995. **11**(8): p. 1549-60.
3. DeKoter, R.P., J.C. Walsh, and H. Singh, *PU.1 regulates both cytokine-dependent proliferation and differentiation of granulocyte/macrophage progenitors.* Embo J, 1998. **17**(15): p. 4456-68.
4. McKercher, S.R., et al., *Targeted disruption of the PU.1 gene results in multiple hematopoietic abnormalities.* Embo J, 1996. **15**(20): p. 5647-58.
5. DeKoter, R.P. and H. Singh, *Regulation of B lymphocyte and macrophage development by graded expression of PU.1.* Science, 2000. **288**(5470): p. 1439-41.
6. Rosenbauer, F., et al., *Acute myeloid leukemia induced by graded reduction of a lineage-specific transcription factor, PU.1.* Nat Genet, 2004. **36**(6): p. 624-30.
7. Walter, M.J., et al., *Reduced PU.1 expression causes myeloid progenitor expansion and increased leukemia penetrance in mice expressing PML-RARalpha.* Proc Natl Acad Sci U S A, 2005. **102**(35): p. 12513-8.
8. Cook, W.D., et al., *PU.1 is a suppressor of myeloid leukemia, inactivated in mice by gene deletion and mutation of its DNA binding domain.* Blood, 2004. **104**(12): p. 3437-44.
9. Fialkow, P.J., *Stem cell origin of human myeloid blood cell neoplasms.* Verh Dtsch Ges Pathol, 1990. **74**: p. 43-7.

10. Lizardi, P.M., et al., *Mutation detection and single-molecule counting using isothermal rolling-circle amplification.* Nat Genet, 1998. **19**(3): p. 225-32.

11. Nilsson, M., et al., *Padlock probes: circularizing oligonucleotides for localized DNA detection.* Science, 1994. **265**(5181): p. 2085-8.

12. Akashi, K., et al., *A clonogenic common myeloid progenitor that gives rise to all myeloid lineages.* Nature, 2000. **404**(6774): p. 193-7.

Figures

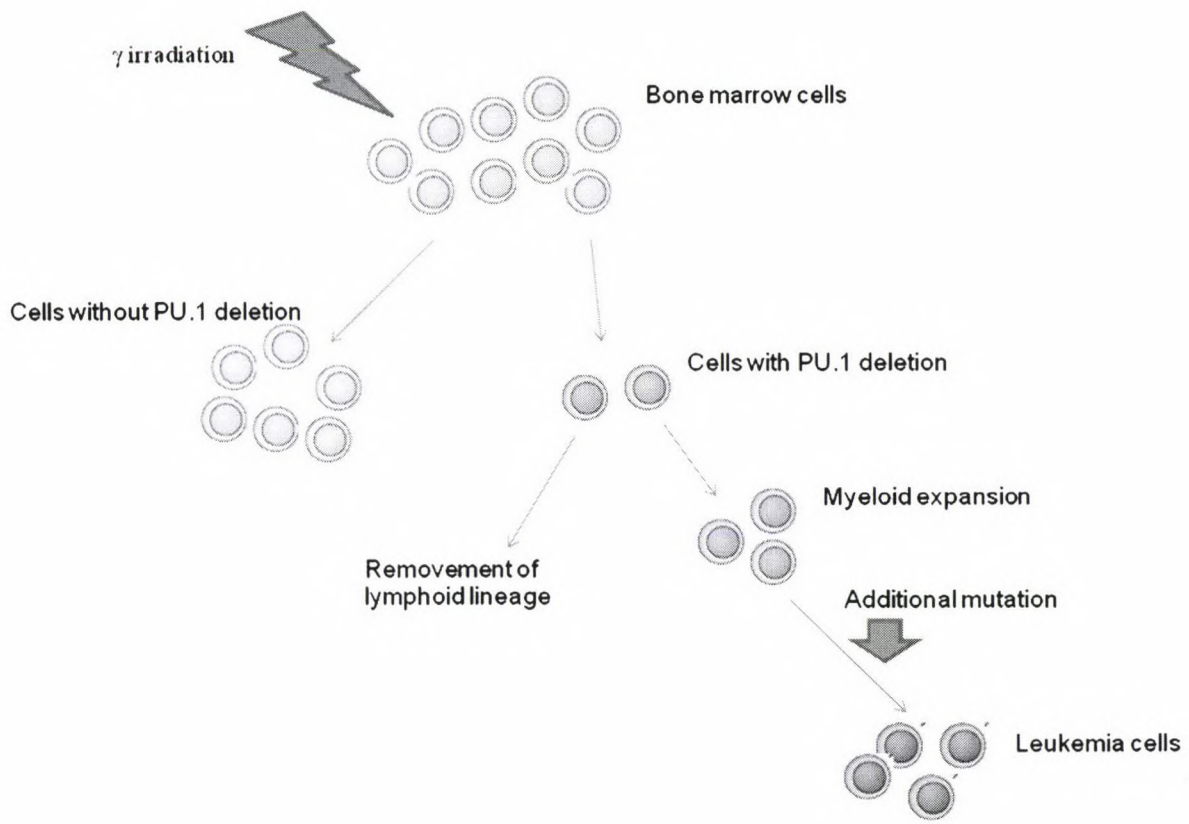


Figure 6.1. Illustration of the development of mouse radiation-induce AML.