Brief Communication

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Acute Lymphoblastic Leukemia with t(1;19)(q23;p13)/TCF3-PBX1 Fusion in an Adult Male with Down Syndrome

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The chromosomal abnormality t(1;19)(q23;p13), leading to the production of the TCF3-PBX1 fusion transcript, is one of the common translocations in pediatric B precursor acute lymphoblastic leukemia (ALL) [1], but it is rarer in adults [2]. Although Down syndrome (DS) patients have a high risk of developing ALL in the first three decades of life [3], few reports have described adults with DS and ALL [4, 5]. We here report the first case of an adult patient with DS who was diagnosed as having ALL with t(1;19)(q23;p13)/TCF3-PBX1 fusion, complicated by coagulopathy and cerebral infarction.

A 28-year-old male with DS was admitted to our hospital because of high fever. He had no history of blood disorders or cardiovascular anomaly. Physical examination showed cervical lymphadenopathy of 2 cm in diameter. Hepatosplenomegaly was absent. Hemogram findings were as following: hemoglobin, 14.2 g/dl; white blood cell count, 16.9 × 10^9/l with 59% blasts, and platelet count, 66 × 10^9/l. The LDH value was 3,446 IU/l. The bone marrow examination revealed marked hypercellularity with 85% blasts, which were positive for CD10, CD19, cytoplasmic CD79a, HLA-DR, and TDT but negative for CD3, CD13, CD20, CD33, and CD34. Chromosomal analysis of bone marrow cells revealed 47, XY, +21 [6]/49, idem, t(1;19)(q23;p13.3), –5, –7, –13 [6]. Reverse transcription-polymerase chain reaction analysis confirmed the presence of the TCF3-PBX1 rearrangement. This patient was then diagnosed as having B-precursor ALL with t(1;19)(q23;p13)/TCF3-PBX1. Janus kinase 2 mutation was not detected. Central nervous system (CNS) involvement was absent. An induction chemotherapy regimen based on vincristine, prednisolone (PSL), cyclophosphamide, L-asparaginase (L-ASP), and pirarubicin was administered. The initial PSL response was poor. On day 14 of induction therapy, the coagulation test showed the following results: platelet count, 34 × 10^9/l; prothrombin time ratio, 1.18 (normal: 0.9–1.1); fibrinogen, 56 mg/dl (normal: 175–430 mg/dl); fibrin degradation products, 71 mg/l (normal: <4.0 mg/l); D-dimer, 50.5 mg/l FEU (normal: <0.5 mg/l FEU), and antithrombin III (AT III) activity level, 87% (normal: 80–120%). A diagnosis of disseminated intravascular coagulation (DIC) was made according to the diagnostic criteria of the International Society of Thrombosis and Hemostasis, and intravenous administration of recombinant thrombomodulin (rTM) was started. The administration of L-ASP on day

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15 was postponed. He recovered from the DIC after 6 days of the administration of rTM, but had clouding of consciousness and left-sided hemiparesis on day 20. Magnetic resonance imaging (MRI) on day 21 showed a subacute cerebral infarction of the right temporal lobe. Since AT III activity was low (37%) on day 21, we judged that the hypercoagulation status might induce thromboembolism. Then, anticoagulation therapy was begun, and his neurological symptoms, except mild left hemiparesis, were improved 10 days later. After that, he received MEC (mitoxantrone, etoposide, and cytarabine) [7] and hyper CVAD (cyclophosphamide, vincristine, Adriamycin, and dexamethasone) [8] treatment but did not achieve complete remission (CR). CNS involvement was observed 7 months after the initial diagnosis, and he died of the primary disease 2 months later.

Our experience has some implications for the treatment of ALL, especially with t(1;19)(q23;p13)/TCF3-PBX1, in adult DS patients. First, ALL with t(1;19) may be more resistant to chemotherapy in adult DS patients. Our patient did not achieve CR despite conventional chemotherapies equal to those for non-DS patients, although adult ALL with fusion of TCF3-PBX1 was reported to show a high CR rate but a short remission duration [2]. Since leukemic cells in our patient had an extraordinary karyotype in addition to trisomy 21 and t(1;19)(q23;p13), such as monosomy 7, there is a possibility that additional genetic abnormalities might make them more resistant. In any case, we need improvement in chemotherapy for ALL with TCF3-PBX1 fusion in DS patients. Second, we should pay more attention to thromboembolism in the treatment of ALL in adult DS patients, even though they have no cardiovascular anomaly. Although it has been shown that the incidence of macroangiopathy is low in adults with DS [9], several cases have been reported of thromboembolism developed in DS patients without cardiovascular anomaly [6, 10]. Together with these reports, the present case suggests that thromboembolism can occur in a hypercoagulated status in the treatment of ALL even in adults with DS.

This is the first report on ALL with t(1;19)(q23;p13)/TCF3-PBX1 in an adult with DS. The patient had a cerebral infarction during the induction therapy. He never achieved CR despite intensive chemotherapies, and died of the primary disease. Our experience suggests difficulty in the treatment of adult DS patients with ALL, especially with TCF3-PBX1 fusion. Therefore, we need improvement in chemotherapy, and careful supportive therapy for them.

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CORRESPONDENCE

Eltrombopag in Refractory Aplastic Anemia

TO THE EDITOR: As described by Olnes et al. (July 5 issue),[1] one possible concern with regard to eltrombopag therapy is that it stimulates c-MPL, which enhances clonal evolution to myelodysplasia or leukemia in patients with aplastic anemia. For example, the risk of progression from myelodysplastic syndromes to acute myeloid leukemia has been observed in a clinical trial of romiplostim, a c-MPL agonist.[2] Olnes et al. reported that as many as 3 of 25 patients with aplastic anemia (12.0% [95% confidence interval, 2.5 to 31.2]) had clonal evolution, including two cases of monosomy 7 and one case of myeloid leukemia. Since a 12-week observation period appeared to be too short for a new tumor to develop, treatment with eltrombopag might have enhanced the expanded clones that already existed before the treatment. Thus, clinical researchers who administer c-MPL agonists should carefully assess patients with aplastic anemia before treatment is begun. It should be noted that a conventional metaphase cytogenetic test and a cytomorphologic examination of bone marrow by light microscopy may not be sensitive enough to detect very minor clones. Could the authors share any techniques, such as newer cytogenetic detection methods, for detecting hidden malignant clones?[3]

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TO THE EDITOR: Olnes et al. report that treatment with eltrombopag led to clinically significant hematologic responses in 11 of 25 patients with severe aplastic anemia. However, in this trial involving patients who did not have a response to prior immunosuppressive therapy, 47 patients were screened for enrollment, 21 of whom were not enrolled. It is not clear from the study what made nearly half the screened patients ineligible. Did these patients have characteristics that made the authors think they would be less likely to have a response to eltrombopag? Because of the positive

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results of this study, would it now be appropriate to consider treating some of the patients who were not enrolled? In a few of the treated patients, it is possible that eltrombopag might have caused prolonged remissions. How do the authors explain this effect? Do they have data regarding the use of eltrombopag in combination with immunosuppression that show enhanced results in patients with aplastic anemia or in laboratory models?

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No potential conflict of interest relevant to this letter was reported.

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THE AUTHORS REPLY: Oshima and colleagues restate the concerns raised in our article. The risk of progression to myelodysplastic syndromes or acute myeloid leukemia may be particularly increased in patients with aplastic anemia that is refractory to immunosuppressive treatment. Although there are reports of progression to acute myeloid leukemia in patients with myelodysplastic syndromes who are treated with romiplostim, an alternative thrombopoietin agonist, there is as yet no evidence that the progression rate is higher than expected.[1] How best to monitor for preexisting or evolving clonal hematopoiesis in aplastic anemia is not clear. A limitation of any test is the profound hypocellularity of the marrow. There are no prospective trials comparing cytogenetic analysis with other methods. Cytogenetic detection can be unsuccessful in at least 10% of patients at presentation, and such analysis cannot detect minor clones. Fluorescence in situ hybridization is a more sensitive test,[2,3] but it screens for only a few abnormalities, and distinguishing a minor monosomy clone from background false positive nuclei is difficult. Array-based technologies such as single-nucleotide polymorphism (SNP) whole-genome arrays are exciting new approaches. Afable et al. reported copy-number abnormalities or loss of heterozygosity in 10 of 33 pretreatment samples with normal cytogenetic characteristics in patients with aplastic anemia.[4] Some abnormalities detected by SNP arrays disappeared on follow-up and may reflect nonpathogenic oligoclonal hematopoiesis. We are prospectively comparing metaphase cytogenetics with array-based methods to detect clonal populations in sequential bone marrow samples obtained from patients enrolled in ongoing trials of eltrombopag for the treatment of aplastic anemia.

In response to Akard: we excluded screened patients on the basis of protocol-defined criteria, most commonly elevated liver enzyme levels due to iron overload, dysplastic changes, or cytogenetic abnormalities on screening marrow examination, or because their blood counts were insufficiently abnormal for inclusion. The fact that a complete response was maintained in one patient after eltrombopag was discontinued was interesting and suggests that normalization of stem cells may persist once achieved, even in the absence of the drug. To further investigate this finding, we have begun to taper this drug in patients enrolled in our extension trial. We have initiated a trial of eltrombopag in combination with antithrombocyte globulin and cyclosporine in patients with severe aplastic anemia of new onset (ClinicalTrials.gov number, NCT01623167) and a trial of eltrombopag as a single agent in patients with moderate aplastic anemia (NCT01328587). However, until data are more abundant regarding clonal progression in patients with aplastic anemia who are treated with thrombopoietin agonists, we would continue to urge that all patients with bone marrow failure who are treated with eltrombopag be enrolled in clinical trials.

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Since publication of their article, the authors report no further potential conflict of interest.

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ORIGINAL ARTICLE

Association of hepatitis B with antirheumatic drugs: a case–control study

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Abstract
Background Though concern of hepatitis B virus (HBV) reactivation by antirheumatic agents has limited therapeutic opportunities in HBV-infected rheumatoid arthritis (RA) patients, the relative risks (RR) among such agents have not been clarified.
Objective We compared the reporting of antirheumatic-agent-associated hepatitis B.
Patients We assessed 92 hepatitis B cases and 98,069 controls from a population of 98,161 RA patients registered into the US Food and Drug Administration's (FDA's) adverse event database between 2004 and 2010.
Measurements A reporting odds ratio (ROR), a signal suggesting a risk for hepatitis B among antirheumatic agents, was measured.
Results Treatment with corticosteroids [ROR 2.3 (95 % confidence interval 1.3–4.0)], methotrexate [4.9 (3.9–6.0)], rituximab [7.2 (5.3–9.9)], tacrolimus [4.2 (1.5–11.9)], or reporting from Japan [2.2 (1.1–4.2)] were associated with higher signal, whereas adalimumab had a lower ROR [0.2 (0.1–0.4)].
Limitations There are known limitations of spontaneous reporting, such as underreporting, the Weber effect, reporting bias, indication bias, and limited clinical information such as HBV status.
Conclusions Adalimumab's low reporting rate is most likely be due to notoriety. However, the possibility that adalimumab might suppress reactivation of HBV cannot be denied. Until the possibility is clarified in well-designed clinical studies, physicians should use adalimumab cautiously in patients with HBV.

Keywords Hepatitis B · Rheumatoid arthritis · Antirheumatic drug · Adverse event reporting system (AERS) · Spontaneous report

Introduction

Progresses in pathophysiological knowledge, especially in cytokine cascades and their effector cells in rheumatoid arthritis (RA), have brought various developments of new antirheumatic agents. The classes of therapeutic agents directed against specific cytokines or effector cells in the disease process of RA, are: (1) disease-modifying antirheumatic drugs (DMARD), such as methotrexate (MTX), hydroxychloroquine (HCQ), leflunomide (LEF), and sulfasalazine (SSZ); (2) biological DMARDs, such as adalimumab (ADA), etanercept (ETA), infliximab (IFX), and rituximab (RTX); and (3) immunosuppressants, such as tacrolimus (TAC), azathioprine (AZT), cyclosporine (CSA), and mizoribine (MZB). These antirheumatic agents have greatly improved and expanded therapeutic options for RA. However, RA patients infected with hepatitis B virus (HBV) have been excluded from the benefit of therapeutic opportunities with these new agents. Reports about severe hepatitis case with increasing HBV-DNA after methotrexate (MTX) and corticosteroid therapy [1, 2]

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alerted the medical community about the use of antirheumatic agents in patients with HBV carrier status and proposed an algorithm for assessment and prevention of HBV reactivation in RA patients. The American College of Rheumatology (ACR) made recommendations on the use of DMARDs and biologics based on hepatitis type, Child–Pugh grade, and whether or not antiviral agents to treat hepatitis had been initiated. The college also asked physicians to consider the risks and benefits of all DMARDs [3]. The Japan College of Rheumatology (JCR) more strictly limited the use of tumor necrosis factor-alpha (TNF-α)-blocking biologics and MTX in HBV-carrying RA patients in 2008 (http://www.ryumachi-jp.com/english/index.html).

Serum hepatitis B surface antigen (HBsAg) is infrequent (0.1–0.5 %) in the normal population in the United States and western Europe. However, a prevalence of up to 5–20 % has been found in the Far East and in some tropical countries in patients with Hodgkin's disease, polyarteritis nodosa, and chronic renal disease [4]. Since HBV reactivation was reported not only in HBV carriers but also in RA patients with resolved or past HBV infection [5, 6], and the prevalence of concurrent and resolved HBV infection among RA patients in Japan was reported to be 0.8 % and 25.1 %, respectively [6], approximately one fourth of RA patients appear to be at risk for reactivation of HBV in Japan. As the reasons for restricting the use of certain DMARDs in HBV carriers by the colleges are mainly based on case series, case reports, and reviews of them, and the relative strength of risks still remain to be clarified. Recent reports suggest that screening for HBV infection and careful monitoring during the use of nonbiologic and biologic DMARDs may ameliorate the risk of severe hepatitis [5, 7, 8].

Guideline for the use of immunosuppressants and chemotherapy for malignant neoplasm in patients with HBV carriers is available in Japan [9]. The guidelines are not restricted to antirheumatic agents, but those who want to treat HBV-carrier RA patients may refer to the recommendations mentioned in the guidelines. The JCR also released recommendations regarding immunosuppressant use for RA patients with HBV infection in 2010 (http://www.ryumachi-jp.com/english/index.html). They describe HBV screening and the use of nucleoside analogs prior to immunosuppressive therapies. However, their recommendations lack a description regarding selection of antirheumatic agents. In Japan, one of the HBV-epidemic areas, no useful information appeared to be available for selecting antirheumatic agents for treating HBV-infected patients from the standpoint of relative risk (RR) for HBV reactivation.

There is no doubt that results from prospectively randomized clinical trial yield high-level evidence comparing risk of drugs. In order to assess risk level, prospective intervention studies using randomly assigned nonbiologic DMARDs or biologics to patients selected based on eligibility criteria and standardized assessment of occurrence of hepatitis are, of course, useful. However, ethical limitations and time/cost may make such a study unfeasible. Testing the risk of drugs with concern for severe adverse reactions may not be ethically acceptable. As clinical trials require exhaustive efforts and extensive costs and time, they may not provide timely information with a reasonable cost. Here, we propose the use of an adverse event reporting system (AERS) to rapidly estimate possible risks in these patients, as mentioned elsewhere [10, 11]. Despite limitations of the AERS, it may provide timely information with fewer costs [12, 13]. In this study, we compared reporting odds ratio (ROR) as a signal of risk for HBV reactivation associated with antirheumatic agents use. One can more effectively design clinical trials with less ethical concern to clarify crucial points based on the estimated results from AERS research.

Methods

Study design

A nested case–control analysis of antirheumatic-agent-associated HBV reported to the FDA between January 2004 and December 2010 was conducted. The subcohort study participants were individuals registered in the FDA AERS, with RA as an indication for drug use. Cases and controls were respectively defined as individuals with and without drug-associated HBV among the subcohort. The analyses included the number of unique cases and ROR among antirheumatic agents. In addition to monovariate analyses, a multivariate assessment by unconditional logistic regression was performed.

Datasource

The AERS database was downloaded from the FDA AERS Web page (http://www.fda.gov/), between first quarter 2004 and fourth quarter 2010.

Case identification

Drugs used to treat RA were identified as follows: First, the table for therapeutic indications was searched, which recorded an individual drug identifier (drug code) and corresponding indication for its use. As reported indications in the AERS database are coded according to the Medical Dictionary for Regulatory Activities (MedDRA) (Maintenance and Support Services Organization, Chantilly, VA, USA), we identified drugs with RA as their

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Imatinib mesylate directly impairs class switch recombination through down-regulation of AID: its potential efficacy as an AID suppressor

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Brief report

Imatinib mesylate directly impairs class switch recombination through down-regulation of AID: its potential efficacy as an AID suppressor

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Activation-induced cytidine deaminase (AID) is essential for class switch recombination and somatic hypermutation. Its deregulated expression acts as a genomic mutator that can contribute to the development of various malignancies. During treatment with imatinib mesylate (IM), patients with chronic myeloid leukemia often develop hypogammaglobulinemia, the mechanism of which has not yet been clarified. Here, we provide evidence that class switch recombination on B-cell activation is apparently inhibited by IM through down-regulation of AID. Furthermore, expression of E2A, a key transcription factor for AID induction, was markedly suppressed by IM. These results elucidate not only the underlying mechanism of IM-induced hypogammaglobulinemia but also its potential efficacy as an AID suppressor. (*Blood*. 2012;119(13): 3123-3127)

Introduction

Activation-induced cytidine deaminase (AID) is essential for class switch recombination (CSR) and somatic hypermutation.[1] Deregulated expression of AID acts as a genomic mutator and can contribute to tumorigenesis through genomic recombination and aberrant somatic hypermutation.[2-4] E2A, which harbors 2 binding sites in the AID promoter, is the crucial transcription factor for induction of AID.[5] Imatinib mesylate (IM) has diverse immunomodulatory effects,[6,7] including reduction of T-cell proliferation and inhibition of T-cell effector functions.[8,9] Previously, we reported that serum titers of IgG and IgA, but not IgM, were significantly lower in chronic myeloid leukemia patients treated with IM versus those treated with IFN-α,[10] suggesting that IM impairs CSR. In the present study, we investigated the effects of IM on CSR both in vitro and in vivo. Here, we present evidence that IM inhibits CSR through down-regulation of AID expression in splenic B cells.

Methods

Mouse immunization

Eight-week-old mice were immunized as previously reported,[1] with or without 50 mg/kg imatinib mesylate. The experiments were approved by the Committee of Animal Care at the Institute of Medical Science, University of Tokyo.

Immunohistochemistry

Immunostaining for AID was performed on frozen sections following the manufacturer's instructions using an AID antibody (H-80; Santa Cruz Biotechnology).

Primer sequences, reagents, and more detailed methods are shown in supplemental Methods (available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article).

Results and discussion

CSR is induced in splenic B cells by stimulation with IL-4 and lipopolysaccharide (LPS).[11] After stimulation with IL-4 and LPS for 72 hours, IM decreased the proportion of IgG1-positive B cells dose-dependently. The proportion of B cells expressing surface IgG1 was approximately 16% without IM but was significantly reduced to approximately 3% with 10μM IM (Figure 1A). In the present culture system, only B cells can survive and proliferate,[1] suggesting that IM may act directly on B cells and inhibit their CSR.

Next, we examined expression of the germline transcript directed by the I promoter of IgG1 and AID, both of which are essential for CSR after B-cell stimulation.[12] Expression of AID was suppressed by IM dose-dependently (Figure 1B), whereas the IgG1 germline transcripts were not decreased by IM (Figure 1C). Likewise, IgA CSR in CH12F3-2A cells was impaired by IM in a dose-dependent manner (Figure 1D). These results

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Scientific Research

Leukemogenic Fusion Gene (p190 BCR-ABL) Transduction into Hematopoietic Stem/Progenitor Cells in the Common Marmoset

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ABSTRACT

Patients with Philadelphia chromosome (p190 BCR-ABL fusion gene)-positive acute lymphoblastic leukemia have a poor prognosis despite intensive therapeutic intervention. In this study, we attempted to develop a leukemia nonhuman primate model that mimics various human systems. Hematopoietic stem/progenitor cells in the common marmoset were transduced with a lentiviral vector containing the p190 BCR-ABL fusion gene by *ex vivo* transduction or *in vivo* direct bone marrow injection. In the latter model, BCR-ABL gene expression was maintained for more than one and a half years. One marmoset unexpectedly developed myelofibrosis-like disease. However, none of the marmosets have developed leukemia to date. In conclusion, we successfully achieved sustained p190 BCR-ABL gene expression *in vivo*. However, a genetic mutation in addition to p190 BCR-ABL may be required for the malignant transformation of hematopoietic stem/progenitor cells in the common marmoset during the short observation period. This novel *in vivo* approach will help develop a marmoset leukemia model in the future.

Keywords: Leukemia; Lentiviral Vector; Myelofibrosis; Common Marmoset

1. Introduction

Many preclinical *in vivo* studies have been conducted in mice because they are easy to breed and their biology and genetics are well-characterized. However, humans and mice differ genetically, pathophysiologically and pharmacokinetically, which makes it difficult to extrapolate the results from mouse models for direct clinical applications in humans. Large animals, especially nonhuman primates, are more closely related to humans. Moreover, because of their long life span, nonhuman primates can be treated and monitored over a long period, which presents opportunities for time-varying sampling of their blood and bone marrow. Thus, the development of non-human primate models that mimic human pathophysiology and pharmacokinetics will significantly further our understanding of human diseases. Particularly, genetically modified primates will be a powerful human disease model that can be used to preclinically assess the safety and efficacy of developing drugs.

Currently, Old World primates, such as the rhesus monkey (*Macaca mulatta*) and cynomolgus monkey (*Macaca fascicularis*), are commonly used for research [1-3]. However, these primates have several disadvantages, such as a slow sexual maturation period of approximately three years, fewer offspring over the female lifespan, and difficulty in handling.

The common marmoset (*Callithrix jacchus*) is a small New World primate that has attracted considerable attention as a potential animal for biomedical research [4,5]. The common marmoset is small, weighing approximately 350 - 400 g, relatively easy to breed, has a short gestation period of approximately 144 days, reaches sexual maturity at 12 - 18 months, and produces 40 - 80 offspring during the female lifespan. Thus, although marmosets are

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not as closely related to humans as apes or Old World primates, they are valuable as a potential primate model of human disease.

In this study, we attempted to establish a marmoset leukemia model by introducing a fusion gene that causes leukemia in humans. The Philadelphia chromosome (Ph) contains one of several forms of BCR and c-ABL gene fusions, and these fusions substantially contribute to the pathogenesis of chronic myelogenous leukemia (CML) and acute lymphoblastic leukemia (ALL). The p190 BCR-ABL fusion gene, in which BCR exon 1 is joined to ABL exon 2 (e1a2) and produces the p190 protein, is detected in 20% - 35% of ALL patients, and the prognosis of these patients is particularly poor [6,7]. Several treatments, such as allogeneic hematopoietic stem cell transplantation and novel small molecules that directly target the p190 BCR-ABL fusion gene have been developed to treat this refractory disease [8]. Transduction of the p190 BCR-ABL fusion gene is reportedly sufficient to cause leukemia in mice [9-11]. However, to date, there are no reports of a primate leukemia model. Establishing a marmoset model of this disease will be useful to test the efficacy of current and future treatments. Therefore, we transduced the p190 BCR-ABL fusion gene into marmoset $CD34^+$ hematopoietic stem/progenitor cells using a lentiviral vector and examined the occurrence of leukemogenic events.

2. Materials and Methods

2.1. Cell Lines

Ba/F3 cells, a mouse interleukin-3 (mIL-3)-dependent hematopoietic cell line, were maintained in RPMI (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) and mIL-3 (10 ng/mL) at 37°C in 5% CO_2 and passaged twice every week.

2.2. Animals and Preparation of Bone Marrow and Peripheral Blood Mononuclear Cells

Common marmosets were purchased from the Division of Animal Experimentation, Central Institute for Experimental Animals (Kawasaki, Japan), and bred at the animal center at our institute. In this study five animals were used including one control marmoset. No. 591 (male, 2 years and 6 months old) and No. 2338 (female, 4 years and 9 months old) were used as *ex vivo* BCR-ABL transduction models. No. 2129 (female, 5 years and 7 months old) and No. 2223 (female, 5 years and 3 months old) were used as BCR-ABL direct *in vivo* injection models. The study protocol was approved by the animal ethical committee of the University of Tokyo.

Bone marrow samples were collected by flushing the femurs of euthanized animals or aspirating the femoral bone marrow with an aspiration needle (Task, Tochigi, Japan). Peripheral blood samples were collected with heparin. Mononuclear cells (MNCs) in each sample were isolated by density-gradient centrifugation with Lymphoprep (Axis-Shield, Oslo, Norway). The cells were frozen in liquid nitrogen until further use.

2.3. Construction, Production and Transduction of Lentiviral Vector

Third generation, VSV.G pseudotyped lentiviral vectors were produced by transiently cotransfecting four plasmids into 293T cells as previously described [12]. Briefly, the p190 BCR-ABL fusion gene driven by a CMV or PGK promoter (HIV-CMV/PGK-BCR-ABL) was inserted into the transfer vector [13]. This plasmid was cotransfected into 293T cells using the calcium-phosphate method. The viral supernatant was harvested 48 and 72 hrs post transfection. The viral pellet was collected by ultra-centrifuging the supernatant and then stored at −80°C. The DNA titer, which is known to reflect the amount of transducible vector genome, was determined by real-time quantitative PCR as previously described [14]. For transduction, a cell pellet (2×10^5) was mixed and incubated with concentrated viral supernatant (20 μL) for 2 hrs in a 37°C incubator. The cells were generally infected *in vitro* with an MOI (multiplicity of infection) of 2.

2.4. Detection of p190 BCR-ABL Transgene Expression

RNA extraction and reverse transcription were performed as previously described using an RNA/DNA extraction kit (Qiagen, Hilden, Germany) and SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). Nested PCR amplification of p190 BCR-ABL was performed using a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA) with primers that specifically amplify the e1a2 transcripts. A 50 μL reaction mixture containing 2 mM of each dNTP, 25 mM $MgCl_2$, 10× PCR buffer, 0.5 μM primers, 1.25 U AmpliTaq Gold (Applied Biosystems) and 10 ng cDNA was subjected to 40 cycles of denaturation (95°C, 30 sec), annealing (61°C, 30 sec), and extension (72°C, 30 sec) and another 40 cycles with the inner primer set of denaturation (95°C, 30 sec), annealing (57°C, 30 sec), and extension (72°C, 30 sec). The final products were analyzed on a 1% agarose gel stained with ethidium bromide. The outer BCR-ABL primer set was forward primer (5'-CGC TCT CCC TCG CAG AAC TC-3') and reverse primer (5'-GGA GTG TTT CTC CAG ACT GTT GAC TG-3'), while the inner primer set was forward primer (5'-AAC AGT CCT TCG ACA GCA GCA-3') and reverse primer (5'-GCG TGA TGT AGT TGC TTG GGA-

