Development of Chronic Neutrophilic Leukemia

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ABSTRACT

The experimental manipulation of protooncogenes and their gene products is a valuable research tool for the study of human neoplasia. In this study, the recently identified human cervical cancer protooncogene (HccR-2) was expressed in transgenic mice under the control of the tetracycline regulatory system. Mice expressing the HccR-2 transgene showed an altered myeloid development characterized by an increased percentage of mature and band-form neutrophils in the peripheral blood, liver and spleen. This phenotype is similar to human chronic neutrophilic leukemia (CNL) in many ways, which is a rare chronic myeloproliferative disorder (CMD) that presents as a sustained leukocytosis of mature neutrophils with a few or no circulating immature granulocytes, an absence of peripheral blood monocytosis, basophilia, or eosinophilia, and an infiltration of neutrophils into the liver, spleen and kidney. Thus, the HccR-2 transgenic mouse model is imperative not only for investigating the biological properties of the HccR-2 protooncogene *in vivo*, but also for analyzing the mechanisms involved in the progression of CNL.

(Key words : Human cervical cancer, Protooncogene, Transgenic mice, Chronic neutrophilic leukemia)

INTRODUCTION

Chronic neutrophilic leukemia (CNL) is a rare chronic myeloproliferative disorder (CMD) that presents as a sustained leukocytosis of mature neutrophils with few or no circulating immature granulocytes, the absence of peripheral blood monocytosis, basophilia, or eosinophilia, and a normal platelet count (Imbert *et al*., 2001; Elliott *et al*., 2001; You and Weisbrot, 1979). Clinical evaluation fails to reveal an underlying disease process responsible for the leukemoid reaction, such as malignancy or infection. Splenomegaly manifests primarily due to neutrophilic infiltration, and the bone marrow biopsy mainly demonstrates a granulocytic proliferation without any evidence of morphologic dysplasia or striking reticulin fibrosis. CNL generally affects elderly patients of both sexes. Most patients with CNL have a poor prognosis, with a mean survival time of 21 months (Meyer *et al*., 1993). Cytogenetic and molecular analyses are negative for the Philadelphia chromosome and its molecular counterpart, the BCR/ABL fusion gene. These features, together with the absence of basophilia, monocytosis and BCR/ABL transcripts, distinguish CNL from chronic myeloid leukemia (CML), atypical chronic myeloid leukemia (a-CML) and chronic myelomonocytic leukemia (CMML), as defined by the French-American-British Cooperative Group (Bennet *et al*., 1994). Although nearly 100 cases of CNL have been reported since the first case was described about 80 years ago (Tuohy, 1920), the pathogenesis and natural history of the disease have not been fully explored. The literature, which consists primarily of isolated case reports published in a wide range of journals, is often vague and incomplete. Despite these limitations, the steering committee for the World Health Organization (WHO) classification of neoplastic diseases has recently acknowledged CNL as a distinct myeloproliferative disorder (Harris *et al*., 1997).

MATERIALS AND METHODS

Generation of Transgenic Mice

The full-length cDNA of HccR-2 (about 2 kb) was kindly provided by Dr. Jin Woo Kim, Catholic Medical Institute, Catholic Medical College, Seoul, Republic of Korea. The HccR-2 cDNA was ligated to a BamHI/SalI digested pTRE2 vector (Clontech, California, USA), and the resulting 5.8 kb plasmid, named as pTRE-HccR, was confirmed by direct sequencing. To generate the first lineage of transgenic mice (HccR expressing mice), the pTRE-HccR construct was digested with XhoI and SapI to remove the prokaryotic sequence, and the pTRE-HccR fragment (3.7kb) was separated by agarose gel electrophoresis. To generate the second lineage of transgenic mice, the pTeT-off plasmid (Clontech, California, USA)

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was digested with XhoI and HindⅢ to remove the prokaryotic sequence, and the Tet-off fragment (2.248 kb) was separated. These two fragments were then individually microinjected into the male pronucleus of fertilized mouse embryos obtained from C57BL/6×C3H F1 hybrid females as described previously (Brigid *et al*., 1994).

PCR Analysis

Insertion of the transgenes were identified and confirmed by PCR analysis. Genomic DNA was prepared from the tails of 4-week old founder mice as described (Lee *et al*., 2003). The following primers were used: HccR-2 sense 5′-CAA TCG CGG GAT CCA TGG CGC T-3′ (214-3s) and antisense 5′-CGC TCC ATG GTC GAC T-CT GCG CCT-3′ (214-3as); and tTA sense 5′-CCT CGA TGG TAG ACC CGT AA-3′ (tTA932-s) and antisense 5′-CCT CGA TGG TAG ACC CGT AA-3′ (tTA1443-as). Amplifications were carried out in a Takara Thermal Cycler (Takara Bio Inc, Kyoto, Japan), and PCR products were detected with a BIO-RAD Gel Doc 2000 Gel documentation system (BIO-RAD, California, USA).

Southern Blot Hybridization Analysis

Transgene insertion was further confirmed by Southern blot. Mouse genomic DNA was purified from transgenic and nontransgenic mice as described previously (Brigid *et al*., 1994). Mouse-tail DNA (20 μg) was digested with XhoI/HindIII (for detection of tTA) or with XhoI (for detection of HccR-2) followed by electrophoretic separation in a 1% agarose gel. The DNA was blotted onto a nitrocellulose membrane and probed with ^{32}P-labeled HccR-2 and tTa PCR fragments that were prepared as above, gel purified and [^{32}P]-dCTP radiolabeled with the Rediprime Ⅱ Random Prime labeling system (Amersham, Piscatway, NJ, USA). Prehybridization and hybridization were carried out at 65℃ in a solution containing 6×SSC, 0.1% SDS, 5×Denhardt's solution, and 100 mg/ml denatured salmon sperm DNA. After prehybridization for 2 hours and hybridization for 18 hours, the membranes were washed with 2×SSC and 0.5% SDS at room temperature for 15 min, followed by two washes with 0.5×SSC and 0.1% SDS at 65℃ for 15 min. Membranes were then exposed to autoradiography films for 3 days at −70℃.

RNA Analysis by Reverse Transcriptase-PCR (RT-PCR)

Total RNA was extracted from normal and transgenic mice with the Trizol reagent (Gibco-BRL, Rockville, MD, USA) according to the manufacturer's protocol. 2 μg RNA was used for RT-PCR reactions (Promega, WI, USA) according to the manufacturer's instructions. RT- minus controls were run to rule out genomic DNA contamination, and RT-PCR, using primers specific for GAPDH, was carried out to ensure RNA integrity. Signals were quantified with a BIO-RAD Gel Doc 2000 Gel documentation System (BIO-RAD, California, USA), and data were collected across three independent experiments.

Western Blot Hybridization Analysis

Western blot analysis was performed using conventional techniques. HccR-2 protein expression was detected with an HccR-2-specific polyclonal antibody (A-148D2, Keegene Science, Seoul, Korea).

Morphologic and Histologic Analyses

Peripheral blood and bone marrow cells were prepared as previously described (Froberg *et al*., 1998), and stained with a modified Wright's Giemsa (Diff-Quik® stain set, Dade Behring Inc, Newark, USA). Cytospin®4 (Thermo Shandon, Cheshire, UK) was used to make bone marrow slides. The leukemic transgenic mice were sacrificed at the last stages of clinical illness and necropsies were performed. Spleens and livers from either nontransgenic or transgenic mice were isolated, fixed in 10% neutral buffer formalin, and subsequently embedded in paraffin. 5 μm -thick sections of these organs were then deparaffinized, stained with hematoxylin and eosin and examined microscopically.

Statistical Analysis

The results of the cell counts were expressed as the mean SD of groups of 4 members. Statistical comparisons were made with a multiple Duncan test.

RESULTS

Production of the Transgenic Mice Expressing HccR-2

For expression of HccR-2 *in vivo*, we used a Tet system in which the tetracycline-transactivating protein (tTa) mediates the transcription of a transgene placed under the control of the tetracycline-response promoter (tet-o). The presence of tetracycline or doxycycline inactivates the transcription mediated by tTa, allowing constitutive expression of the transgene to be turned off. To regulate HccR-2 transcription *in vivo*, we generated two lineages of transgenic mice. The first contained the HccR-2 cDNA under the control of the tetracycline-responsive minimal promoter (Tet-o-HccR). The second expressed the tTa under the control of the CMV promoter (Fig. 1A, B). These mice developed no leukemia, had a typical life expectancy and body weight, exhibited regular tissue histology, and possessed normal number of mature and differentiated granulocytes (Fig. 2, 3, 4 & data not shown). In contrast, trans-

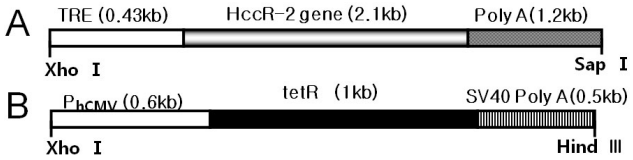

Fig. 1. Production of double transgenic mice. (A) Schematic model of the expression cassettes used to generate HccR-2 double transgenic mice. (B) Tetracycline regulatory system constructs.

genic mice from a cross between pTRE-HccR line 17 and tTa line 52 (i.e. Double Transgenic mice that exhibited abundant HccR-2 expression), that were not treated with doxycycline, developed CNL-like symptoms, with death occurring in 50% of the female mice by 9 months of age.

Clinical and Laboratory Diagnosis

The age of leukemic DTg mice (LTg, doxycycline free condition) at diagnosis ranged from 6 to 10 months (median 8 months). LTg mice presented with weight loss of 2~3 g, coupled with fatigue and illness (Fig. 2). All LTg mice had splenomegaly, palpable at a median of 3.2 cm (range 2.8 to 3.7 cm; data not shown) and demonstrated peripheral leukocytosis with a median white blood cell count at the time of CNL diagnosis of 18.24 K/μl (ranging from 10.26 to 31.50 K/μl, Fig. 3). This increase in white blood cell count (compared

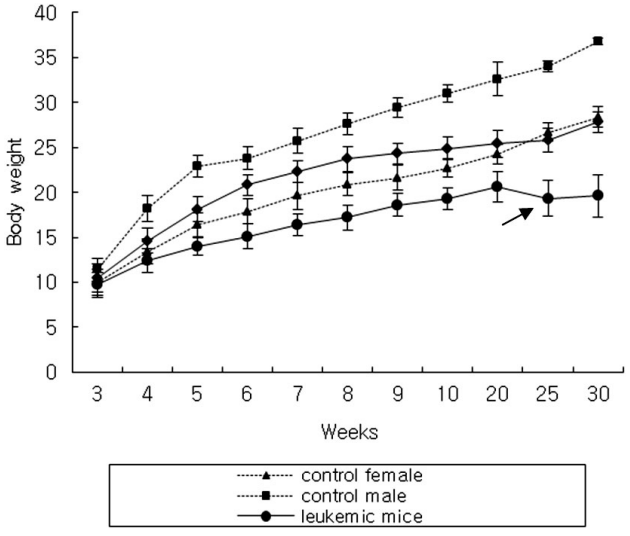

Fig. 2. Body-weight of the transgenic mice [leukemic mice (Doc−), non-leukemic mice (Doc+)] and control mice. The body-weight of leukemic mice decreased over 25 weeks (arrow indicates the time of CNL diagnosis). Under the doxycycline free condition, the double transgenic (DTg) mice developed CNL, as seen through stagnation of body-weight after approximately 20 weeks. Nonleukemic mice (male doxycycline-treated DTg mice) showed a normal increase in body-weight over time, as did control mice. Doc−, doxycycline free condition, Doc+, doxycycline present in drinking water.

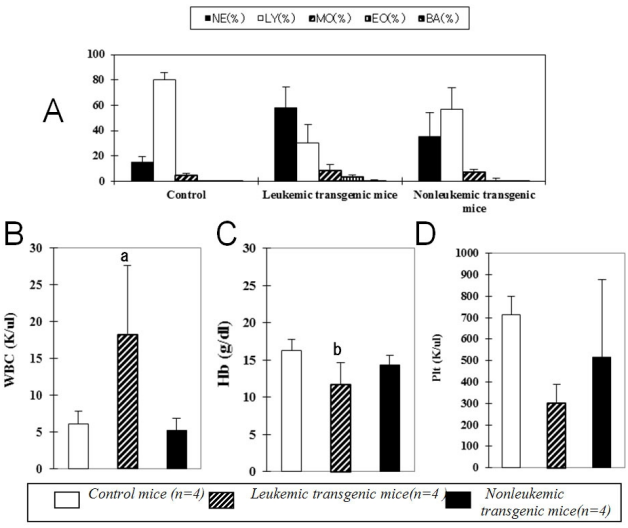

Fig. 3. Comparison of peripheral blood cells. Each value represents the mean SD of white blood cell composition ratio (A), total white blood cell counts (B), hemoglobin content (C) and total platelet counts (D), for control or double transgenic mice with nonleukemic (Dox+) and leukemic (Doc−) phenotypes. Statistically significant differences were observed for the percentage of neutrophils in control mice versus double transgenic (nonleukemic and leukemic) mice, and for the decrease in platelet counts for leukemic transgenic mice (LTg). Total white blood cell count was increased in LTg mice, but there was no significant difference in hemoglobin. NE: neutrophil, LY: lymphocyte, MO: monocyte, EO: eosinophil, BA: basophil, WBC: white blood cell, Hb: hemoglobin, Plt: platelet. a $p<0.05$ in comparison with control and nonleukemic transgenic mice groups. b $p<0.05$ in comparison with control group.

to the normal mouse count of 8.4 K/μl) (Fox *et al*., 2002) was due to neutrophilia, almost exclusively at the segmented and band stages of development. The percentage of white blood cells at or before the metamyelocyte stage was never greater than 2%. No absolute or relative monocytosis, eosinophilia, or basophilia was identified. Some LTg mice were anemic at presentation, with hemoglobin levels ranging from 8.9 to 15.7 g/μl (compared to the normal mouse level of 13.4 g/μl; Fig. 3). Furthermore, LTg mice had mild thrombocytopenia, with platelet counts ranging from 205 to 393 K/μl (compared to the normal mice average of 600 K/μl, Fig. 3) (Fox *et al*., 2002). The clinical features and laboratory data, both at diagnosis and during follow-up, are presented in Table 1.

Development of Chronic Neutrophilic Leukemia (CNL)

When compared with nontransgenic spleen, the white pulp of the leukemic mice was markedly atrophied and showed massive proliferation of neutrophils, while the multinucleated giant cells were seen in the red pulp. Infiltration of progranulocytes was markedly observed around the blood vessels in the liver, lung and

Table 1. Clinical and laboratory data in transgenic mice with chronic neutrophilic leukemia

Name	WBC	NE	LY	MO	EO	BA	NE(%)*	LY(%)*	MO(%)*	EO(%)*	BA(%)*	RBC	Hb	HCT	MCV	MCH	RDW	PLT
L1	13.20	5.44	6.42	0.93	0.34	0.07	41.21	48.65	7.03	2.55	0.55	9.15	11.5	49.7	54.3	15.8	22.1	249
L2	31.50	25.39	4.11	0.58	1.04	0.37	80.61	13.05	1.84	3.31	1.19	7.06	8.9	27.1	38.4	12.6	26.2	355
L3	17.98	9.64	4.62	2.67	0.96	0.10	53.63	25.67	14.83	5.33	0.54	10.98	15.7	60.7	55.3	17.9	24.0	393
L4	10.26	5.72	3.37	0.87	0.29	0.02	55.73	32.80	8.49	2.78	0.20	9.88	10.9	50.3	50.9	14.1	22.2	205
C1	7.74	1.32	6.10	0.30	0.02	0.01	17.02	78.84	3.83	0.20	0.11	9.33	16.8	50.8	54.4	18.0	19.4	716
C2	5.94	0.79	5.00	0.13	0.00	0.01	13.38	84.23	2.17	0.04	0.18	9.15	16.3	48.7	53.2	17.8	18.6	692
C3	7.02	1.38	5.18	0.45	0.01	0.01	19.67	73.72	6.38	0.14	0.08	9.47	17.8	52.0	54.9	18.8	19.0	619
C4	3.84	0.39	3.27	0.17	0.01	0.01	10.09	85.05	4.51	0.36	0.00	8.94	14.1	42.3	47.3	15.8	20.0	828
NL1	4.88	0.47	3.89	0.52	0.00	0.01	9.69	79.71	10.56	0.00	0.04	8.39	12.5	41.5	49.5	14.9	16.9	223
NL2	4.62	1.53	2.77	0.29	0.04	0.01	33.01	59.90	6.22	0.80	0.08	10.10	14.5	50.2	49.7	14.4	18.5	187
NL3	7.56	3.74	3.31	0.37	0.13	0.01	49.49	43.81	4.94	1.67	0.09	10.69	15.3	56.6	52.9	14.3	19.5	779
NL4	3.84	1.88	1.65	0.22	0.08	0.01	48.93	43.03	5.78	2.17	0.10	8.37	15.1	48.0	57.3	18.0	19.1	874

* White blood cell distribution. L: Leukemic double transgenic mice (Doxycycline free condition, female), C: control (Nontransgenic mice), NL: Nonleukemic transgenic mice (Offered doxycycline in drinking water, female), WBC: Total white blood cell count, NE: Neutrophil count, LY: Lymphocyte count, MO: Monocyte count, EO: Eosinophil, BA: Basophil count, RBC: Red blood cell count, Hb: Hemoglobine count, HCT: Hematocrit, MCV: Mean corpuscular volume, MCH: Mean corpuscular hemoglobin, RDW: RBC distribution width, PLT: Platelet count.

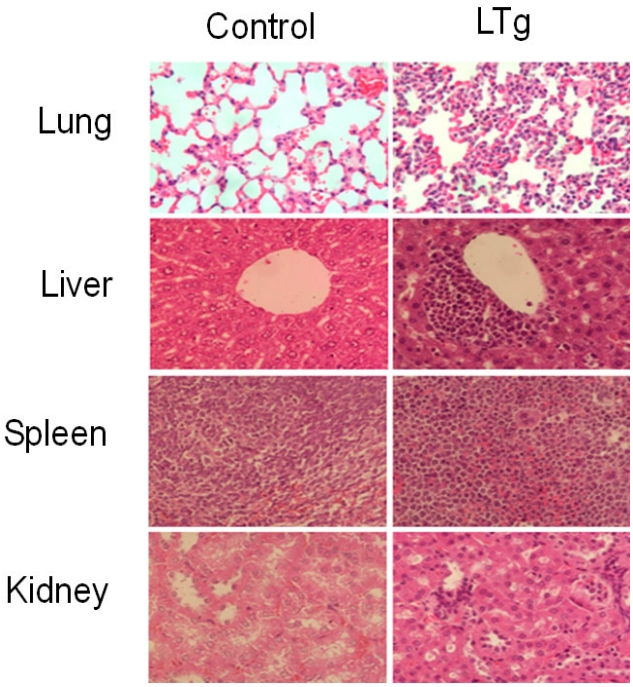

Fig. 4. H & E-stained sections of the liver, spleen, lung, and kidney of a double transgenic Doc⁻mouse (leukemic) and a nontransgenic control. Leukemic cells proliferated around the blood vessels of the liver, lung and kidney in leukemic double transgenic mice (LTg). In the spleen, the white pulp was characterized by markedly atrophic progranulocytes and the red pulp was characterized by markedly increased infiltration of megakaryocytes and lymphocytes. LTg: Leukemic transgenic mice, Control: nontransgenic mice.

kidneys (Fig. 4). None of the LTg mice presented with bleeding diatheses, and disseminated intravascular coagulation (DIC) was not apparent at autopsy in any of the mice with the leukemic phenotype. Male DTg (Doc⁻) and DTg (both sexes, Doc+) mice did not contract CNL. Male DTg (Doc⁻) mice had the same phenotype as the CNL females, but the clinical blood examination did not support the diagnosis of CNL.

DISCUSSION

Thrombocytopenia, mild anemia and splenomegaly, which are characteristic of human CNL, were consistently observed in leukemic double transgenic (LTg) mice. Both human CNL patients and leukemic mice showed high percentage of the band forms in the peripheral blood sample (Bohm and Schaefer, 2002). Many mature and band-form neutrophils were detected in the bone marrow, peripheral blood, spleen and liver of human CNL patients and leukemic mice. However, a common characteristic of the CNL patients is the presence of toxic granulation and Döhle bodies in blood smears, which were not observed in the leukemic mice (Relly, 2002). These differences suggest that the transgenic expression of HccR-2 in the early myeloid cells of the transgenic mice was not sufficient to fully recapitulate the human CNL phenotype and/or that there is a species difference.

Molecular studies for the BCR/ABL rearrangement, which must be negative before this diagnosis can be made, have been performed only in cases described since 1992 accounting for fewer than 10 reported cases (Froberg *et al*., 1998; Hasle *et al*., 1996; Kojima *et al*., 1999; Kwong and Cheng, 1993; Matano *et al*., 1997; Meyer *et al*., 1993; Storek, 1992; Yanagisawa *et al*., 1998;). CNL is characterized by the presence of mature neutrophils with few or no circulating immature granulocytes, splenomegaly and bone marrow hypercellularity with marked granulocytic hyperplasia, and mild reticulin fibrosis (Fox *et al*., 2002). In the LTg mice from this study, we did not observe the characteristics of the more common CMD such as reactive leukocytosis, leukemoid reaction, chronic myelogenous leukemia, or myelodysplastic syndromes, including CMML.

In this work, all LTg cases showed exclusive neutrophilia without granulocytic immaturity or evidence of proliferation of any other myeloid lineage. From a morphologic basis, dacryocytes, basophilia and immature granulocytes were absent in the peripheral blood of LTg mice. In contrast to the bone marrow findings in agnogenic myeloid metaplasia, polycythemia vera and essential thrombocythemia, the bone marrow from LTg mice with CNL consistently showed relatively smaller, atypical megakaryocytes, megakaryocytic clustering or significant reticulin fibrosis (Kojima *et al*., 1999). The peripheral blood in LTg mice showed mature and band-form neutrophilia. Left-shifted granulocytosis associated with eosinophilia or basophilia characterizes CML and helps to distinguish it from CNL. Most LTg from this study did not show a left shift, which is consistent with CNL. In practice, it may be impossible to make a diagnosis of CNL solely on morphologic grounds if the marrow is not packed and if the degree of reticulin fibrosis is not convincing. Some previously reported cases of supposed CNL were diagnosed in association with other disease processe, such as polycythemia vera, agnogenic myeloid metaplasia, myelodysplastic syndromes, monoclonal gammopathy and multiple myeloma (Di Donato *et al*., 1986; Orazi *et al*., 1990; Pane *et al*., 1996; Standen *et al*., 1990; Standen *et al*., 1993). These may not represent true cases of CNL as the presence of another clonal hematologic malignancy would certainly confound this diagnosis (Elliott *et al*., 2001). In such cases, it is difficult to distinguish whether CNL is a reactive leukocytosis or a discrete disease. The clonal nature of CNL has been the subject of controversy. Unlike CML, no characteristic clonal, chromosomal or molecular marker has been identified. Similar to other CMD, e.g. polycythemia vera and essential thrombocythemia, distinguishing a clonal hematologic disorder from a reactive process might be difficult, particularly early in the course of the disease. However, as seen in the cases presented here, CNL is associated with a sustained progressive neutrophilia, splenomegaly, a packed bone marrow with marked granulocytic proliferation, an absence of an underlying disorder and evolution to a terminal course due either to blastic transformation or a refractory leukocytosis. Taken together, these findings argue against a reactive process and are consistent with CNL representing a clonal, myeloproliferative disorder.

Histological analysis of LTg mice showed remarkable changes in the liver, kidneys, lung and spleen (Fig. 4). The overall decrease in splenic lymphocytes indicated that the expression of HccR-2 might impair normal lymphocyte development in both T and B lineages, which in turn could explain the reason for the high mortality rate observed in the LTg (Doxycycline free) mice. Reduction in the lymphocyte number would render the transgenic mice immunodeficient and highly susceptible to fatal infections (Hiroaki *et al*., 1999). The finding that transgenic mice treated with doxycycline survived much longer than those maintained under doxycycline-free conditions supports this idea. Interestingly, the phenotypic changes (emaciation and death) were not observed before weaning, possibly because the Ig transmitted through the mother's milk was capable of rescuing the immunodeficiency of the transgenic mice, as suggested in a previous report (Shull *et al*., 1992). This needs further investigation.

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