Novel Disease Model 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. The phenotype observed was similar in many respects to human chronic neutrophilic leukemia (CNL). Thus, the HccR-2 transgenic mouse model is important 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 myeloproliferative disorder that features a persistent neutrophilia in peripheral blood, myeloid hyperplasia in bone marrow, hepatosplenomegaly, and an absence of the Philadelphia chromosome or a BCR/ABL fusion gene (Jaffe *et al*., 2001). This is a rare disease, with less than about 100 reported cases so far., Characteristically, an equal male:female ratio was observed in majority of these cases (You and Weisbrot, 1979), usually involving older adults (Zittoun *et al*., 1994). The etiology of CNL is currently unidentified. Based on the observation that 20% of the CNL cases also involve myeloma an association between CNL and multiple myeloma has been suggested (Standen *et al*., 1993). However, a clonal genetic abnormality has not been detected in these myeloma-associated cases of CNL, suggesting the possibility that the neutrophilia is a reaction due to the neoplastic myeloma cells (Jaffe *et al*., 2001). The postulated cell of origin is a limited-potential, marrow-derived stem cell (Yanagisawa *et al*., 1998). The most common clinical finding is hepatosplenomegaly with occasional Pruritus, gout and mucocutaneous bleeding, Peripheral blood neutrophilia (> 25×10^9/L) with myeloid precursors (promyelocytes, myelocytes, metamyelocytes) comprising less than 5% of leukocytes (You and Weisbrot, 1979; Zittoun *et al*., 1994). Peripheral blood, bone marrow, spleen and liver are the most common sites, however, any other organ or tissue could also be infiltrated by neutrophils (You and Weisbrot, 1979; Zittoun *et al*., 1994). Splenic infiltrates are typically found only in the red pulp (You and Weisbrot, 1979; Zittoun *et al*., 1994).

The new human cervical cancer protooncogene (HccR-2) was originally identified as a 523 bp cDNA fragment (CC214) expressed in CUMC-6 cervical cancer cells (Kim *et al*., 1996), but not in normal cervical tissue. Previously, a full-length HccR-2 cDNA (2118 bp; GenBank #AF441865) was cloned from human lung fibroblast cDNA library and found to encode a predicted 360 amino acid polypeptide with a putative 25 amino acid leader sequence and a hydrophobic transmembrane domain of 20 amino acids. Although HccR-2 has been characterized as a protooncogene in the lung, liver and blood, the exact cellular function of this protein are not yet understood. In this study, we sought to elucidate the biological function of HccR-2 gene by generating transgenic mice expressing it under the control of Tet-off system.

MATERIALS AND METHODS

Production of Transgenic Mice

The full-length cDNA of HccR-2, about 2 kb, was kindly provided by Dr. Jin Woo Kim at the 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-

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HccR, was confirmed by direct sequencing. To generate the first lineage of transgenic mice, the pTRE-HccR construct was digested with XhoI and SapI to remove the prokaryotic sequence, and the pTRE-HccR fragment (3.7 kb) was separated by agarose gel electrophoresis. To generate the second lineage of transgenic mice, the p-TeT-off plasmid (Clontech, California, USA) was digested with XhoI and HindⅢ to remove the prokaryotic sequence, and the Tet-off fragment (2.248 kb) was microinjected into the male pronucleus of fertilized mouse embryos obtained from C57BL/6×C3H F1 hybrid females as previously described (Brigid *et al*., 1994).

PCR Analysis

Insertions 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 TCT 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 (Lee *et al*., 2003). 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. Two micrograms of RNA were analyzed by RT-PCR (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 (A148-D2, 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. Five micron-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

Generation 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. Two lineages of transgenic mice were generated in order to regulate HccR-2 transcription *in vivo*. The first contained the HccR-2 cDNA under the control of the tetracycline-responsive minimal promoter (Tet-o-HccR), while, the second expressed the tTa under the control of the *CMV* promoter (Fig. 1A, B). The transgenic founders from each lineage were crossed to yield the pro-

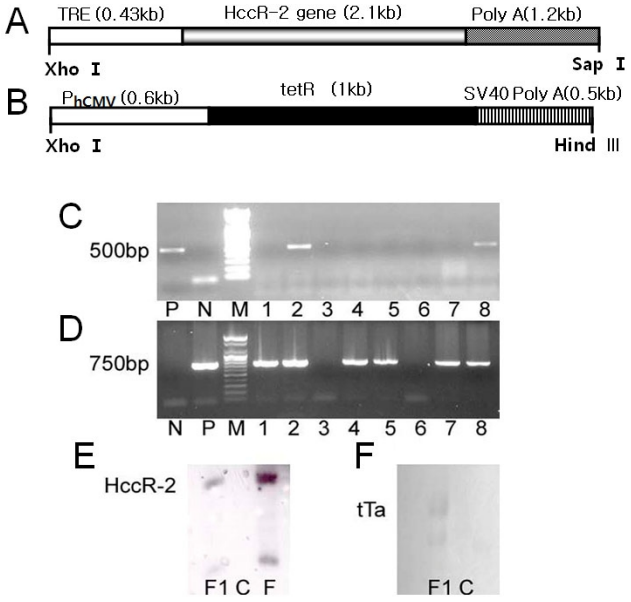

Fig. 1. Generation of double transgenic mice. (A) Schematic model of the expression cassettes used to generate HccR-2 double transgenic mice. (B) Tetracycline regulatory system constructs. PCR-identification of double transgenic mice from tail biopsies. Genomic PCR using the primers (tTa932-s & tTa1443-as, 214-3s & 214-3as) allowed identification of double transgenic mice. (C) PCR for tTa (PCR product is 500 bp). (D) PCR for HccR-2 (PCR product is 7,500 bp). (E,F) Genotyping by Southern blot analysis. (E) Tail tip DNA (10 ug) from offspring of HccR-2 tTa (F1) was digested with Xho1/Hind Ⅲ, size fractionated, transferred to a membrane, and probed with 32P-labeled HccR-2 cDNA. (F) Southern blot for tTa. N: negative control (nontransgenic), P: positive control using the transgene expression vector, M: molecular marker (100 bp ladder), 1~8: founder mice, F: TRE-HccR founder mice, F1: double transgenic mice, C: nontransgenic mice.

geny that constitutively expressed the HccR-2 transgene (Fig. 1C, D, 2A) in the absence of doxycycline or tetracycline. Both PCR and Southern blotting was used to identify the double transgenic (DTg) mice (Fig. 1C, D, E, F). RT-PCR showed that DTg mice expressed HccR-2 in the absence of doxycycline, and the fragment was not detected in nontransgenic mice (Fig. 2A). Mice transgenic for either tTa or tet-o-HccR alone or DTg mice continuously treated with doxycycline, appeared normal.

Peripheral Blood and Bone Marrow Features

Next, we examined the hematological change by blood count. The peripheral blood smears of LTg mice showed a prominent neutrophilia without a left shift (Fig. 3). The peripheral blood and the bone marrow of these mice contained high percentages of neutrophils that were primarily at the band-form stage of development, or occasionally at the segmented stage. No blastocytes or micromegakaryocytes were seen in any of the cases. Importantly, no dysplastic features were noted in the granulocytic series. Further, the red blood cells sh-

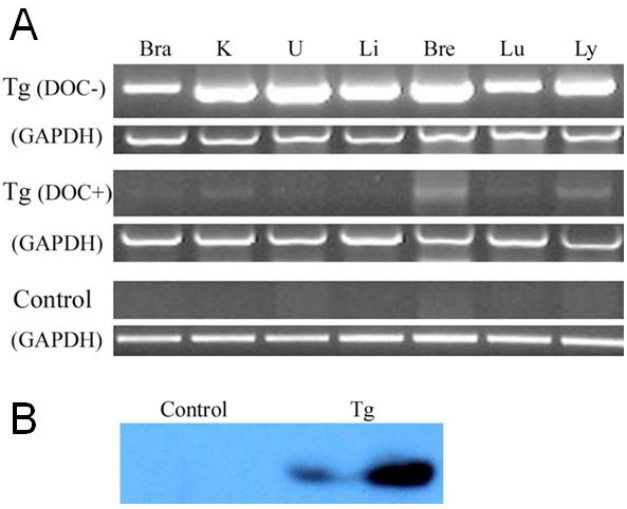

Fig. 2. Conditional transgene expression of HccR-2. (A) RT-PCR analysis of mRNA from HccR-2 double transgenic mice (DTg). The cDNA was amplified using primer pair 214-3s & 214-3as. Tg (Doc−) mice were kept under doxycycline free condition (resulting in transgene expression) and developed the CNL-like phenotype. Tg(Doc+) mice were treated with doxycycline, thus suppressing transgene expression. (B) Western blot analysis of transgene (HccR-2) expression in the spleens of Doc- and nontransgenic mice (Control). Tg, double transgenic mice;, Doc-, Doxycycline-free conditions; Doc+, treated with doxycycline; C & Control, nontransgenic mice; Bra, brain; K, kidney; U, uterus; Li, liver; Bre, breast; Lu, lung; Ly, lymph node.

owed, at most, mild anisopoikilocytosis. Moreover, no Dacryocytic, or teardrop-shaped cells were found in transgenic mice and smears from some transgenic mice showed a few scattered burr cells. Platelets were normal with no hypogranular or large, atypical forms being observed. The bone marrow cytospin smears in LTg cases consistently showed marked granulocytic hyperplasia, with more than 80% granulocytic precursors (Fig. 3). These smears showed effective granulocytic maturation, the majority of granulocytes being at the metamyelocyte to segmented stages, with no observable increase in the number of blasts. No dysplastic features such as hypogranulation or pseudo-Pelger-Huët changes were seen, and no Auer rods were identified. Erythroid precursors had normoblastic maturation, and no significant monocyte, eosinophil, or basophil populations were seen. Megakaryocytes in the spleen increased progressively along with the disease. However, the presence of megakaryocytes was not prominent as one would expect in CML. The bone marrow cytospin smears were uniformly hypercellular, with all cases showing an essentially packed marrow.

Development of Chronic Neutrophilic Leukemia (CNL)

Approximately 50% of female DTg (Doc-) mice were developed a severe leukemic phenotype by 8 months (range 6 to 10 months). These mice generally presented with severe leukocytosis, usually associated with mild

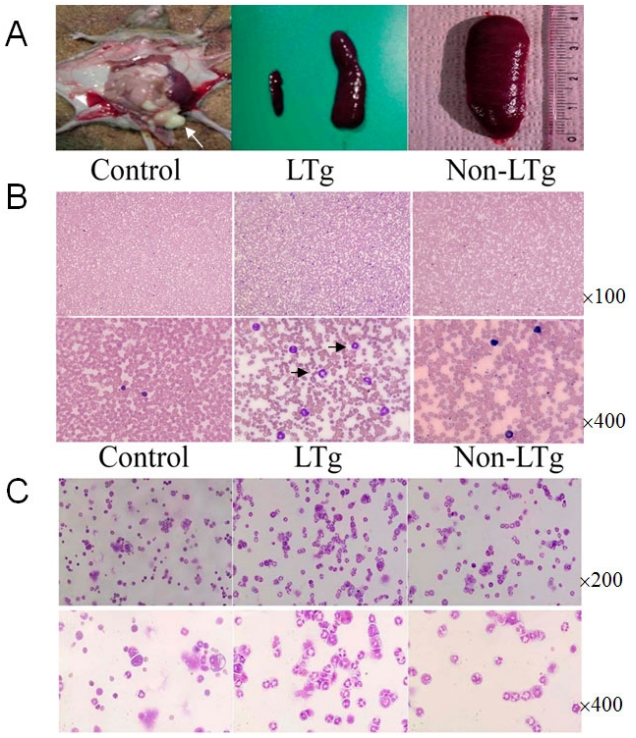

Fig. 3. Macroscopic and microscopic pictures of the leukemic double transgenic mice. (A) LTg mice exhibit splenomegaly. Microabscesses, detected in all LTg cases, were accompanied by tissue necrosis and infiltration of granulocytes (A, white arrow). (B) Wright's Giemsa stained peripheral blood smears. Increased numbers of banded or segmented-form neutrophilia were seen in leukemic mice (black arrow). Banded neutrophils were rarely seen in control mice. Nonleukemic transgenic mice (Doc+) had a phenotype that was intermediate between that of leukemic mice and control mice, with a few band-form neutrophils apparent. In leukemic transgenic mice the platelet count was decreased, red blood cells were mildly burred, and neutrophils were markedly increased. No monocytosis, eosinophilia, or basophilia was observed. (C) Wright's Giemsa staining of cytospin bone marrow preparations. Marked granulocytic proliferation was detected in LTg mice. No megakaryocyte clustering was present. Nonleukemic transgenic mice (Doc+) had slightly increased granulocyte proliferation, while leukemic transgenic mice (Doc-) had increased numbers of neutrophils. Control mice had normal phenotype. LTg: leukemic transgenic mice Control: nontransgenic mice, Non-LTg; nonleukemic transgenic mice.

thrombocytopenia and/or anemia. Massive splenomegaly was consistently observed in these animals (Fig. 3). In addition, the peripheral blood and the bone marrow of these mice contained high percentages of neutrophils that were primarily at the band forms or occasionally at the segmented stage of development (Fig. 3).

Leukemic Transgenic Mice were Susceptible to Infection

Under conventional maintaining conditions (i.e. doxycycline-free, under which condition the HccR-2 transgene was constitutively expressed), transgenic mice grew normally until weaning (4 weeks of age), then gradually exhibited progressive emaciation. Many transgenic mice died within 6~9 months after birth (50%). In contrast, nontransgenic littermates (100%) survived during the same period. Autopsies of the dead animals revealed abscesses, which strongly suggested that the transgenic mice were susceptible to infection. To address this issue, newly weaned transgenic littermates (4 weeks of age) were divided into two groups. One group of mice were kept under a doxycycline-free condition (transgene expression turned on, Doc-), whereas doxycycline (transgene expression turned off, 0.2 mg/ml, Doc+) was administered in the second group. After 9 months of observation, we found that the Doc- transgenic mice showed a high mortality rate (5 of 10 transgenic mice died), whereas the Doc+ transgenic mice exhibited a much better survival rate (9 of 10 transgenic mice survived past 9 months) (Fig. 4, right). During this period, all nontransgenic mice survived under both conditions (Fig. 4, left). To investigate the cause of death of the Doc-transgenic mice, tissues of dead or moribund mice were pathologically examined. In most cases, macroscopic abscesses were found in the uterus. Microabscesses were detected in all cases and were accompanied by tissue necrosis and infiltration of granulocytes. Staining of stamp specimens of the abscesses showed proliferation of bacteria. These results suggest that transgenic mice were susceptible to infection and that the major cause of transgenic mouse mortality was bacterial infection.

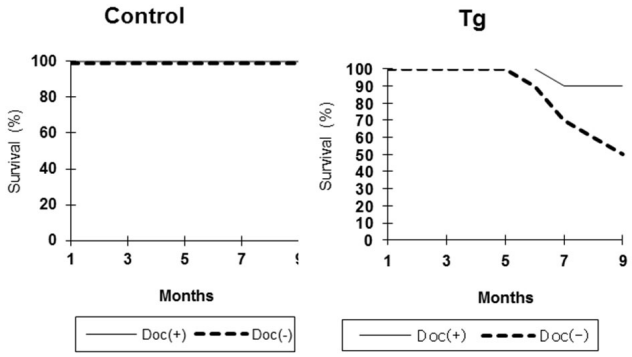

Fig. 4. Survival curves of the transgenic mice (Tg, right) and the nontransgenic control (C, left) that were kept doxycycline-free (Doc+, dotted lines) or were treated with doxycycline (Doc−, solid lines). The Doc+ double transgenic (DTg) mice exhibited a good survival rate (90% over 9 months), but the Doc- DTg mice had a decreased survival rate (50%, 9 months). Control mice (Doc+ and Doc-) showed a normal survival rate over the 9 months.

DISCUSSION

In this study, we describe the production of DTg mice containing a Tet-controlled HccR-2 expression casse-

tte. These DTg mice developed an accumulation of granulocytes in the bone marrow, which presumably led to the development of extramedullary hematopoiesis in the spleen. In addition, the liver and kidneys of DTg mice showed accumulation of neutrophils. This altered neutrophil infiltration increased the susceptibility of these animals to develop CNL after a middle latent period ranging from 6 to 10 months. The leukemic phenotype of these transgenic mice exhibits both similarities as well as certain differences with that of human patients with CNL.

Although CNL is commonly considered a chronic myeloproliferative disorder, fewer than 100 cases have been reported since its original description in 1920, with most being case reports (Piliotis *et al*., 2002). Moreover, it is likely that not all of the reported cases represented true CNL.

In humans, CNL is molecularly diagnosed by the absence of the BCR/ABL fusion gene. However, such a test is not yet available in mice. Therefore, for differential diagnosis of CNL, scoring for the presence or absence of particular morphologic features is crucial. Dysgranulopoiesis and dyserythropoiesis should not be present; i.e. cytochemical stains for ringed sideroblasts, marrow monocytes and dual esterase-positive cells must be negative in bone marrow specimens for exclusion of myelodysplastic processes (Elliott *et al*., 2001). More importantly in considering CNL, other CMD must be excluded.

It is unclear why the mice in this study showed a sex-specific disease phenotype in which female LTg mice showed disease latency of 6～10 months. This might be related to the observation that CNL is a disease of older adult humans, with females being affected later (79 years in females vs. 60 in males) (Bohm and Schaefer, 2002). The 6 to 10 months latency of the disease, taken together with disease phenotype, suggests that leukemogenesis by HccR-2 requires additional genetic changes. We believe that expression of HccR-2 alone is not sufficient to cause leukemia because the leukemic phenotype combines incomplete penetrance with delayed onset.

In summary, we produced a novel, transgenic mouse in which expression of the HccR-2 protooncogene leads to the development of a CNL-like phenotype with splenomegaly and neutrophilia. This model will be valuable not only for investigating the biological properties of HccR-2 *in vivo*, but also aid in characterizing the underlying mechanism involved in the progression of CNL.

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