Immunohistochemical Analysis for Excessive Splenomegaly in Transgenic Mice Expressing Dimeric Erythropoietin

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ABSTRACT

Erythropoietin (EPO), a glycoprotein hormone produced from primarily cells of the peritubular capillary endothelium of the kidney, is responsible for the regulation of red blood cell production. We have been investigating the roles of glycosylation site added in the biosynthesis and function of recombinant protein. In this study, we analyzed by immunohistochemical methods adaptive mechanisms to excessive erythrocytosis in transgenic (tg) mice expressing dimeric human erythropoietin (dHuEPO) gene. Splenomegaly was observed over 11~21 times in the tg mice. The 2,672 candidate spleen-derived genes were identified through the microarray analysis method, and decreased genes were higher than increased genes in the spleen. The specific proteins in the increased and decreased genes were analyzed by immunohistochemical methods. Our results demonstrate that problems of abnormal splenomegaly would solve in tg mice overexpressing dHuEPO gene.

(Key words : Erythropoietin, dHuEPO, Transgenic mice, Splenomegaly)

INTRODUCTION

Erythropoietin (EPO) is a glycoprotein produced from a 193-amino acid gene product after an N-terminal leader sequence containing 27 amino acids is cleaved. A carboxy-terminal arginine is lost from this 166-amino acid residue during passage into the circulation leaving a circulating hormone with 165 amino acids. The molecular mass of the EPO peptide is about 18 kDa (Lai *et al.*, 1986). EPO is mainly synthesized in the adult kidney and circulates in blood plasma, and a small portion of it is synthesized by the liver, and possibly by macrophages in the bone marrow (Benjamin and Franklin, 1999). Physiologically, EPO is produced in the fetal liver and adult kidney and primarily stimulates proliferation, differentiation, and maturation of erythroid progenitor cells (erythropoiesis) (Jelkmann, 1992; Schuster and Caro, 1993). In addition, EPO is also expressed in neuronal cells (Digicaylioglu *et al.*, 1995), can cross the blood-brain barrier (Brines *et al.*, 2000), and displays a protective effect against cerebral stroke and light-induced retinal degeneration (Brines, 2002; Ehrenreich *et al.*, 2002).

To follow the consequences of excessive erythrocytosis with a suitable *in vivo* model, transgenic (tg) mouse line generated. Constitutive overexpression of human EPO cDNA reaches hematocrit values up to 0.89 during the first eight to nine postnatal weeks (Ruschitzka *et al.*, 2000; Wagner *et al.*, 2001). Tg mice show a 10-to 12-fold elevation of EPO plasma levels. Plasma volume was not altered, whereas blood volume in tg mice was nearly doubled compared with wild-type siblings (Vogel *et al.*, 2003). The use of microarray and other global profiling technologies has led to a significant number of exciting new biological discoveries, and important correlations between gene-expression patterns and disease states. Nonetheless, it is important that investigators continue to optimize array methodologies and develop new approaches to producing accurate and experimentally valid data (Chuaqui *et al.*, 2002).

In earlier studies, we have been studying the recombinant glycoproteins (eCG, hFSH, TPO and EPO). It was found that deglycosylated sites were affected the expression and biological activity of these recombinant derivatives. Thus glycosylation sites play a pivotal role of the function and roles (Lee *et al.*, 2003; Min, 2000; 2001; Min *et al.*, 1996, 1997, 2004; Park *et al.*, 2005; Kim *et al.*, 2007; Naidansuren and Min., 2009). In the presented study, we analyzed immunohistochemical methods by using the increased and decreased genes for excessive splenomegaly in tg mice expressing dHuEPO gene.

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MATERIALS AND METHODS

Materials

CHO-K1 cells were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). General PCR reaction cocktail (Top-Taq™ Polymerase, 10×buffer, d-NTP mixture) purchased from Core Bio System (Seoul, Korea). Restriction enzymes used were bought from Takara and Toyobo (Osaka and Tokyo, Japan). Ham's F-12, Opti- MEM I, serum free CHO-S-SFM II, neomycin analogue G418 and lipofectamine 2000 reagents were bought from Gibco BRL (MD, USA). Fetal bovine serum was from Hyclone laboratories (Utah, USA). The EPO ELISA assay kit purchased from R&D systems Inc (MN, USA. ICR (Institute of Cancer Research) mice purchased from Korea Animal Technology (Koatech) (Seoul, Korea). EPO Elisa kit was from R & D system Inc. (MM, USA). All the other reagents used were from Wako Pure Chemicals (Osaka, Japan).

Construction of the dHuEPO Gene

The N-terminal EPO domain of the human EPO dimer-encoding construct was amplified by polymerase chain reaction (PCR) with a plasmid containing the human EPO cDNA (Park *et al.*, 2005), which contains the Asp-Ile restriction enzyme sites that were used to ligate 2 EPO molecules. The dimeric EPO molecule was constructed as previously reported (Min *et al.*, 2004). The dHuEPO gene was inserted into the expression vector pBC1 under the control of the goat β-casein promoter (designated as pBC1-dHuEPO). The direction of the ligated fragment was confirmed by restriction mapping using *Xho*I and *Sal*I. The sequence of the entire dHuEPO cDNA was verified by automated DNA sequencing performed using a previously reported method (Min *et al.*, 2004).

Immunohistochemistry of Spleen Section

We used the ABC complex (Vectastain ABC kit; Vector Laboratories, Inc., Burlingame, CA) for immunohistological staining. Paraffin-embedded sections (thickness, 4 μm) were deparaffinized with xylene and rehydrated with ethanol. Then, the sections were boiled in 10 mM sodium citrate for 10 min and placed on ice for 20 min. The sections were washed in phosphate-buffered saline (PBS) and incubated in 3% hydrogen peroxide for 10 min to block endogenous peroxidase activity. After washing in PBS, the sections were incubated overnight at 4℃ with the respective primary antibody diluted in blocking buffer (Elastase, CKR-6 and CD40, 1:250; TRIB3, PLEK2, ITPR2 and cathelicidin, 1:1,000). Then, the sections were incubated with the respective biotinylated secondary antibody (rabbit anti-rat or rat anti-goat IgG, 1:1,000 dilutions). Antibody binding was visualized by incubation of the membranes with ABC for 30 min and development in DAB solution. The sections were counterstained with hematoxylin and mounted with a cover glass. Finally, the sections were observed under a light microscope.

RESULTS

Immunohistochemistry of Spleen Section

Visual inspection of tg mice at necropsy revealed a dramatically enlarged spleen, suggesting increased extramedullar erythropoiesis (Fig. 1). Histologic examina-

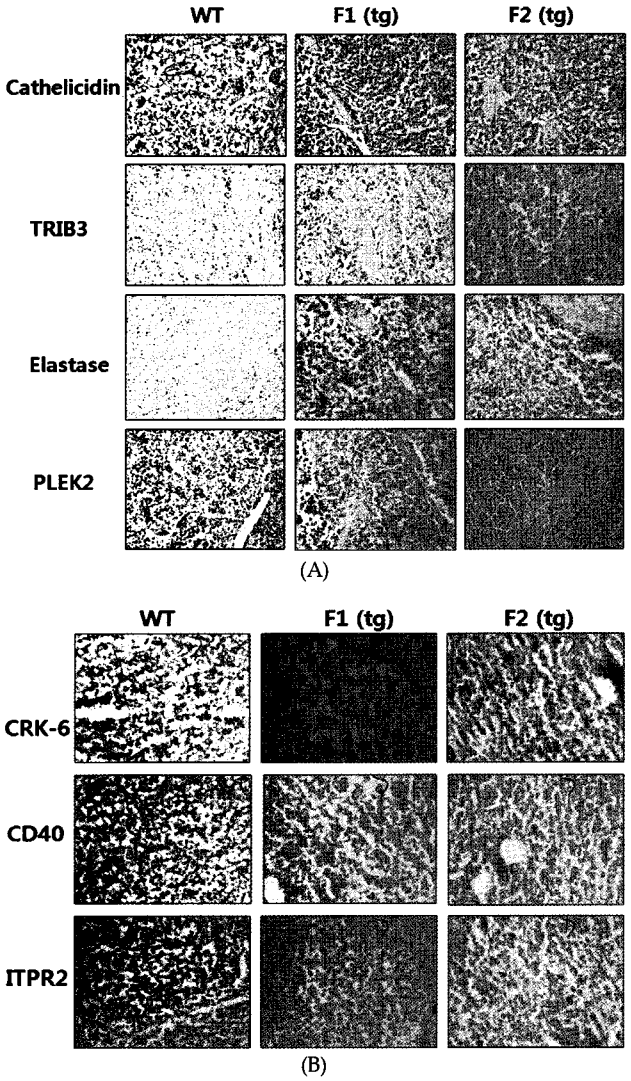

Fig. 1. Immunohistochemical analyses in splenomegaly of tg mice overexpressing dHuEPO gene. (A) The spleen sections obtained from wild type and tg mouse were stained with up-regulated gene antibodys, cathelicidin, TRIBs, elastase, and PLEK2. (B) The sections were stained with the down-regulated gene antibodys, CKR- 6, CD40, and ITPR2. Magnification, × 100.

tion confirmed extramedullar erythropoiesis in the spleen. Whereas a clear separation of white pulp and red plup (physiologically degrading senescent erythrocytes) was observed in wild type spleen, the tg spleen revealed an increased red pulp area with dissected white pulp structures. We validated expression of selected up-regulated and down-regulated genes using two approaches: RT-PCR and real-time PCR using RNA isolated from wild type and tg mice' spleen (data now shown). By the microarray results, the increased genes, cathelicidin, TRIB3, Elastase, and PLEK2, in tg mice spleen were shown in Fig. 1A. The down-regulation genes, CRK-6, CD40, and ITPR2, in tg mice spleen were shown Fig 1B. Up-regulation' result was consistent with the microarray expression profiling analysis, although the levels of fold-change are not the same as in the microarray analysis and would not be expected to be the same. However, it was not consistent to microarray analysis in the down-regulation genes.

DISCUSSION

By generating the dHuEPO over expressing mouse line, we aimed to establish a unique animal model to study the impact of elevated EPO plasma levels and excessive erythrocytosis. We observed severe degenerative processes in spleen that in concert resulted in markedly reduced life expectancy of mice (unpublished data). We analyzed the expressing gene of the abnormal splenomegaly in tg mice of dHuEPO gene. Splenomegaly was observed over 11~21 times in the tg mice. The 2,672 candidate spleen-derived genes were identified through the microarray analysis method, and decreased genes were higher than increased genes in the spleen. The specific proteins in the increased and decreased genes were analyzed by immunohistochemical methods.

Bogdanova *et al.* (2007) reported that macrophages of EPO tg mice are more active than wt macrophages and that tg erythrocytes are more attractive for macrophages than wt ones. Thus, in tg mice erythrocyte aging is accelerated, which results, together with an increased number and activity of their macrophages, in enhanced erythrocyte clearance. Moreover, they suggest that a new mechanism down-regulating red cell mass in excessive erythrocytosis in mice. They also insist that CD47 loss of the whole cell population would facilitate erythrophagocytosis in tg mice. However, a reduction of CD47 level per surface area on the red cells could be in principle also is due to a gain of surface area after the erythrocytes have left the bone marrow. This has been observed in patients suffering from cirrhosis of the alcoholic (Cooper *et al.*, 1972) or in dogs after feeding with a cholesterol-rich diet (Cooper *et al.*, 1980) and is mediated by cholesterol incorporation into the erythrocyte membrane. This phenomenon could be confirmed *in vitro* by incubating red cells with cholesterol-rich lipid dispersions (Cooper *et al.*, 1975). EPO Tg mice have a liver pathology since their liver is enlarged and shows hemosiderin dispositions and inflammatory foci (Bogdanova *et al.*, 2007). But EPO tg mice do not show any signs of liver cirrhosis (Heinicke *et al.*, 2006). Some reports show that a given elevation in hematocrit levels enhances exercise performance (Ekblom and Berglund, 1991). However, EPO-induced excessive erythrocytosis in tg mice does not (Heinicke *et al.*, 2006).

Several genes and gene families are up-regulated and down-regulated in the tg spleen expressing *dHuEPO* gene. Tribbles homologoue 3 (TRIB3) is the most abundantly (44-fold) up-regulated gene in tg spleen. It negatively interferes with insulin-mediated phosphorylation and activation of v-akt murine thymoma viral oncogene homologue (Oberkofler *et al.*, 2010). Although, in mice, deletion of TRIB3 did not produce major disturbances in insulin signalling and glucose homeostasis (Okamoto *et al.*, 2007), and in cultured rat hepatocytes adenoviral TRIB3 overexpression failed to affect insulin mediated AKT1 phosphorylation (Lynedjian, 2005). Cathelicidin (LL-37), the second most abundantly (16-fold) up-regulated gene in the tg mice is an antimicrobial peptide produced by neutrophils and respiratory epithelial cells that has similar roles in lung immunity as the defensins (Tecle *et al.*, 2010). In general, defensins and LL-37 have two major functions in host defense; direct inhibition of pathogens and modulation of other innate and adaptive immune responses. LL-37 plays key roles in host responses to infection (Mayer and Hancock, 2010). Of interest in the spleen of tg is the finding of expression and marked (5-fold) up-regulation of neutrophil elastase (NE). NE decreases the endothelial production of prostacyclin (PGI_2) through the inhibition of endothelial nitric oxide synthase (NOS) activation and thereby contributes to the development of ischemia-induced liver injury (Kawai *et al.*, 2010). NE induces MUC5AC gene expression in airway epithelium via a pathway involving reactive oxygen species (Fisher and Voynow, 2002). Pleckstrin-2 (PLEK2) is a 353 amino acid protein identified by the similarity to PLEK1. Both PLEK1 and PLEK2 contain two PH domains in their amino- and carboxyl-terminals (Hu *et al.*, 1999). PLEK2 has been implicated to be regulated by phosphatidylinositol (PI) 3-kinase, while PLEK1 has been suggested to be a major PKC substrate in platelets (Hamaguchi *et al.*, 2007).

In the down-regulated gene in tg mice spleen expressing dHuEPO gene, CRK-6, cdc2-related kinases (CRKs), 1, 2, 3, 4, and 6, were identified in the T. brucei genome (Hammarton *et al.*, 2003). An RNAi knockdown of CRK3 expression reduced the growth of the procyclic form by 91% and the bloodstream form by 69%

with an enrichment of cells in the G2/M phases in both forms (Tu and Wang, 2005). CD40 is a 48 kDa integral membrane protein expressed by cell of B cells, origin, dentritic cells, monocytes, epithelial cells, endothelial cells and tumor cells including carcinomas (Gruss *et al.*, 1997). The CD40/CD40 ligand system has been linked to the pathogenesis of atherothrombotic complications in cardiovascular disease. CD40-CD40L interactions are crucially involved in development of autoimmune disease in a number of animal models (Laman *et al.*, 1996). Inositol-1,4,5-triphosphate receptors (ITPR2) are two families of proteins located on membranes of cellular organelles that store calcium (Hertle and Yeckel, 2007). There are three known isotypes of ITPRs. ITPR1 was observed in pyramidal cells and granule cells, ITPR2 immunoreactivity was observed in perivascular astrocytes and endothelial cells (Hertle and Yeckel, 2007). The data presented herein offer the opportunity to develop a spleen database of genes expressed in tg mice expressing dHuEPO gene. The current study validates using microarray technology to investigate global changes in gene expression in mice spleen and can be extrapolated to defining the genetic profiles. Finally, this study sets the stage to develop a screen for candidate genes in patients with splenomegaly.

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