Extracellular Superoxide Dismutase (EC-SOD) Transgenic Mice: Possible Animal Model for Various Skin Changes

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ABSTRACT

We have generated transgenic mice that expressed mouse extracellular superoxide dismutase (EC-SOD) in their skin. In particular, the expression plasmid DNA containing human keratin K14 promoter was used to direct the keratinocyte-specific transcription of the transgene. To compare intron-dependent and intron-independent gene expression, we constructed two vectors. The vector B, which contains the rabbit -globin intron 2, was not effective for mouse EC-SOD overexpression. The EC-SOD transcript was detected in the skin, as determined by Northern blot analysis. Furthermore, EC-SOD protein was detected in the skin tissue, as demonstrated by Western blot analysis. To evaluate the expression levels of EC-SOD in various tissues, we purified EC-SOD from the skin, lungs, brain, kidneys, livers, and spleen of transgenic mice and measured its activities. EC-SOD activities in the transgenic mice skin were approximately 7 fold higher than in wild-type mice. These results suggest that the mouse overexpressing vector not only induces keratinocyte-specific expression of EC-SOD, but also expresses successfully functional EC-SOD. Thus, these transgenic mice appeared to be useful for the expression of the EC-SOD gene and subsequent analysis of various skin changes, such as erythema, inflamation, photoaging, and skin tumors.

(Key words : Transgenic mouse, EC-SOD, hk14 promoter, Antioxidant enzymes)

INTRODUCTION

Superoxide dismutases (SODs: EC 1.15.1.1) are key antioxidant enzymes in metabolism of reactive oxygen species (ROS). These enzymes catalyze the reaction, $O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$ (Crapo *et al.*, 1978; Folz *et al.*, 1997). Three different isoforms of SOD exist in mammals (Marklund *et al.*, 2002). A copper- and zinc-containing form of superoxide dismutase (Cu/Zn-SOD or SOD-1) was the first enzyme to be discovered and to be extensively characterized. This enzyme is a homodimer with 15.6 kDa subunits (Steinman *et al.*, 1974) and is widely distributed in the cytosol and nucleus, but it is noticeably absent from mitochondria and secretory compartments (Crapo and Tierney, 1974). Second, manganese SOD (Mn-SOD or SOD-2) is a manganese-containing enzyme localized in the mitochondrial matrix. It is a homotetramer with 22 kDa subunits (Folz *et al.*, 1997). Finally, extracellular SOD (EC-SOD, secreted SOD, s-SOD, or SOD-3) is the most recently discovered SOD family member (Buchman and Berg, 1988). In contrast to intracellular SOD1 and SOD2, the expression of SOD3 appears restricted to only a few cell types in several tissues (Zelko *et al.*, 2002). This isoform is a copper- and zinc-containing enzyme like Cu/Zn-SOD and is a homotetrameric glycoprotein with a molecular weight of about 165 kDa in mice (Fattman *et al.*, 2000). Unlike Cu/Zn- and Mn-SODs, EC-SOD is found in the extracellular matrix of tissue and extracellular fluids, such as serum, cerebrospinal, ascite, and synovial fluids (Ookawara *et al.*, 1998 Marklund, 2002). EC-SOD may play an important role in diseases such as insulin-independent diabetes mellitus (IDDM), systemic lupus erythematosus (SLE), reye syndrome, epilepsy (Ookawara *et al.*, 2000) and coronary artery disease (CAD) (Horinig *et al.*, 2001). However, EC-SOD is only present at low levels in extracellular fluids and it is difficult to obtain the purified protein thus, less is known about the physiological and biochemical properties of EC-SOD than of other SODs (Fattman *et al.*, 2000).

In the skin, there are many sources of ROSs; such as ultraviolet radiation, hyperbaric oxygen, and photosensitizing agent (psoralens, porphyrins, and some tetracyclines), as well as traumatic injuries (Pugliese, 1998). These sources promote production of ROSs and conse-

*This study was supported by a grant of the BioGreen21 (200203706), Republic of Korea.

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quently induce tissue damage by ROSs (Freeman and Carpo, 1982; Pugliese, 1995). Seven major enzymatic antioxidant systems defending againstdamage by ROSs have been identified in the skin, and the SOD family is one of the major antioxidant enzymes. Especially, SODs scavenge the superoxide anion radical, and consequently inhibit the formation of highly cytotoxic hydroxyl radical and peroxynitrite anion in the skin. The SOD level in the epidermis is low compared to the livers, heart, kidneys, lungs, and skeletal muscles (Carraro and Pathak, 1988) and the epidermal level of SOD was 5- to 10-fold lower than in the other organs mentioned above (Marklund, 1980). Despite previous physiological and biochemical studies, little is known about the detailed function and expression levels of EC-SOD in the skin.

In the current study, to evaluate the expression levels of EC-SOD in various mouse tissues, we generated transgenic mice that expressed mouse extracellular superoxide dismutase (EC-SOD) in their skin. In particular, the expression of plasmid DNA containing human keratin K14 promoter was used to direct the keratinocyte-specific transcription of the transgene.

MATERIALS AND METHODS

Animals

C57BL/6×C3H hybrid mice were purchased from Daehan Biolink, Korea. The animals were provided with a commercial diet (CJ, Korea) and water *ad libitum* under 22±2℃ temperature, 50±5% relative humidity, and 12 hr of artificial lighting from 07:30 to 19:30.

Transgene Construction and Production of Transgenic Mice

A diagram illustrating the DNA cassette used to generate transgenic mice is shown in Fig. 1. In order to induce the skin-specific expression of mouse EC-SOD (756 bp), human keratin 14 promoter (1,952 bp), which has been described as a keratinocyte-specific promoter (Staggers *et al.*, 1995), was cloned from the pBS KS^+ vector with a human keratin 14 promoter region. To compare intron-dependent and intron-independent gene expression, a rabbit-globin intron 2 (573 bp) was used. Two expression vectors were constructed by recombination of the EC-SOD gene into the pCR II TOPO vectors (Invitrogene, USA). Chicken ovalbumin poly A (192 bp) was also inserted. Plasmid DNA containing the sense-strand transgene vector was purified using a Qiagen Midi kit, and digested with *Hind* III and *Xho* I in order to produce a linear construct consisting of human K14 skin promoter, mouse EC-SOD cDNA, and a polyadenylation sequence. The DNA construct was then microinjected into fertilized C57BL/6×C3H embryos. Eleven founder mice expressing mouse EC-SOD were established by breeding them with C57BL/6×C3H hybrid mice. The transgenic mice were confirmed by PCR using the following primers: hk14-2F, TTG TCT CTA ATA GAG GGT C; SOD-2R, TCA AGC CTG TCT ATC TTC T (95℃ for 1 min; 51℃ for 1 min; 72℃ for 1 min, 30 cycles). The line with the highest transgene expression level was selected for further analysis.

RT-PCR

The total RNA was isolated from the skin and various tissues of the transgenic mice using the TRI reagents (M.R.C, USA) according to the manufacturer's protocol. 1 g of the total RNA was reverse-transcribed by AMV reverse transcriptase (Promega, USA). PCR was performed using the forward primer hk14-2F: TTG TCT CTA ATA GAG GGT C and the reverse primer SOD-2R: TCA AGC CTG TCT ATC TTC T. After denaturation for 5 min at 94℃, 30 cycles of PCR (94℃ for 1 min, 51℃ for 1 min, and 72℃ for 1 min) were then performed. The mouse GAPDH gene was amplified from the first-strand cDNA by PCR using primers GAPDH-F 5V-AAT GCA TCC TGC ACC ACC AA-3V, and GAPDH-R, 5V-GTA GCC ATA TTC ATT GTC ATA-3V (95℃ for 1 min; 55℃ for 1 min; 72℃ for 1 min, 30 cycles), which extended the DNA fragments of the mGAPDH gene, respectively, and yielded a 500 bp fragment. The reaction conditions were the same as the ones listed above.

Northern Blot Analysis

Total RNA was isolated from skin tissues according to standard procedures. Samples of 20 μg total RNA were resolved by electrophoresis on 1% agarose-formaldehyde gels and transferred to hybond nylon membranes according to the manufacturer's instructions (Amersham Pharmacia Biotech, UK). After ultraviolet (UV) cross-linking, the blots were pre-hybridized at 65℃ for 2 hr followed by the hybridization with the same solution plus the EC-SOD/hk14 cDNAprobe for 20 hr. After hybridization, the membranes were washed once in 2× SSC/1% SDS at 65℃ for 15 min and once in 1×SSC/1% SDS at 65℃ for 30 min. Autoradiography was performed at -70℃ using intensifying screens. To obtain sufficient signals the blots were exposed to X-ray film for 2 days.

Western Blot Analysis

Homogenized skins were used to determine EC-SOD expression in the transgenic mice. The protein concentrations were determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA) and BSA was used as a standard. Equal amounts of protein from each cell homogenate were subjected to 8% SDS-PAGE, and then transferred to a nitrocellulose membrane. This filter was blocked

in Tris-buffered saline containing 5% BSA, 50 mM Tris-HCl, pH 7.5, 0.15 M NaCl and 0.1% Tween-20 (TBST) at room temperature for 3 hr, washed with TBST, and then blotted with a monoclonal antibody against EC-SOD.

Measurement of EC-SOD Activities in Various Tissues

F1 transgenic line 27 (3 mice of each gender) were sacrificed and skin, lungs, brain, kidneys, livers and spleen were obtained. These tissues were homogenized in 10 volumes of ice-cold 50 mM potassium phosphate buffer (pH 7.4) with 0.3 M KBr, 0.5 M phenyl methylsulfonyl fluoride, 3 mM diethylenetriaminepentaacetic acid, and 100,000 IU/ml aprotinin, as described by Fattman *et al.* (2000). The homogenate were sonicated for 3 min followed by centrifugation at 20,000 ×g for 20 min at 4℃. To separate EC-SOD from total SODs, the supernatant was passed over a concanavalin A-sepharose column (Amersham Pharmacia, UK) as described by Fattman *et al.* (2000) and Marklund (1990). Briefly, a 1 ml concanavalin A-sepharos column was prepared at 4℃ and equilibrated with 10 volumes of 20 mM Tris-HCl solution (pH 7.4) containing 0.5 M NaCl. The supernatant (0.5 ml) was applied to the column. After 5 min, the column was washed with 10 volumes of the same equilibration solution. Finally, 2.5 ml of elution buffer (pH 7.4) composed of 0.5 M-methylmannoside, 20 mM Tris-HCl, and 0.5 M NaCl was applied to the column. The eluted EC-SOD solution was stored at −70℃. EC-SOD activity was measured by inhibition of xanthine plus xanthine oxidase-mediated cytochrome-*C* reduction at pH 10, as described by Fattman *et al.* (2000) Briefly, three stock solutions were prepared; the first contained 50 mM of sodium carbonate (pH 10), the second was the same as the first solution, but supplemented with 0.1 mM of cytochrome-*C* (Calbiochem, USA), and the third was the same as the first, but supplemented with 0.5 mM of xanthine (Sigma-Aldrich, USA). Prior to the assay, the reaction mixture was prepared by adding 0.3 ml of the 2nd and 3rd solutions to 2.4 ml of the 1st stock solution in a 3 ml cuvette, which was then adjusted to 25℃. The reaction was initiated by adding 10 μl of autoclaved H_2O and 10 μl xanthine oxidase (Sigma-Aldrich, USA), and the increase in absorbance at 550 nm per minute was determined using a spectrophotometer (DU-70, Beckman, USA). The EC-SOD activity was calculated with % inhibition of the sample rate against the standard rate. A unit of EC-SOD was defined as the quantity of EC-SOD required to produce 50% inhibition of reduction rate of cytochrome-*C*. The total protein was determined by measuring absorption at 750 nm using a DC Protein Assay Kit (Bio-Rad, USA).

Statistical Analysis

Data were analyzed by Chi-square analysis to determine differences between groups.

RESULTS

Generation of Transgenic Mice

In order to induce the skin-specific expression of EC-SOD, human keratin K14 (hK14) promoter vector was used. This promoter has been shown to drive the expression of a transgene in the skin. A two-transgene construct, hK14-EC-SOD (Fig. 1A,B), was microinjected into mouse embryos and the progenies were examined as the founders of the new transgenic mouse lines. Total genomic DNA extracted from tail biopsies of founder candidates were screened using PCR analysis for the presence of the EC-SOD transgene (Table 1). Of eleven transgenic mice generated, eight were germ-line transgenic, three were non-germ lines (data not shown). When each founder mouse was mated with a non-transgenic mouse, all were able to produce offspring and the transgene was inherited in a Mendelian manner (data not shown). The newborn transgenic mice grew up without any health problems and became sexually mature.

Expression of EC-SOD Transgene

The mRNA levels of the EC-SOD gene and tissue-specific expression were analyzed by RT-PCR. Fig. 2 shows a representative band indicating that 4 lines of eleven transgenic mice overexpressed the EC-SOD gene. The EC-SOD mRNA levels of line 16, 26, and 27 in the founder mice had a highly skin-specific pattern of transgene expression (Fig. 2). To compare intron-dependent and intron-independent gene expression, we constructed two

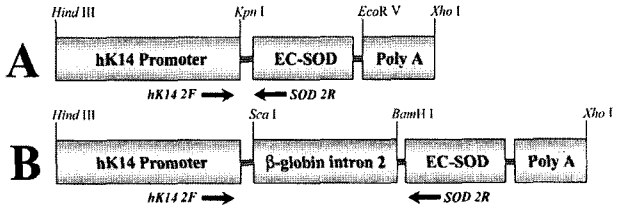

Fig. 1. Transgenic construct for skin-specific expression of mouse EC-SOD. Diagram illustrating the DNA cassette used for injection. The primers were used for tail biopsy PCR are indicated as arrows. hK 14 promoter; human keratin 14 promoter (1,952 bp), mouse EC-SOD; mouse extracellular superoxide dismutase (756 bp), β -globin intron 2; rabbit β -globin intron 2(585 bp), poly A; chicken ovalbumin poly A (212 bp).

Table 1. Efficiency of the transgenic mice production

Vector type	No. of embryos injected	No. of embryos transferred	No. of recipients	No. of (%) mice born	No. (%) of transgenic mice
Type A	694	463	23	33	6(18.1)
Type B	569	368	18	21	5(23.8)

Type A; without β -globin intron 2, Type B; with β -globin intron 2.

vectors (Fig. 1). Vector B induced rabbit-globin intron 2, whereas the vector A did not. Vector A induced that fourlines of six transgenic mice overexpressed the EC-SOD mRNA in the transgenic mice skin. In contrast, vector B was not detected in any other founders (Fig. 2). Fig. 3 shows the EC-SOD mRNA levels of line 27 in various tissues, demonstrating that transgenic mice have a highly skin-specific pattern of transgene expression. The skin specific expression of the EC-SOD transgene was further confirmed by Northern blot analysis of total RNAs extracted from various tissues of mice of the transgenic line 27 (Fig. 4). The EC-SOD mRNA was detected only in skin and was not seen in any other tissues, demonstrating that EC-SOD expression is tissue- specific.

EC-SOD Exists as a Dimer Composed of Disulfide-linked Monomers

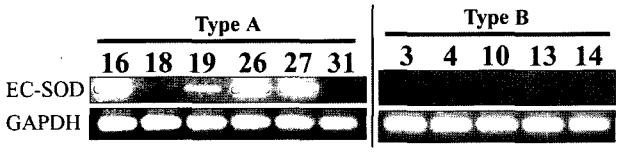

Fig. 2. Messenger RNA levels for the mouse EC-SOD in skin of transgenic mice. RT-PCR performed using 1 μg per lane of total RNA isolated from skin of offspring mice. Type B transgenic mice; 3, 4, 10, 13, 14, Type A transgenic mice; 16, 18, 19, 26, 27, 31.

Fig. 3. Messenger RNA levels for the mouse EC-SOD in skin and various tissues of transgenic mice (27 line) by RT-PCR. RT-PCR was performed using 1 μg per lane of total RNA isolated from skin and various tissues of offspring mice. Sk-C normal mouse skin, Sk-D; transgenic mouse dorsal skin, Sk-V; transgenic mouse ventral skin, Li; livers, He; heart, Lu; lungs, Br; brain, Ur; ureter, Ut; uterus, Ki; kidneys, Sp; spleen.

Analysis of EC-SOD proteins under non-reducing conditions revealed two major bands, 31.5 and 63 kDa, and also a truncated 51 kDa minor form, respectively (Fig. 5).

EC-SOD Activities in Various Tissues

EC-SOD activities in skin, lungs, brain, kidneys, livers, and spleen of the transgenic line 27 and wild-type mice were measured (Table 2). At 8 weeks in both the transgenic and wild-type mice, EC-SOD activities in measured

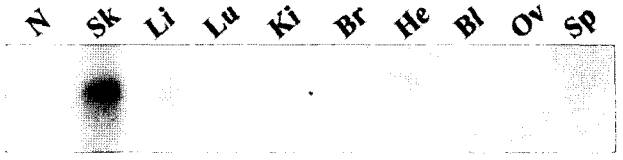

Fig. 4. Messenger RNA levels for the mouse EC-SOD in skin and various tissues of transgenic mice (line 27). Northern-blot analysis was performed using 20 μg per lane of total RNA isolated from skin and various tissues of offspring mice. N; normal skin, Sk; skin, Li; livers, Lu; lungs, Ki; kidneys, Br; brain, He; heart, Bl; bladder, Ov; ovary, Sp; spleen.

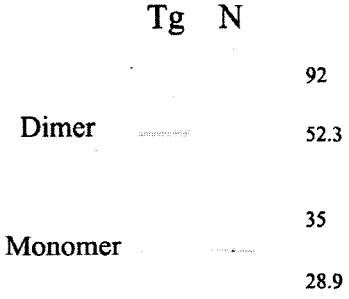

Fig. 5. Western blot analysis for mouse EC-SOD using a monoclonal antibody against EC-SOD in mouse skin. Tg; EC-SOD transgenic mice (line 27), N; non-transgenic mice.

Table 2. EC-SOD activities in various tissues of the transgenic mice

Tissue	Wild-type		Transgenic mouse (27 line)	
	Male	Female	Male	Female
Skin	6.14±0.42[a*]	6.11±0.67[a]	45.20±3.87[b]	45.40±4.82[b]
Spleen	5.90±1.60	5.30±0.82	5.47±0.60	5.39±1.12
Kidney	4.03±0.03	4.09±0.14	4.21±0.09	4.12±0.40
Brain	5.60±0.06	5.54±0.18	5.72±0.55	5.62±0.14
Lung	11.19±1.70	10.12±0.41	11.16±1.53	11.32±0.70
Liver	5.34±0.07	5.60±0.18	5.60±0.40	5.14±0.11

* Means±SD. The values show the EC-SOD activity units per mg of total protein.
[a,b] Significantly different at $p<0.01$ compared to the transgenic and wild-type mice by Chi-square analysis.

tissues did not different between females and males. The EC-SOD activities in the skin of transgenic and wild-type of male and female mice were 45.20±3.87, 45.40 ±4.82 and 6.14±0.42, 6.11±0.67 units per mg of total protein, respectively. The EC-SOD activities in the transgenic skin were approximately 7 fold higher than in the wild-type skin ($p<0.01$). In the transgenic and wild-type mice, lung EC-SOD activities of males and females were 11.16±1.53, 11.32±0.70 and 11.60±1.70, 10.12±0.41 units per mg of total protein, respectively. In the present study, mice contained high activities of EC-SOD in their lungs as compared to other tissues. In fact, the lung EC-SOD activities in measured tissues, except in the transgenic mouse skin, were approximately 2 to 3 fold higher than in other tissues.

DISCUSSION

We have generated transgenic mice that expressed mouse extracellular superoxide dismutase (EC-SOD) in their skin in this study. To obtain detailed information about EC-SOD, many scientists have resorted to genetic engineering techniques, such as knock-out (Carlsson *et al.*, 1995 Jonsson *et al.*, 2002) and transgenic (Oury *et al.*, 1992 Bowler *et al.*, 2001),to investigate the function of EC-SOD. However, there are no transgenic studies of EC-SOD in the skin.

According to Buchman and Berg (1988), the recombinant vector carrying rabbit-globin cDNA failed to express β -globin unless an intron was induced in the transcription unit. However, our results indicate that vector B, which contains rabbit-globin intron 2, is not effective for mouse EC-SOD overexpression.

Our result suggests that the difference in molecular weights of the EC-SOD bands is due to truncation of the carboxyl-terminal region of EC-SOD, as has previously been shown for human EC-SOD (Oury *et al.*, 1996). Notably, our result indicates that EC-SOD transgenic mice expressing protein are more stably dimerized in skin extracts. The covalent interaction of the dimeric EC-SOD is due to an interchain disulfide bond. These results are observed in Western blots of total skin homogenates probed with an antibody to mouse EC-SOD, indicating that the interchain disulfide bonds depend on EC-SOD expression in tissues.

The EC-SOD activity results in this study suggest that the vector may not only induce keratinocyte-specific expression of EC-SOD but that it also expresses successfully functional EC-SOD. Our results indicate that EC-SOD may be expressed massively in lungs and that this enzyme may play a physiologically important role in lungs, although more detailed information is still needed. Therefore, to clarify the detailed function of EC-SOD in the skin, we will in further studies develop transgenic mice overexpressing mouse EC-SOD skin-specifically.

Thus, these transgenic mice appear to be useful for the expression of EC-SOD gene and subsequent analysis of various skin changes, such as erythema, imflamation, photoaging and skin tumors.

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(Received: 5 August 2006/ Accepted: 11 September 2006)