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Gene Transfer of Extracellular Superoxide Dismutase to Atherosclerotic Mice

MIKKO O. LAUKKANEN,[1] PIA LEPPÄNEN,[1] PÄIVI TURUNEN,[1]
ELINA PORKKALA-SARATAHO,[2]
JUKKA T. SALONEN,[2] and SEPPO YLÄ-HERTTUALA[1,3]

ABSTRACT

Clinical and epidemiological studies have provided circumstantial evidence that oxidized low-density lipoprotein (LDL) and antioxidants are involved in the pathogenesis of atherosclerosis. Superoxide dismutases (SODs) have been shown *in vitro* to protect LDL from deleterious effects of superoxide anions. In the present study, we have used adenoviral gene transfer to determine effect of extracellular SOD (EC-SOD) on atherogenesis in LDL receptor −/− mice. Intravenous administration of EC-SOD adenovirus (2×10^9 plaque forming units) into tail vein targeted transgene mainly to liver and induced a 3.5- to sevenfold increase in plasma total SOD activity. EC-SOD was secreted into circulation for 2–3 weeks mostly in a truncated B-form, suggesting that endogenous proteolytic mechanisms control the level and distribution of the enzyme. Therapeutic potential was determined by measuring plasma resistance against copper oxidation and analyzing atherosclerotic lesion areas in aortas of LDL receptor −/− mice. Mice were kept on a cholesterol diet for 10 weeks before gene transfer and 3 or 6 weeks after the gene transfer. Results showed a tendency for a reduction in the overall lesion area after EC-SOD gene transfer as compared with LacZ transduced control mice, but the difference did not reach statistical significance. It is concluded that short-term overexpression of EC-SOD *in vivo* does not affect atherogenesis in LDL receptor −/− mice. Antioxid. Redox Signal. 3, 397–402.

INTRODUCTION

SUPEROXIDE ANION (O_2^-), the production of which is increased in atherogenesis, may not itself be highly atherogenic, but can lead to the production of more harmful compounds, such as peroxynitrite ($ONOO^-$) and hydroxyl radical ($^{\cdot}OH$) (3, 7). The action of reactive oxygen species (ROS) causes increased uptake of low-density lipoprotein (LDL) into macrophages through macrophage scavenger receptors, leading to foam cell formation and reduction of lumen size (25). Synthetic antioxidants, like BO-653 and CGP 2881, have been developed to protect LDL from oxidation with promising results (2, 6).

Superoxide dismutases (SODs) are antioxidant enzymes that protect tissues against detrimental effects of O_2^-. Extracellular SOD (EC-SOD) as an extracellular protein is a potential tool for gene therapy. The half-life of the protein can be up to 20 h (12) in the vascular system, which is markedly longer than the half-life of intracellular copper/zinc-SOD in circulation (7 min) (9). *In vitro* EC-SOD has been shown to reduce endothelial cell-mediated

[1]A.I. Virtanen Institute for Molecular Sciences, [2]Research Institute of Public Health, and [3]Department of Medicine, University of Kuopio, Kuopio, Finland.

LDL oxidation as determined by macrophage degradation assay and thiobarbituric acid reactive substances (TBARS) (10).

In the present study, we have used systemic adenoviral gene transfer in mice to determine whether EC-SOD can attenuate LDL oxidation and lesion formation in cholesterol-fed LDL receptor −/− mice. Gene transfer tended to reduce lesion formation as compared with controls, but did not reach a statistically significant level. The results suggest that short-term overexpression of EC-SOD does not protect arteries against atherosclerosis.

MATERIALS AND METHODS

Animals

LDL receptor −/− mice (10) were kept on western-type, 0.15%, cholesterol diet (Harlan Teklad, Madison, WI, U.S.A.) for 2 months prior to adenovirus injection [2×10^9 plaque forming units (pfu)] into the tail vein. Plasma samples were taken 3 days after gene transfer for EC-SOD activity and cholesterol analysis. Cholesterol levels were analyzed by Preciset Cholesterol kit (Boehringer-Mannheim, Mannheim, Germany). EC-SOD and LacZ control adenovirus (AdBglII) were produced in 293 cells and analyzed to be free of microbiological contaminants, mycoplasma, endotoxin, and replication-competent viruses (14).

EC-SOD activity analysis

The efficiency of adenovirus gene transfer was determined by measuring total SOD activity from the plasma. The assay is based on the ability of EC-SOD to catalyze the disproportionation of O_2^- in alkaline aqueous solution. The disproportionation was measured spectrophotometrically (Lambda Bio, Perkin–Elmer, Boston, MA, U.S.A.) using KO_2 as a substrate. One unit in the assay is defined as the activity that brings about a decay in O_2^- concentration at a rate of 0.1 s^{-1} in 3 ml of buffer and corresponds to 8.6 ng of human EC-SOD (18).

Histological analysis

To determine the distribution of control LacZ adenovirus *in vivo,* tissue samples were dissected from mice after perfusion–fixation for 2 min with 4% paraformaldehyde/phosphate buffer. The fixation of the samples was continued for 2 h in the same fixative, rinsed in 0.15 *M* sodium phosphate overnight, and embedded in O.C.T. compound (Miles Scientific, Elkhart, IL, U.S.A.) (26). β-Galactosidase activity was analyzed by 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; Sigma, St. Louis, MO, U.S.A.) staining of samples for overnight at 37°C (26).

Affinity chromatography

Plasma from a subset of EC-SOD (n=4) and LacZ (n=4) adenovirus-transduced mice was analyzed on heparin Sepharose affinity chromatography using Hi-trap columns (Pharmacia, Freiburg, Germany). Pre- and postheparin (1,000 U/kg) plasma samples were collected 3 days after transduction with 2×10^9 pfu/ml AdEC-SOD. Aprotinin (80 μg/ml), leupeptin (10 μg/ml), and phenylmethylsulfonyl fluoride (0.5 m*M*) (Sigma) were added to avoid degradation of EC-SOD. The column was equilibrated with 10 volumes of 15 m*M* cacodylic acid/50 m*M* NaCl, pH 6.5, before application of 100 μl of plasma. EC-SOD was eluted using a step gradient (0.2 *M* NaCl, 0.45 *M* NaCl, and 0.7 *M* NaCl in 15 m*M* cacodylic acid, pH 6.5) (21). The amount of EC-SOD in 500-μl eluates was determined by EC-SOD activity as described above.

Analysis of conjugated dienes

Mice were killed and plasma was collected by heart puncture 3 days after the gene transfer. The antioxidant capacity of EC-SOD (n = 9) was determined by measuring serum resistance to oxidation and compared with LacZ controls (n = 7). Oxidation was initiated by adding of 100 μl of 1 mmol/L $CuCl_2$ into serum samples (100 μl). The formation of conjugated dienes was followed by monitoring the change in 234 nm absorbance (20).

Analysis of atherosclerotic lesions

Mice were killed 3 weeks (AdEC-SOD, n = 9; AdLacZ, n = 10) or 6 weeks (AdEC-SOD, n = 16; AdLacZ, n = 16) after the gene transfer. Aortas were cut longitudinally, and a percent-

age of the endothelium area covered by lesions was determined by image analysis (MCID/M4, Imaging Research Inc., Toronto, Ontario, Canada). The result was confirmed by determining the lesion size from histological cross-sections after hematoxylin–eosin staining (15).

RESULTS

Systemic delivery of adenovirus commonly targets transgene to the liver. Accordingly, the expression of systemically transferred LacZ gene in mice was detected mainly in liver and spleen (Fig. 1) and to a lesser extent also in kidney. Plasma total SOD activity was increased by 3.5- to sevenfold as measured 3 days after the gene transfer.

Heparin can be used to release endothelial bound EC-SOD into plasma, which results in a rapid increase in plasma total SOD activity (11). However, injection of heparin into AdEC-SOD transduced mice did not result in any marked increase in total SOD activity, but the activity stayed mostly at the preheparin level (Fig. 2A). To clarify these findings, plasma samples were analyzed 3 days after the gene transfer by heparin affinity chromatography and shown to contain mainly the B-form of EC-SOD, which does not bind to heparan sulfate proteoglycans with high affinity. The A-form was also present, whereas the C-form was clearly underrepresented (Fig. 2B).

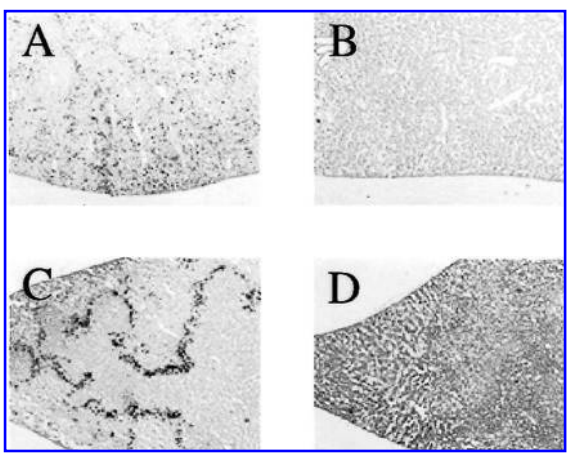

FIG. 1. Tissue distribution of adenovirus after gene transfer. (**A**) X-Gal staining AdLacZ transduced liver. (**B**) X-Gal staining AdEC-SOD transduced liver. (**C**) X-Gal staining AdLacZ transduced spleen. (**D**) X-Gal staining AdEC-SOD transduced spleen. Original magnification was 10×.

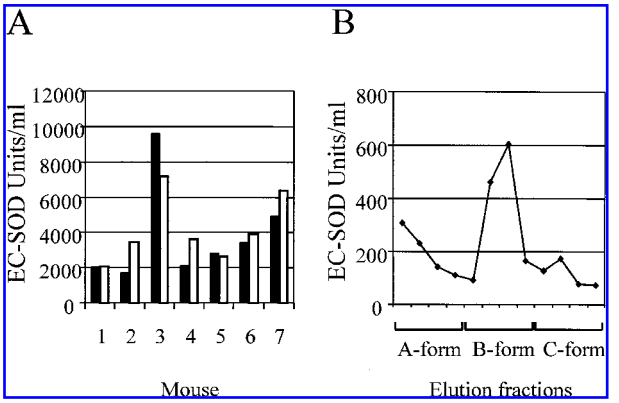

FIG. 2. (**A**) Effect of heparin injection on plasma total SOD activity after AdEC-SOD gene transfer. Three days after the gene transfer, 1,000 U/kg heparin was given subcutaneously. Plasma samples were taken 15 min after the heparin injection, and total SOD activities (open columns) were compared with preheparin values (black columns). (**B**) Heparin Sepharose chromatography of plasma samples. Three days after the gene transfer, plasma was collected by heart puncture. Different EC-SOD isoforms were separated from 100-μl plasma samples by step gradient elution in heparin affinity column. The A-form was eluted with 0.2 *M* NaCl, the B-form with 0.45 *M* NaCl, and the C-form with 0.7 *M* NaCl. Most of the SOD activity in plasma is in the B-form, which has a relatively weak affinity to heparan sulfate proteoglycans, or A-form, which has no affinity to heparin.

To evaluate the effect of EC-SOD on LDL oxidation *in vivo*, we measured plasma resistance against copper-induced oxidation. Previously, we have shown that EC-SOD is capable of inhibiting LDL oxidation by endothelial cells *in vitro* (14). However, determination of the formation of conjugated dienes from plasma samples showed that EC-SOD gene transfer did not increase protection of plasma against copper-mediated oxidation (Table 1).

Cholesterol-fed mice were injected with AdEC-SOD and AdLacZ, and lesion areas were determined at 3- and 6-week time points (Fig. 3). AdEC-SOD transduced mice did not show any significant differences in lesion formation at either time point even though there was a tendency toward a reduction of lesion size.

DISCUSSION

Several antioxidant defense mechanisms protect LDL from lipid peroxidation. These include

TABLE 1. COPPER-INDUCED OXIDATION OF PLASMA

	Activity (U/ml)	*Lag time (min)*	*Total cholesterol (mmol/L)*
EC-SOD 1	2,385	180	39.14
EC-SOD 2	4,789	215	33.56
EC-SOD 3	3,510	260	28.97
EC-SOD 4	1,200	225	43.88
EC-SOD 5	878	220	40.14
EC-SOD 6	5,842	265	36.69
EC-SOD 7	1,870	265	35.83
EC-SOD 8	916	400	37.84
EC-SOD 9	3,087	295	31.95
Mean	2,719	258	36.44
LacZ 1	563	75	23.57
LacZ 2	614	135	34.85
LacZ 3	755	245	35.88
LacZ 4	555	255	41.5
LacZ 5	1,076	170	43.47
LacZ 6	749	375	42.05
LacZ 7	917	430	52.66
Mean	747	243	39.14

Mice 1–9 were injected with AdEC-SOD and mice 10–16 with AdLacZ adenovirus. Three days after gene transfer, plasma total SOD activity and oxidation resistance, as well as total cholesterol level, were determined. The average increase in total SOD activity was fourfold as compared with controls. The average time of resistance for the EC-SOD group was 258 min and for the LacZ group 243 min, showing 6.5% longer resistance for the EC-SOD plasma.

lipid- and water-soluble antioxidants and enzymes like glutathione peroxidases, catalase, and SODs. As EC-SOD has a prominent expression in atherosclerotic lesions (8, 17, 23) and is affected by methylation changes found in atherosclerotic lesions (13), it is suggested that EC-SOD plays a significant role in atherogenesis.

LDL oxidation in vascular wall can be divided into two phases: LDL peroxidation and the modification of LDL apoprotein B (apoB). An important step in LDL peroxidation is the enrichment of LDL with lipid hydroperoxides, which are then transformed into intermediate products, like malondialdehyde and 4-hydroxynonenal, responsible for apoB modification. The latter phase is characterized by apoB breakdown and covalent binding of reactive aldehydes to lysine residues of apoB (5, 24). These reactions change LDL properties in a way that it is metabolized through macrophage scavenger receptors, which then leads to accumulation of lipids into vascular wall macrophages and foam cell formation (22).

EC-SOD is abundantly present in the vascular wall and synthesized in atherosclerotic lesions by smooth muscle cells and macrophages (8, 17, 23). Recently, it has been reported that the level of low-heparin affinity forms of EC-SOD are increased in atherosclerotic patients, whereas the amount of high-heparin affinity C-form is decreased (1). It has also been shown that proteolytic processing leading to the appearance of truncated EC-SOD can occur both intracellularly and in the extracellular space (4).

The systemic gene transfer resulted in the production of partially truncated EC-SOD B-form, which has reduced affinity to endothelial cell heparan sulfate proteoglycans (19). Present results together with the previously reported posttranscritional regulation seen *in vitro* (14) suggest that EC-SOD synthesis is regulated at several steps. Adenovirus-mediated gene transfer resulted mainly in the synthesis of the EC-SOD B-form, which has lowered affinity to heparan sulfate proteoglycans and could explain the low antiatherogenic property of the transgene. Truncation of the overexpressed EC-SOD may change the physiological localization and also tissue distribution of the enzyme. This

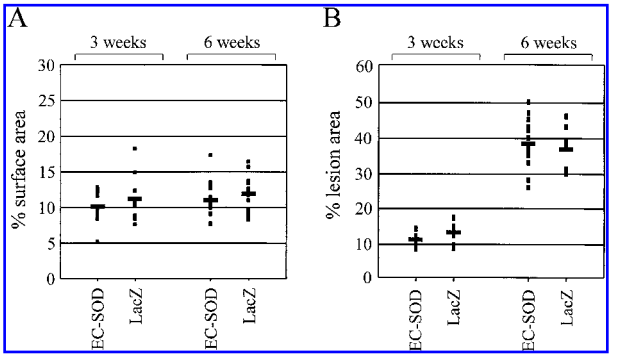

FIG. 3. Area covered by atherosclerotic lesions in aorta. LDL receptor −/− mice were kept on the cholesterol diet for 10 weeks before gene transfer and killed 3 or 6 weeks after the gene transfer. (**A**) Three weeks after the gene transfer, the aorta was isolated and opened longitudinally, and the lesion area covering the endothelium was measured. In the 3-week experiment, the mean value for lesions for the EC-SOD group is 10.1 ± 2.5% and for the LacZ control group 10.9 ± 3.4%. In the 6-week experiment, the mean value of the lesion size for the EC-SOD group is 10.8 ± 2.6% and for the LacZ control group 11.9 ± 2.5%. (**B**) Lesion area determined from cross-sections 3 weeks after gene transfer. The mean values for lesions in EC-SOD and LacZ transduced mice were 11.0 ± 2% and 12.0 ± 3% 3 weeks after gene transfer and 38.5 ± 7% and 37.8 ± 6% 6 weeks after gene transfer.

may have an influence on EC-SOD bioactivity because EC-SOD is most effective when it is bound on the cellular surfaces (16).

In our previous studies, we have shown that EC-SOD is capable of inhibiting LDL oxidation in vitro as detected by macrophage degradation assay, agarose gel electrophoresis, and TBARS analysis (14). In the present study, no effect was found in the resistance against copper-induced plasma oxidation after the AdEC-SOD gene transfer. This may be due to a low increase in plasma total SOD activity after the gene transfer. Also, no inhibitory effect of EC-SOD on the development of lesions may be partially due to the use of adenovirus that generally results in temporary expression for 2–3 weeks. Another reason may be the partial truncation of EC-SOD, which diminishes the amount of EC-SOD present on the endothelial surfaces. The complete evaluation of the role of EC-SOD in atherogenesis would require the use of e.g., the lentivirus vector, which produces much longer expression of the transgene in target tissue.

It is concluded that short-term overexpression of EC-SOD does not affect atherogenesis in LDL receptor −/− mice. Further analysis including local gene transfer into vessel wall with retro- or lentiviral vectors is required to analyze the local role of EC-SOD in the development of atherosclerotic lesions.

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ABBREVIATIONS

apoB, apolipoprotein B; EC-SOD, extracellular superoxide dismutase; LDL, low-density lipoprotein; O_2^-, superoxide anion; pfu, plaque forming unit; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances.

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Address reprint requests to:
Dr. S. Ylä-Herttuala
A.I. Virtanen Institute for Molecular Sciences
University of Kuopio
P.O. Box 1627
FIN-70211 Kuopio
Finland

E-mail: Seppo.Ylaherttuala@uku.fi

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