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Overexpression of EC-SOD Suppresses Endothelial-Cell-Mediated LDL Oxidation

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Reactive oxygen species have been proposed to play important roles in atherosclerosis. To investigate the protective role of extracellular superoxide dismutase (EC-SOD), its inhibition of endothelial-cell-mediated LDL oxidation was examined. We constructed the recombinant adenovirus AxCAEC-SOD expressing human EC-SOD by CAG promoter. Infection of endothelial cells with AxCAEC-SOD resulted in EC-SOD protein secretion in a dose-dependent manner and a decrease of endothelial-cell-derived superoxide production. Moreover, it was proven to coexist with heparan sulfate by immunohistochemical staining. Endothelial-cell-mediated LDL oxidation enhanced by ferric-sodium EDTA was inhibited by 47% in TBARS formation by AxCAEC-SOD infection. In agarose gel electrophoresis, AxCAEC-SOD decreased the negative charge of oxidized LDL by 50% and suppressed fragmentation of apolipoprotein B. These results suggested that human EC-SOD localized in the extracellular space and reduced endothelial-cell-mediated LDL oxidation. In subendothelial space, EC-SOD bound on heparan sulfate might suppress LDL oxidation through reduction of superoxide anion.

Key Words: **extracellular superoxide dismutase; low density lipoprotein; endothelial cell; oxidative stress; superoxides; heparan sulfate; adenovirus.**

Recent advances in studies of oxygen radicals and cell interactions have allowed scientists and clinicians to better comprehend the pathogenesis of atherosclerosis. Of the many kinds of oxidants, oxidized low-density lipoprotein (LDL) is considered to play an important role in the initiation and progression of the atheromatous lesion (1, 2). Many reports revealed that oxidized LDL initiated the activation of endothelial cells, resulting in the expression of cytokines, chemokines or cell adhesion molecules. However, the mechanism of oxidation of LDL is still controversial. The deposition of oxidized LDL was clearly demonstrated in the subendothelial space by immunohistochemical techniques in human atherosclerotic plaque (3). It has also been proposed that superoxide anion and its related derivatives were relevant to the initiation of LDL oxidation by cells, which consisted of arterial wall or atheromatous lesions.

Moreover, it was postulated that the defensive mechanisms against oxidized stress were also elicited to reduce the effect by superoxide radicals. Superoxide dismutase (SOD) is the enzyme to disproportion superoxide anions and three isozymes, namely, Mn-SOD, Cu/Zn-SOD, and extracellular SOD (EC-SOD), are recognized. The former two are located intracellularly to interact with superoxide anions generated in mitochondria and cytosol, respectively. EC-SOD is located in the extracellular space (4) and binds to sulfated glycosaminoglycans (5–7). The extracellular matrix is the place where LDL may be mainly oxidized by endothelial cells, smooth muscle cells or macrophages. Fang *et al.* indicated that overexpression of Mn-SOD or Cu/Zn-SOD partially inhibited endothelial-cell-mediated LDL oxidation (8). Taken together these findings and the fact that arterial wall abundantly contains EC-SOD, it is reasonable to postulate that EC-SOD might antagonize cell-mediated oxidized LDL formation by reducing superoxide anions.

In the present study, we investigated the distribution of EC-SOD in porcine aortic endothelial cells (PAECs) by introducing replication-deficient adenoviral vectors containing human EC-SOD gene and its inhibitory effect against endothelial-cell-mediated LDL oxidation.

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MATERIALS AND METHODS

Materials. Materials were purchased from the following sources; RPMI 1640 medium, Ham's F-10 medium, penicillin and streptomycin solution and amphotericin B from GIBCO BRL; fetal bovine serum (FBS) from Bio Whittaker; Adenovirus Expression Vector Kit and restriction enzymes of *Eco*RI and *Xho*I from TaKaRa Biochemicals; human embryonic kidney (HEK) 293 cells from the American Type Culture Collection; mouse anti-heparan sulfate (10E4 epitope) antibody from Seikagaku Kogyo; fluorescein-5-isothiocyanate (FITC) conjugated goat $F(ab')_2$ fragment to rabbit IgG and tetramethylrhodamine-5-(and-6)-isothiocyanate (TRITC)-conjugated goat $F(ab')_2$ fragment to mouse IgM from ICN Pharmaceuticals-Cappel Products; ferric-sodium EDTA, disodium EDTA, bis-*N*-methylacridinium nitrate (lucigenin), Cu/Zn-SOD and β-nicotinamide adenine dinucleotide (NADH) from Sigma, Coomassie brilliant blue (CBB) from Nacalai Tesque. Dispase is a product from Godo Shusei

Endothelial cell culture. PAECs were primary isolated from the descending aorta as previously described (9). Briefly, aorta segments of 20 cm in lengths were washed with 3 liters of PBS containing 0.25 μg/ml amphotericin B and then filled with PBS containing 600 Units/ml dispase. After incubation for 20 min at 37°C, the contents were collected and the vessels were rinsed once with PBS. The solution containing endothelial cells was centrifuged at 200*g* for 3 min and placed into gelatin-coated dishes in RPMI 1640 medium, supplemented with 10% FBS, penicillin (100 units/ml) and streptomycin (10 μg/ml). Cells in culture medium were incubated at 37°C under humidified 5% CO_2 in room air and the medium was exchanged every 12 h for the first 2 days, and every 3 days thereafter. PAECs were subcultured using PBS containing 600 Units/ml dispase and cells from passages 2 through 4 were used for the experiments. Endothelial cells were identified with their cobblestone appearance and angiotensin-converting enzyme activity (10).

Preparation of the adenovirus vector. We generated E1a, E1b, and E3 deleted replication-deficient adenovirus vector as previously described (11). The pLS3 plasmid (12) carrying full-length human EC-SOD cDNA was kindly provided by S. L. Marklund (Umea University, Sweden). The full-length EC-SOD cDNA (1396 bp) was isolated by digestion with restriction enzyme *Eco*RI and placed into cosmid vector pAxCAwt (13) under the transcription control of CAG promoter (cytomegalovirus enhancer, chicken β-actin promoter, rabbit β-globin poly(A)) (14) provided in the adenovirus expression vector kit. This cosmid shuttle vector (pAxCAEC-SOD) was cotransfected with the restriction enzyme digested DNA-terminal protein complex (11) into HEK 293 cells, which provide the E1a gene product, for homological recombination and generation of the adenovirus vector. This adenovirus vector expressing human EC-SOD (AxCAEC-SOD) was purified from infected HEK 293 cells by sonication (ISONATOR 201M, KUBOTA) and ultracentrifugation through a $CsCl_2$ gradient and extensive dialysis. The titer of adenovirus vector was determined by plaque assay on 293 cells. To exclude wild-type adenovirus contamination, polymerase chain reaction with primer for E1a amplification (forward primer, 5′ATGAGACATATTATCTGCCACGGAGGTGTTATTAC3′, reverse primer, 5′CCTCTTCATCCTCGTCGTCACTGGGTGGAAAGCCA3′) were applied to the purified adenovirus vector and digestion with restriction enzyme *Xho*I were applied to the total DNA of infected HEK 293 cells containing next generation the adenovirus vector. The AxCALacZ adenovirus containing the *Escherichia coli* LacZ gene was also generated as the control virus under the transcription control of the CAG promoter.

Adenovirus infection and measurement of EC-SOD. PAECs were subcultured in 24-well plates. After they reached subconfluence, the medium was changed to RPMI 1640 medium supplemented with 5% FBS for 1 day. Then, these PAECs were infected with AxCAEC-SOD at various multiplicity of infection (MOI) in 200 μl of serum-free RPMI 1640 medium for 2 h. After removal of the virus solution, the cells were washed with PBS twice and incubated in 1 ml of Ham's F-10 medium at 37°C under humidified 5% CO_2. The conditioned medium without cells was collected into disodium EDTA containing tube (final concentration 2 mM), then the EC-SOD concentration was determined by enzyme-linked immunosorbent assay (ELISA) as described previously (15).

Superoxide measurement. To estimate the scavenger ability of EC-SOD, NADH-induced superoxide from PAECs was measured using lucigenin-enhanced chemiluminescence (16–18). PAECs were infected with AxCAEC-SOD or AxCALacZ at an MOI of 300 and incubated for 34 h in RPMI 1640 medium. PAECs were washed with PBS and detached with PBS containing 600 Units/ml dispase. Then, cells were centrifuged and resuspended in conditioned medium. Since EC-SOD is secreted into the extracellular space (4), chemiluminescence was applied with conditioned medium. After addition of lucigenin to the cells-containing medium (final concentration 250 μmol/l), chemiluminescence was measured for 10 min, to allow adaptation to the dark, in Lumat LB 9507 (Berthold) luminometer. Then, NADH (100 μmol/l) was added and scintillation count was recorded every 15 s for 20 min, the respective background count was subtracted. Superoxide production was expressed as relative light units (RLU) per minute per number of cells determined with a Coulter counter CDA-500 (Sysmex).

Immunofluorescence. PAECs were plated at a density of 3 to 4 $\times$ $10^4/cm^2$ on a gelatin-coated chamber slide (Lab-Tek II, Nalgen Nunc International) and infected with AxCAEC-SOD at an MOI of 75 for 2 h and incubated for 34 h in RPMI 1640 medium. Then, the cells were washed quickly with PBS and fixed with 4% paraformaldehyde in 0.08 M phosphate buffer for 15 min at room temperature. For blocking, it was incubated with 5% skim milk containing PBS for 30 min at room temperature. As the primary antibody, rabbit anti human EC-SOD antibody and mouse anti heparan sulfate antibody were coincubated at 4°C overnight. After rinsing with PBS, FITC-conjugated goat $F(ab')_2$ fragment to rabbit IgG and TRITC conjugated goat $F(ab')_2$ fragment to mouse IgM were incubated for 1 h at room temperature. FITC and TRITC fluorescence were visualized under confocal laser microscopy (LSM 410,Carl Zeiss Microscope Systems).

Lipoprotein preparation. Blood was drawn from the healthy normolipidemic men, after 12 h fasting, into disodium EDTA containing tube (final concentration 2 mM). Then, the LDL fraction, the part with a specific gravity of 1.020 to 1.055, was separated by gradient ultracentrifugation method (19). To remove potassium bromide, LDL containing solution was dialyzed four times for 36 h against 100 vol of phosphate buffer (PBS) containing 1 mM disodium EDTA (pH 7.4) in a dark room at 4°C. The purity of lipoprotein was verified by agarose gel electrophoresis. It was preserved at 4°C and dialyzed twice against 100 vol of PBS to remove EDTA and sterilized by filtration through a 0.45-μm filter (Millex-HV, MILLIPORE) before each experiment. The apolipoprotein B (apo B) concentration of LDL was determined by anti-human apo B goat antibody (APO B AUTO N "DAIICHI," Daiichi Pure Chemicals), using COBAS MIRA PLUS Systems (Roche Diagnostics).

Endothelial cells-mediate LDL modification. The medium of subconfluent PAECs cultured in 24-well plates was changed to RPMI 1640 medium supplemented with 5% FBS. After incubation for 24 h, the cells were infected with AxCAEC-SOD or AxCALacZ at various MOIs in 200 μl of serum-free RPMI 1640 medium for 2 h. After removal of adenovirus containing medium, cells were washed twice and incubated in Ham's F-10 medium for 34 h. In some experiments, PAECs were incubated with Cu/Zn-SOD without adenovirus infection. Then, human LDL (200 μg apo B protein/ml medium) and ferric-sodium EDTA (final concentration 5 μmol/l) were added to the medium and incubated for 18 h for cell-mediated LDL oxidation. As a positive control, $CuSO^4$ (final concentration 5 μmol/l) was added to the same amount of human LDL in PBS without endothelial cells and concurrently incubated for 18 h.

Assay of LDL oxidation. Thiobarbituric acid reacting substances (TBARS), described by Buege and Aust (20), were used to estimate lipid peroxidation products. Freshly diluted tetramethoxypropane was used as the standard and the result was expressed as nmol of malondialdehyde (MDA) equivalents per milligram apo B protein. Agarose gel electrophoresis was performed to estimate LDL modification using Paragon Electrophoresis System (Beckman Instruments). Electrophoresis of the sample was carried out at 100 V for 30 min on 0.5% agarose gel with barbital buffer (pH 8.6), then, proteins were stained with CBB. Electrophoretic mobility of LDL was expressed as the distance from origin of loading to the center of the dense deposit. Percentage LDL modifications were calculated from the relative mobility of endothelial-mediated modified LDL without infection versus native LDL as a reference.

Statistics. Data are presented as the mean value ± standard deviation (SD). Statistical analysis were performed by analysis of variance (ANOVA), followed by Bonferoni's test or unpaired Student's *t* test, as appropriate. A P value of <0.05 was considered to be statistically significant.

RESULTS

Expression of Recombinant Functional Human EC-SOD in PAECs

To examine the ability of AxCAEC-SOD to express EC-SOD protein on PAECs, cells were infected either at various MOIs or for various time intervals. As shown in Fig. 1A, EC-SOD protein increased according to the MOI values. At an MOI of 30, 100, or 300, the expression of EC-SOD was sufficiently high to be equal to the human physiological level. To determine the time-dependent expression of EC-SOD in the extracellular space (Fig. 1B), EC-SOD protein in the medium was measured during incubation every 24 h after AxCAEC-SOD. The concentration of EC-SOD was the lowest at 24 h, and the maximal rate of increase in EC-SOD expression was observed from 24 to 72 h. To investigate the functional aspect of EC-SOD, lucigenin-enhanced chemiluminescence assay was performed. A markedly reduction of superoxide production was confirmed in AxCAEC-SOD-infected cells compared to vehicle or AxCALacZ-infected cells (Fig. 1C).

Immunohistochemical Localization of EC-SOD and Heparan Sulfate

Figure 2 shows immunofluorescence microscopy performed with visualization by confocal laser microscopy. EC-SOD was confirmed in 98% of PAECs infected with AxCAEC-SOD (Fig. 2, left). Moreover, recombinant human EC-SOD protein localized not only in the intracellular space but also in the extracellular space. On the other hand, heparan sulfate was diffusely stained in the extracellular space (Fig. 2, middle). In merged image of A and B, the extracellular expression of EC-SOD was in accordance with that of heparan sulfate (Fig. 2, right). This indicated that EC-SOD in the extracellular space colocalized with heparan sulfate around endothelial cells.

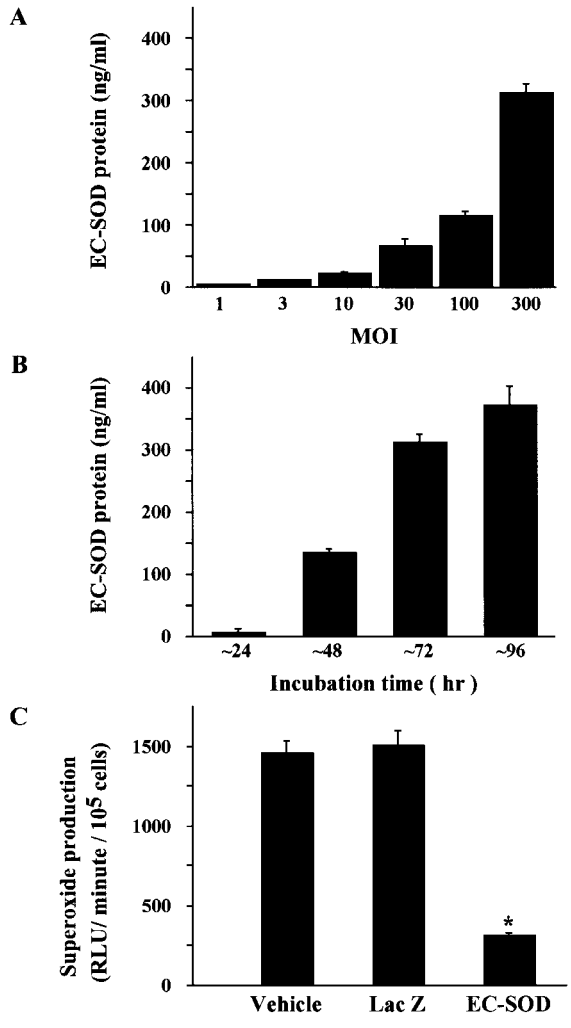

FIG. 1. Effect of AxCAEC-SOD infection on the expression of functional human EC-SOD in the extracellular space. (A) EC-SOD protein expression; Dose response: PAECs grown in 24-well plates were infected with AxCAEC-SOD at various MOIs as described under Materials and Methods. After 72 h, incubation medium was collected and human EC-SOD concentration was determined by ELISA. (B) EC-SOD protein expression; Time course: Incubation after gene transfer was ended every 24 h and human EC-SOD protein in the incubation medium was determined by ELISA. (C) Superoxide production from endothelial cells: PAECs were infected with AxCAEC-SOD or AxCALacZ at an MOI of 300 and incubated in RPMI 1640 Medium for 2 days. Cells were added lucigenin (250 μmol/l) and NADH (100 μmol/l) as described under Materials and Methods. Superoxide production was expressed as relative light units (RLU) per minute per cell number. Data are the mean value ± SD (bars) obtained from three separate experiments (A and B) or four separate experiments (C). *$P < 0.05$ compared with vehicle (noninfected cells) or infected with AxCALacZ.

Endothelial-Cell-Mediated LDL Oxidation

To confirm PAECs-mediated LDL oxidation, cells were incubated with LDL in some conditioned medium. In Ham's F-10 medium, TBARS value in oxidized LDL was increased from 6.7 ± 1.0 nmol MDA/mg apo B

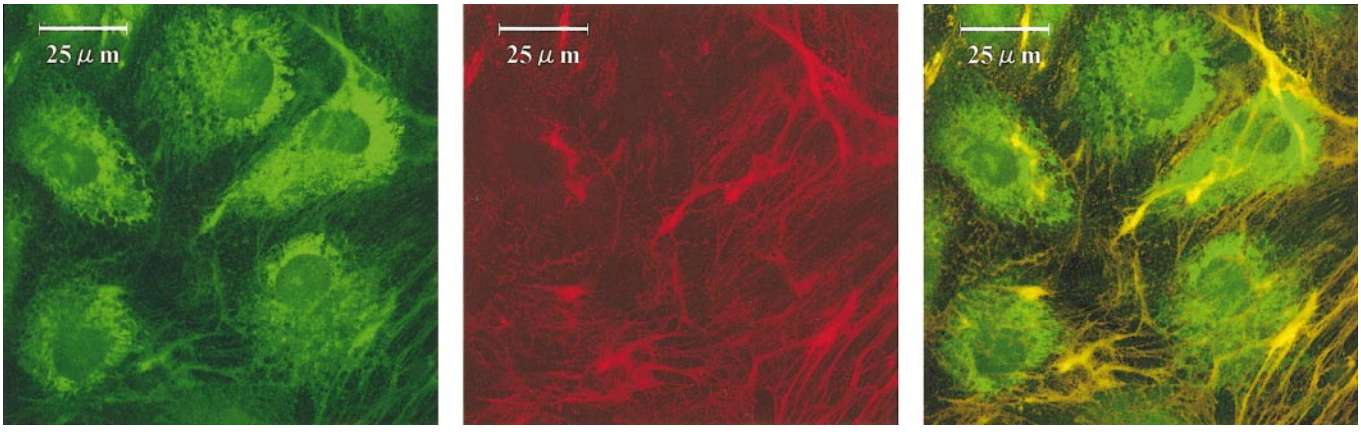

FIG. 2. Immunofluorescence image of PAECs infected with AxCAEC-SOD. PAECs were infected with AxCAEC-SOD at an MOI of 75 and EC-SOD expression and heparan sulfate were analyzed 36 h after infection by immunofluorescence as described under Materials and Methods. EC-SOD protein labeled with green fluorescence (left) and heparan sulfate protein labeled with red fluorescence (middle). The merged image of green fluorescence and red fluorescence (right) showed the distribution of immunoreactive EC-SOD and heparan sulfate.

protein without PAECs to 9.9 ± 1.0 with PAECs. Moreover, in the addition of 5 μM ferric-sodium EDTA to Ham's F-10 medium, TBARS was markedly increased from 14.9 ± 0.9 nmol MDA/mg apo B protein without PAECs to 45.2 ± 1.6 with PAECs. Therefore, we examined the inhibitory effect of SOD in PAECs cultured in Ham's F-10 medium containing 5 μM ferric-sodium EDTA in following experiments. In this condition, addition of 1 or 10 μg/ml Cu/Zn-SOD resulted in 16.2 and 30.9% decrease in TBARS formation, respectively.

Effect of AxCAEC-SOD Gene Transfer on LDL Oxidation

To examine the effect of EC-SOD on LDL oxidation, PAECs were infected with 3 to 300 MOIs of AxCAEC-SOD or AxCALacZ (Fig. 3). At an MOI of 1, TBARS formation did not differ in AxCAEC-SOD infection compared to AxCALacZ. At more than 3 MOI, TBARS formation was significantly inhibited by AxCAEC-SOD infection when compared with AxCALacZ. The degree of TBARS inhibition was proportional to the MOI of AxCAEC-SOD. However, no influences were observed with AxCALacZ.

Time Course of Effects of AxCAEC-SOD on LDL Oxidation

After infection with 300 MOI of AxCAEC-SOD, AxCALacZ or vehicle, LDL incubation was continued up to 32 h (Fig. 4). In vehicle or AxCALacZ infected PAECs, TBARS formation increased in a time-dependent manner up to 24 h. In AxCAEC-SOD-infected PAECs, TBARS formation was significantly inhibited compared to those in vehicle or AxCALacZ at all time intervals.

Effects of AxCAEC-SOD on LDL Electrophoretic Mobility

Electrophoretic mobility was also examined to evaluate the extent of LDL oxidation (Fig. 5). Native LDL indicated low mobility and no protein fragmentation (lane 1). Copper induced LDL oxidation, resulting in much more mobility and fragmentation showing smear-like appearance (lane 7). The mobility of PAECs-mediated LDL was almost halfway between native LDL and Cu^{2+}-induced oxidized LDL (lane 3). Incubation of LDL with PAECs infected with AxCALacZ

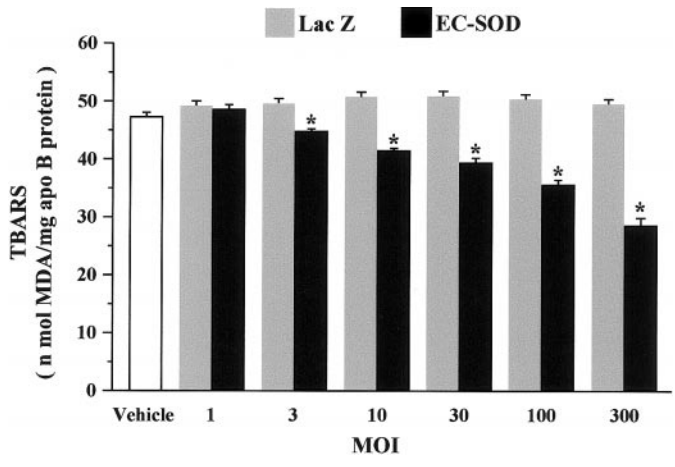

FIG. 3. Effect of AxCAEC-SOD infection on endothelial cells-mediated LDL oxidation on TBARS formation. PAECs were infected with AxCAEC-SOD or AxCALacZ at various MOIs and incubated with human LDL (200 μg apo B protein/ml medium) in Ham's F-10 medium containing ferric-sodium EDTA for 18 h, for cell-mediated LDL oxidation as described under Materials and Methods. TBARS formation was used to estimate lipid peroxidation products in the medium. Data are the mean value ± SD (bars) obtained from four separate experiments. *$P < 0.05$ compared with vehicle (noninfected cells) or cells infected with AxCALacZ.

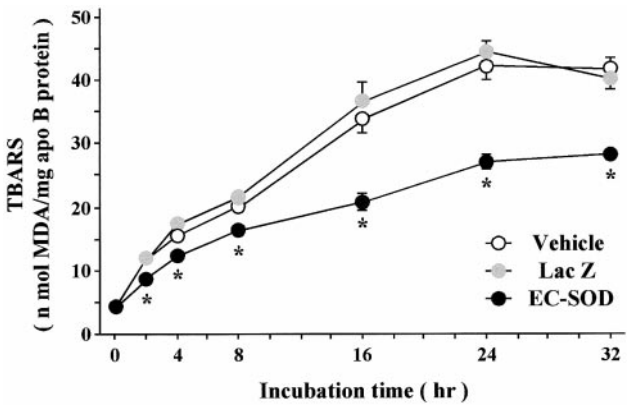

FIG. 4. Time course of the effects of AxCAEC-SOD infection on endothelial cell-mediated LDL oxidation on TBARS formation. PAECs were infected with AxCAEC-SOD or AxCALacZ at an MOI of 300 and incubated with human LDL (200 μg apo B protein/ml medium) in Ham's F-10 medium containing ferric-sodium EDTA as described under Materials and Methods. Incubations for cell-mediated LDL oxidation were ended at the indicated time. Data are the mean value ± SD (bars) obtained from four separate experiments. *$P < 0.05$ compared with vehicle (noninfected cells) or cells infected with AxCALacZ.

at an MOI of 300 resulted in an increased mobility similarly to that of vehicle (lane 6). On the contrary, inhibitory effects of AxCAEC-SOD infection were observed on mobility and fragmentation (lanes 3, 4, and 5) and this effect was suggested to be dose-dependent. Percentage LDL modification with AxCAEC-SOD was decreased by 50% at an MOI of 300 compared with AxCALacZ (Table 1).

TABLE 1

Effect of AxCAEC-SOD Infection on Endothelial-Cell-Mediated LDL Oxidation on Electrophoretic Mobility

	Electrophoretic mobility (mm)	LDL modification (%)
Native LDL	4.00 ± 0.00	0.0
Vehicle	13.19 ± 0.25	100.0
EC-SOD (MOI = 3)	11.36 ± 0.52*	88.0*
EC-SOD (MOI = 30)	10.38 ± 0.26*	69.4*
EC-SOD (MOI = 300)	8.74 ± 0.42*	50.3*
LacZ (MOI = 300)	13.56 ± 0.44	104.0
Cu^{2+}-induced oxidized LDL	24.75 ± 0.48*	225.8*

Note. Values are the mean value ± SD obtained from four separate experiments. Percentages of LDL modification were calculated from the relative mobility of modified LDL with PAECs (vehicle) against native LDL.

* $P < 0.05$ compared with vehicle.

DISCUSSION

In the present study, we clearly demonstrated that infection of endothelial cells with an adenovirus vector containing the cDNA for human EC-SOD resulted in overexpression of EC-SOD in the extracellular space and coincided with the presence of heparan sulfate proteoglycans. Moreover, we also clarified that this recombinant EC-SOD could effectively abolish superoxide and suppress cell-mediated LDL oxidation.

Advanced atherosclerotic lesions, it is characterized by the focal accumulation of lipoprotein in the arterial

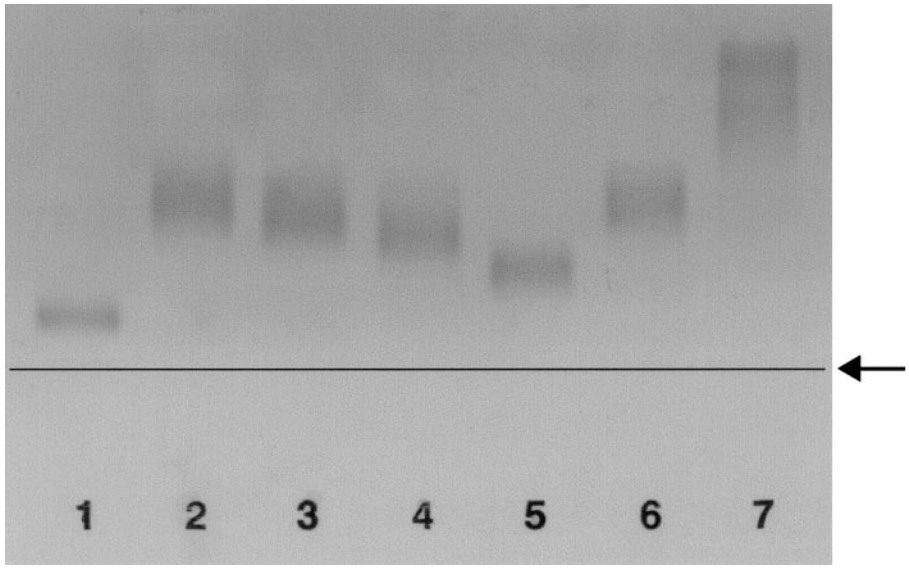

FIG. 5. Effect of AxCAEC-SOD infection on endothelial cells-mediated LDL oxidation on electrophoretic mobility. PAECs were infected with AxCAEC-SOD or AxCALacZ at various MOIs and incubated with human LDL (200 μg apo B protein/ml medium) in Ham's F-10 medium containing ferric-sodium EDTA as described under Materials and Methods. Electrophoresis of the samples was carried out at 100 V for 30 min on 0.5% agarose gel. An example of electrophoresis is shown in this figure: lane 1 native LDL; lane 2, vehicle; lane 3, AxCAEC-SOD (MOI = 3); lane 4, AxCAEC-SOD (MOI = 30); lane 5, AxCAEC-SOD (MOI = 300); lane 6, AxCALacZ (MOI = 300); lane 7, 5 μM of Cu^{2+} induced Ox LDL. Arrow indicates the origin of LDL loading.

intima (21). *In vivo* experiments using rabbit models revealed that the earliest excessive deposition of lipoproteins started in the subendothelial space several days after a cholesterol-rich diet (22). Collagen fibers and elastic fibers coexist in the matrix of the subendothelial space and embed proteoglycans, hyaluronan and glycoproteins. Among these substances, binding to glycosaminoglycans mostly contributes to the retention of lipoprotein (23). Actually, it was reported that complexes of lipoproteins with GAGs were isolated from atherosclerotic lesion (24). This LDL retention with glycosaminoglycans was considered to let LDL particles stay in the arterial wall and to make them susceptible to oxidative modification (25). In this study, we immunohistochemically proved coexistence of EC-SOD and heparan sulfate in the extracellular matrix. These were anticipated that EC-SOD might be positioned very closely to LDL particle, which retained in arterial intima. An excessive amount of extrinsic Cu/Zn-SOD, which had no binding ability to heparan sulfate, was needed to inhibit LDL oxidation, similar to previous report (26–28). On the other hand, the concentration of EC-SOD in our experiments was much lesser. Therefore we guess the close positioning between EC-SOD and LDL particle via heparan sulfate was favorable for EC-SOD to antagonize superoxide effectively in the extracellular matrix. Moreover, it has been clarified that modified LDL binds more strongly to glycosaminoglycans than native LDL (29) and subsequently produces superoxide from endothelial cells (30). The presence of EC-SOD around endothelial cells might be reasonable to protect atherosclerosis progression.

We used the model of LDL oxidation, which seemed to be catalyzed by radical oxygen species with iron and EDTA. Cell-mediated LDL oxidation was augmented in the presence of 5 μM ferric-sodium EDTA. However, the addition of NADH or angiotensin II, which could produce superoxide anion, resulted in only a little increase of LDL oxidation (data not shown). In the meantime, adding ferric-sodium EDTA did not show a clear enhancement in superoxide production by lucigenin chemiluminescence (data not shown). Thus, LDL oxidation with iron and EDTA seemed to be based on hydroxyl radicals generated by Fenton's reaction (31, 32). It has been proven that the reaction through iron is greatly promoted by the presence of a chelater such as EDTA (33, 34). Although superoxide itself does not show high reactivity, the hydroxyl radical from superoxide shows very high reactivity, enough to damage tissues (35). Moreover, hydroxyl radical derives only through superoxide generation and there is not found the peculiar mechanism to eliminate it *in vivo.* In this study, we demonstrated that disproportion of superoxide by EC-SOD effectively reduced LDL oxidation even in hydroxyl radical production might be accelerated.

In recent year, many studies have revealed that superoxide production plays an important role in atherosclerosis. In several models of atherosclerosis, even in early stage, superoxide production in the vascular wall is largely produced by endothelial cells (36, 37) and its predominant source is membrane-bound NAD(P)H oxidase (38). In endothelial cells (39, 40) but not smooth muscle cells (41, 42), NAD(P)H oxidase transfer electron across the membrane to oxygen and produce extracellular superoxide like leukocytes (43). Therefore, it is presumed that extracellular superoxide is mainly increased around endothelial cell in atherosclerosis lesion, it may promote consecutive atherosclerosis progression as oxidized stress. As defensive mechanism against extracellular superoxide, the role of EC-SOD in the vessel wall has also been clarified. Some studies showed that EC-SOD existed abundantly in the arterial wall (44, 45), and also suggested that it played an important role in defense against extracellular superoxide in the arterial wall (46). In recent clinical investigations, it was indicated that a decrease of EC-SOD might account for the progression of coronary atherosclerosis. Wang *et al.* reported that low plasma EC-SOD was independently associated with an increase of history of myocardial infarction (47). Moreover, EC-SOD activity but not that of Cu/Zn-SOD or Mn-SOD was reduced in coronary artery segments in patients with coronary artery disease compared with normal subjects (48). We have reported that the EC-SOD affinity to endothelial surface heparan sulfate was decreased in coronary artery disease (49). The amount of EC-SOD in our experiments was from 20 to 400 ng/ml in medium (Figs. 1A and 1B), which was postulated to be equilibrated in the extracellular space and heparan sulfate (7). The physiological level in human plasma also ranged from 60 to 200 ng/ml in our report (49). Thus, the EC-SOD concentration in our experiment was similar to the physiological level. Therefore, the reduction in LDL oxidation by EC-SOD in this study was considered helpful to explain the mechanisms in these clinical reports.

In conclusion, we manifested that co-localization of EC-SOD and heparan sulfate on the surface of endothelial cells and the effective suppression of LDL oxidation by introducing AxCAEC-SOD into endothelial cells. We postulate that this close location among EC-SOD, LDL particle and endothelial cells, which might be supplier of extracellular superoxide, is important for the effective inhibition of LDL oxidation in arterial wall. Since EC-SOD is only superoxide dismutase in extracellular space, the presence of EC-SOD may be one of key mechanisms of defense against the initiation and the progression of atherosclerosis.

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