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Size and targeting to PECAM vs ICAM control endothelial delivery, internalization and protective effect of multimolecular SOD conjugates

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ABSTRACT

Controlled endothelial delivery of SOD may alleviate abnormal local surplus of superoxide involved in ischemia-reperfusion, inflammation and other disease conditions. Targeting SOD to endothelial surface vs. intracellular compartments is desirable to prevent pathological effects of external vs. endogenous superoxide, respectively. Thus, SOD conjugated with antibodies to cell adhesion molecule PECAM (Ab/SOD) inhibits pro-inflammatory signaling mediated by endogenous superoxide produced in the endothelial endosomes in response to cytokines. Here we defined control of surface vs. endosomal delivery and effect of Ab/SOD, focusing on conjugate size and targeting to PECAM vs. ICAM. Ab/SOD enlargement from about 100 to 300 nm enhanced amount of cell-bound SOD and protection against extracellular superoxide. In contrast, enlargement inhibited endocytosis of Ab/SOD and diminished mitigation of inflammatory signaling of endothelial superoxide. In addition to size, shape is important: endocytosis of antibody-coated spheres was more effective than that of polymorphous antibody conjugates. Further, targeting to ICAM provides higher endocytic efficacy than targeting to PECAM. ICAM-targeted Ab/SOD more effectively mitigated inflammatory signaling by intracellular superoxide in vitro and in animal models, although total uptake was inferior to that of PECAM-targeted Ab/SOD. Therefore, both geometry and targeting features of Ab/SOD conjugates control delivery to cell surface vs. endosomes for optimal protection against extracellular vs. endosomal oxidative stress, respectively.

1. Introduction

In pathological conditions including inflammation and ischemia-reperfusion, both insufficiency of antioxidants and/or surplus of reactive oxygen species (ROS, e.g., superoxide anion), lead to vascular oxidative stress, which further aggravates injury and ignites the vicious cycle of tissue damage [1,2]. Design of drug delivery systems for antioxidants including polymeric and liposomal carriers, fusion proteins, antibody and other protein conjugates, nanozymes and other approaches is an active and promising area of current drug delivery research [3–5].

Endothelial cells represent the key therapeutic target for antioxidant interventions in these conditions [2]. Indeed, endothelial targeting of antioxidants provides potent protective effects in animal models of vascular oxidative stress unrivaled by untargeted enzymes [6–8]. However, better mechanistic understanding and design of delivery systems are necessary in order to convert these encouraging results into an approach providing tangible medical benefits.

Among other aspects, the spatial control of endothelial targeting of SOD at sub-cellular level is warranted. It has long been known that ROS released from activated leukocytes attack endothelium from the milieu [9]. More recently, it has been found, however, that ROS produced by endothelium itself in response to pathological stimuli play an important role in the pathogenesis of inflammation and ischemia-reperfusion [2,10,11]. In particular, cytokines activate NADPH-oxidase in endothelial endosomes to flux superoxide in the lumen of these vesicles and local intracellular surplus of this ROS ignites an “autocrine” signaling leading to pro-inflammatory endothelial changes [11–13].

SOD conjugated with PECAM antibody (Ab/SOD) enters endothelial endosomes and quenches superoxide, thereby intercepting this unusual pro-inflammatory pathway and conferring anti-inflammatory effects [14,15]. However, the design parameters that favor surface retention vs endosomal delivery of Ab/SOD have not been defined. These distinct localizations would be preferential for protection against extracellular ROS attack vs endosomal ROS signaling, respectively.

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Size and binding specificity are among the most influential parameters of design that modulate endosomal delivery of diverse drug delivery systems [16]. However, the role of these parameters in endothelial targeting, internalization and effect of Ab/SOD is not known. Accordingly, here we studied how size of Ab/SOD targeted to endothelial molecules PECAM and ICAM regulate intracellular delivery and effect of Ab/SOD.

2. Materials and methods

2.1. Materials and cell cultures

Cu, Zn-superoxide dismutase (SOD) from bovine erythrocytes is from Calbiochem (San Diego, CA). Succinimidyl-6-[biotinamido] hexanoate (NHS-LC-biotin), 4-[*N*-maleimidomethyl] cyclohexane-1-carboxylate (SMCC), *N*-succinimidyl-*S*-acetylthioacetate (SATA) were from Pierce Biotechnology (Rockford, IL). Anti-human PECAM monoclonal antibodies used were mAb 62. Anti-human ICAM monoclonal antibodies were R6.5 from ATCC (Manassas, VA; hybridoma R6–5-D6) and LB-2 from Santa-Cruz Biotech. (Dallas, TX). Anti-murine PECAM monoclonal antibodies were MEC13.3 (BD Parmingen, SanJose, CA). Anti-murine ICAM monoclonal antibodies were YN1 from ATCC. Anti-human VCAM-1 goat polyclonal antibodies were from R&D systems (Minneapolis, MN; cat# BBA19), anti-mouse VCAM-1 goat polyclonal antibodies were from Santa-Cruz Biotech.; anti-actin antibody HRP-conjugated was from Abcam (Cambridge, MA). Ab/SOD conjugates were prepared via amino-chemistry as described earlier [15]. Tumor necrosis factor (TNF) was from BD Biosciences (Bedford, MA). Lipopolysaccharide (*Escherichia coli* O55:B5, γ-irradiated) was from Sigma (St. Louis, MO). FITC-labeled polystyrene microspheres (0.05, 0.1, 0.2, 0.5, and 6.0 μm diameter) were purchased from Polysciences (Warrington, PA). SOD was radiolabeled with sodium iodide radioisotope $Na^{125}I$ (PerkinElmer; Wellesley, MA) using Iodogen.

2.2. Cell culture and treatment

Human umbilical endothelial cells (HUVEC; Lonza Walkersville, Walkersville, MD) were maintained in EGM-2 BulletKit medium (Lonza) with 10% fetal bovine serum supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin (GIBCO) in Falcon tissue culture flasks (BD Biosciences, San Jose, CA) precoated with 1% gelatin for 1 h. Cells were activated by addition of TNF for 4–5 h.

2.3. Conjugate preparation

Antibodies were conjugated with SOD using amino-chemistry as described [15]. Protected sulfhydryl groups were introduced in the molecule of antibody via primary amines using SATA at a molar ratio antibody:SATA 1:100 at room temperature for 30 min. Further sulfhydryl groups were de-protected using 50 mM hydroxylamine for 2 h. Maleimide groups, which specifically react towards SH-group, were introduce into enzyme molecule using hetero-bifunctional cross-linker SMCC. The reaction was performed at a molar ratio SOD:SMCC 1:20 for 1 h at room temperature. Preliminary experiments showed that at 1:1 Ab:SOD molar ratio the resulting conjugates did not reach 50 nm, probably due to small size of SOD molecule (31 kDa) compared to IgG molecule (150 kDa). To prepare a series of conjugates with different sizes the molar ratio between antibody and SOD were varied from 1:1.5 to 1:5. Conjugation reaction was performed during 1 h on ice. Unreacted components were removed using Spin Protein Columns (G-25 Sephadex, Roche Applied Science, Indianapolis, IN) after a reaction is completed. The effective diameter of the obtained conjugates was measured by Dynamic light scattering (DLS) using Zetasizer Nano ZSP (Malvern Instruments Ltd., Malvern, UK) or 90Plus Particle Sizer (Brookhaven Instruments Corp., NY, US). Achievable size of the particles was from 50 to 350 nm; larger particles tended to aggregate. Conjugates were complimented with 7% sucrose (final concentration) as cryoprotector, frozen, and stored at −80 °C before use. Test conjugation was always done when new batch of either protein or cross-linker were used before preparation of stock conjugates to adjust experimental conditions. For binding studies SOD was radiolabeled with $Na^{125}I$ using Iodogen (Pierce Biotechnology, Rockford, IL) as recommended by the manufacturer.

2.4. Conjugate fractionation

To differentiate the protective effects of 'small' vs. 'large' particles we fractionated the preparation of ~300-nm conjugates. Conjugates were centrifuged for 10 min at 10,000 g yielded the supernatant containing Ab/SOD with a narrower size distribution and smaller average size. The supernatant fraction was compared with total material in binding and functional assays.

2.5. Preparation of Ab/nanoparticles

FITC-labeled polystyrene microspheres (50, 100, 200, 500 nm and 6 μm) were coated with anti-PECAM or anti-ICAM using incubation at room temperature for 1 h as described elsewhere [17], centrifuged to remove unbound materials, then resuspended in 1% bovine serum albumin (BSA)-PBS and microsonicated for 20 s at low power. The effective particle size was controlled by dynamic light scattering using a BI-90 Plus particle size analyzer with BI-9000AT Digital autocorrelator (Brookhaven Instruments, Brookhaven, NY).

2.6. Transmission electron microscopy (TEM) of conjugates

Conjugate preparations were applied on TEM grid (Formvar Film 200 mesh; Electron Microscopy Sciences, Hartfield, PA) and subsequently stained with 2% (w/v) uranyl acetate. Images were taken using JEM-1010 Transmission Electron Microscope (JEOL USA, Inc., Peabody, MA).

2.7. Binding of PECAM-targeted SOD to endothelial cells

Ab/SOD conjugates targeted to PECAM carrying 10 mol% of radiolabeled SOD were incubated with HUVEC at 37 °C for 1 h, unbound materials were washed out and cells were lysed with lysis buffer (1% Triton X-100, 1.0 M NaOH). Bound radioactivity was measured using a Wallac 1470 Wizard™ gamma counter (Gaithersburg, MD).

2.8. Western blotting analysis

Cells in 24 well culture dishes were washed twice with phosphate-buffered saline (PBS) and lysed in 100 μl of sample buffer for sodium dodecyl sulfate polyacrylamide gel electrophoresis. Cell proteins were subjected to 4–15% gradient gel. Gels were transferred to PVDF membrane (Millipore) and the membrane was blocked with 3% nonfat dry milk in TBS-T (100 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween 20) for 1 h followed by incubations with primary and secondary antibodies in the blocking solution. The blot was detected using ECL reagents (GE Healthcare).

2.9. Internalization studies

Cells were grown on microscope glass cover slips pre-coated with Fibronectin-like protein polymer (Sigma), treated with Ab/SOD conjugates, and fixed with ice-cold 2% paraformaldehyde for 15 min. For internalization studies, cells were treated first with Alexa Fluor 594 labeled goat anti-mouse IgG, then permeabilized with 0.2% Triton X-100 for 15 min, and treated with Alexa Fluor 488 goat anti-mouse IgG [17]. For studies of internalization of FITC-labeled polysterene beads fixed cells were treated with Alexa Fluor 594 labeled goat anti-mouse IgG.