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T cell activation induces CuZn superoxide dismutase (SOD)-1 intracellular re-localization, production and secretion

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ABSTRACT

Reactive oxygen species (ROS) behave as second messengers in signal transduction for a series of receptor/ligand interactions. A major regulatory role is played by hydrogen peroxide (H_2O_2), more stable and able to freely diffuse through cell membranes. Copper–zinc superoxide dismutase (CuZn-SOD)-1 is a cytosolic enzyme involved in scavenging oxygen radicals to H_2O_2 and molecular oxygen, thus representing a major cytosolic source of peroxides. Previous studies suggested that superoxide anion and H_2O_2 generation are involved in T cell receptor (TCR)-dependent signaling. Here, we describe that antigen-dependent activation of human T lymphocytes significantly increased extracellular SOD-1 levels in lymphocyte cultures. This effect was accompanied by the synthesis of SOD-1-specific mRNA and by the induction of microvesicle SOD-1 secretion. It is of note that SOD-1 increased its concentration specifically in T cell population, while no significant changes were observed in the "non-T" cell counterpart. Moreover, confocal microscopy showed that antigen-dependent activation was able to modify SOD-1 intracellular localization in T cells. Indeed, was observed a clear SOD-1 recruitment by TCR clusters. The ROS scavenger N-acetylcysteine (NAC) inhibited this phenomenon. Further studies are needed to define whether SOD-1-dependent superoxide/peroxide balance is relevant for regulation of T cell activation, as well as in the functional cross talk between immune effectors.

1. Introduction

T cell activation is a complex phenomenon in which intracellular signals, mediated by the engagement of TCR, are integrated by a variety of ligand/receptor interactions whose outcome is to finely tune antigen-dependent T cell response [1]. T lymphocytes play a pivotal role in the orchestration of the immune response and TCR-mediated signaling is a critical event to properly channeling the immune response and to obtain pathogen control and self-tolerance [2].

Several studies have been suggesting that TCR-dependent T cell activation induces ROS production [3–5]. Different enzymatic sources, such as the mitochondrial respiratory chain [6], lipooxygenases, NADPH oxidases NOX2 and DUOX1 [7,8], have been described to contribute to ROS generation upon TCR triggering. In the light of these observations, the involvement of multiple anti-oxidant enzymes in fine tuning of antigen-dependent T cell response can be hypothesized.

TCR stimulation generates both H_2O_2 and superoxide anion [8,10] and antioxidant enzymes specific for H_2O_2 enhance and/or prolong TCR-dependent ERK activation, while those specific for superoxide anion have no effect [11].

ROS include oxygen superoxide, hydrogen peroxides, hydroxyl radicals and peroxides. They represent a normal product of cellular metabolism and play relevant roles in innate defense against pathogens [12]. Several receptor/ligand interactions, as represented by TGF-beta [13], insulin [14], angiotensin II [15] and EGF [16] have been correlated to the presence of ROS. In this context, ROS appear to act as key second messenger regulating several crucial cellular responses, as protein kinase activation, gene expression and cell proliferation/apoptosis [17].

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H_2O_2 is more stable than other short-lived ROS molecules (1 minute half-life). It is electrically neutral and it can diffuse inside the cell and freely through cell membranes. In addition, H_2O_2 can be rapidly generated and easily scavenged by numerous mechanisms, thus sharing several features with well-known second messengers [18–20].

SOD molecules mediate scavenging of ROS, to H_2O_2 and molecular oxygen. They belong to a large family of isoenzymes that mediate cellular response to oxidative stress and represent the main enzymatic source of peroxides [21]. All mammalian cells express both the intra-mitochondrial Mn-SOD and the cytosolic dimeric CuZn-SOD (SOD-1), while the tetrameric extracellular CuZn-SOD isoenzyme seems to be selectively expressed by specific cell populations [22,23]. Hyperoxia and copper availability accelerate both the synthesis and activity of Cu,Zn SOD [24]. Significant control of ROS signaling depends on its spatially restricted production at intracellular sites, where redox-regulated signal occurs [25]. In this context, SOD-1 recruitment has been described in redox-dependent TNF-alpha and IL-1 receptor-induced endosomes [26,27]. In addition, SOD-1 associates with Rac-1-regulated NADPH oxidase complexes in different mouse tissues and cell lines [28].

SOD-1 may be released *in vitro* by fibroblasts, hepatocytes [29], human neuroblastoma cells [30] and Sertoli cells [31]. The extracellular release of such enzyme is related to specific stress conditions [32]. ER/Golgi involvement in SOD-1 secretion has been described [33–36], while it is unclear how this cytosolic protein can be targeted into the ER/Golgi network.

SOD-1 is constitutively secreted by microvesicles in some cell lines through an ATP dependent mechanism [37]. The intracellular increase of the enzyme can be observed in neuroblastoma SK-N-BE cells when they are exposed to oxidative stress [37]. Recently, it has been shown that SOD-1 secretion is induced by high level of extracellular K^+ in GH3 rat pituitary cells [38] and that the enzyme interacts with membrane of neuroblastoma SK-N-BE cells activating a phospholipase/protein kinase C pathway, able to increase intracellular calcium [39,40].

Receptor–ligand interactions, involving members of hematopoietin receptor super family and EGF, have been described to mediate extracellular H_2O_2 generation [41,42]. Moreover, exogenously added H_2O_2 is able to induce signals in the absence of ligands, whereas catalase is able to inhibit such effect [43,44]. A role for SOD-1 in modulating ROS-dependent intra-cellular and inter-cellular signaling might be hypothesized.

Communication between immune cells involves the secretion of several proteins, like the cytokines, and the presence of their receptors on neighboring cells. This type of intercellular "dialog" may involve the release of membrane vesicles, like exosomes. These vesicles can affect cell physiology inducing intracellular signaling and conferring them new biological properties [45,46]. Peripheral blood human T cells, T cell clones and Jurkat T cells are able to release microvesicles in the culture medium. The microvesicle production is finely regulated and, notably, it increases upon TCR triggering [47].

In previous papers, we showed that cytosolic SOD-1 is secreted by several cell types [29,30,37] and it is also released in primary lymphoid organs, as represented by human thymus [48]. These observations suggest a paracrine role for SOD-1.

Multiple cytokines have been observed to regulate the expression of the tetrameric form of extra-cellular SOD-1 [49], while no data are available on the role of dimeric, cytosolic SOD-1 in functional adaptive immune effectors. Therefore, the role for SOD-1 in ROS-dependent signaling as well as in the communication between immune effectors needs to be addressed.

This study is aimed to investigate whether cytosolic SOD-1 might be part of the molecular network involved in TCR triggering. With this purpose SOD-1 intracellular level and localization, as well as SOD-1 microvesicle secretion have been investigated in TCR-triggered human T lymphocytes.

2. Material and methods

2.1. Cells

Peripheral blood mononuclear cells (PBMCs) were isolated from 10 healthy donors, after informed consent, by centrifugation of peripheral blood on Ficoll-Paque cushion (GE Healthcare, Uppsala, Sweden) gradient. T cells have been isolated from PBMC by using a negative isolation kit (Invitrogen Corporation, Carlsbad, CA, USA) and following the manufacturer's instructions. PBMC or T cells (1×10^6/ml) were cultured in 96 well flat-bottomed plates (Falcon) in RPMI 1640 medium with 2% FCS (Invitrogen, Carlsbad, CA, USA). TCR triggering was performed by anti-CD3 mAb (Becton Dickinson, Mountain View, CA, USA) at 5 ng/ml or by using anti-CD3/anti-CD28 beads (Invitrogen), at 0.3 bead/cell. This activation strategy has been largely demonstrated to mimic antigen-dependent T cell triggering. To analyze TCR-dependent SOD-1T cell export, distinct experiments were performed in the presence of Brefeldin-A, (BFA) at 5 μg/ml or of 1 mM methylamine, all purchased from Sigma-Aldrich (Milan, Italy), as described [37]. Cell viability was evaluated by using Propidium Iodide (PI) (Sigma-Aldrich) labeling and flow cytometry detection [37] as well as by analyzing lactate dehydrogenase (LDH) activity in culture supernatants by using the Roche Molecular Biochemical LDH kit (Mannheim, Germany). Written informed consent (model n. 5526 of Azienda Ospedaliera Universitaria "FEDERICO II") was obtained from each donor at the time of venous peripheral blood donation. All the experiments done by using blood donations were performed and analyzed anonymously, without any biographical reference to donors.

2.2. ELISA

The quantitative detection of human SOD-1 in medium of cultured PBMC was carried out using the Bender Med System kit (Bender Med System Diagnostic, Vienna, Austria), as described [37]. Results were always normalized for total protein content of the tested sample. SOD-1 ELISA detection has been always performed on culture supernatants immediately frozen at −80 °C. Protein concentrations were determined according to the method of Lowry et al. [50] using BSA, as standard.

2.3. RNA preparation, semi-quantitative RT-PCR and DNA sequencing

Analysis of SOD-1 specific RNA has been performed, as described [51]. Briefly, total RNA was extracted with High Pure RNA isolation kit (Roche Italia, Milano, Italia), according to the manufacturer's instructions. Traces of contaminated DNA were removed with DNAse I treatment. Quantification was achieved in a single reaction by using the housekeeping β-actin gene as internal standard. To rule out genomic DNA contamination we performed a negative control that contained RNA instead of cDNA. The signal intensities of PCR products were separated on a 1.2% agarose gel and were visualized by ethidium bromide staining. The products' signal intensities were determined by computerized densitometric analysis using Fotoplot software. The expression of SOD-1 was normalized to β-actin mRNA levels. To check the specificity of the amplified products, DNA bands were eluted from the gel and purified; sequence analysis was determined by the Big Dye Terminator Cycle Sequencing method (ABI-PRISM Sequencer 310 Perkin-Elmer).

2.4. Microvesicle isolation and western blotting for SOD-1 detection

To purify the membrane microvesicle-containing fraction, supernatants were collected immediately after culture and treated, as described [52]. Briefly, they were sequentially centrifuged at 500 *g* for 15 min to remove cellular debris and again at 10,000 *g* for 20 min. The obtained supernatant was collected and further centrifuged at 100,000 *g* for

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