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Evidence for secretion of Cu,Zn superoxide dismutase via exosomes from a cell model of amyotrophic lateral sclerosis

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Abstract

A familial form of the neurodegenerative disease amyotrophic lateral sclerosis (ALS), is caused by dominant mutations in the cytosolic Cu,Zn superoxide dismutase (SOD1). There has been evidence for secretion of SOD1, by an unknown mechanism. In this work stable mouse motor neuron-like NSC-34 cells overexpressing human SOD1 wild-type hSOD1wt (NSC-34/hSOD1wt) and mutant hSOD1 G93A (NSC-34/hSOD1G93A) have been used as an ALS cell model. SOD1 was found to be secreted in association with a membrane fraction that pelleted at 100,000 × g. Sucrose density gradient separation of this fraction showed that wild-type and mutant SOD1 were found between 0.5 and 1.16 M sucrose and co-localized with the exosomal marker CD9. Therefore, SOD1 secretion occurred via exosomes. p115 a cytosolic and Golgi apparatus (GA) protein involved in vesicle tethering was also found in exosomes, contrary to the endoplasmic reticulum protein calnexin. SOD1 secretion mediated by exosomes could explain cell-to-cell transfer of mutant toxicity.

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Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease that results from selective dysfunction and death of upper and lower motor neurons (MN) in the spinal cord, brainstem and cortex (reviewed in Bruijn et al. [1]). In 20% of familial ALS patients population more than 100 mutations in the protein Cu,Zn superoxide dismutase (SOD1), that is ubiquitously expressed, have been identified.

There has been evidence for secretion of cytosolic SOD1, by an unknown mechanism [9–11]. Since, SOD1 is a cytoplasmic protein that lacks a signal peptide there must be an alternative mechanism for the extracellular export of this protein.

More recently, Turner et al. [18] have shown that extracellular secretion of mutant SOD1 is impaired in NSC-34 cells modelling familial ALS. Urushitani et al. [20] reported that SOD1 mutants linked to ALS can be secreted by interaction with chromagranins. The authors also showed that extracellular SOD1 mutants can trigger microgliaosis and death of MN in culture, suggesting a pathogenic mechanism based on toxicity of secreted SOD1 mutant proteins [20]. The importance of the MN environment has been emphasized, and it is now believed that toxicity to MN derives from damage developed within cell types beyond the MN. However, the mechanism by which the toxicity of mutant SOD1 may be transferred from one cell to another is still unclear [2].

Concomitant to these reports, there is an increasing body of evidence that different cell types, including neurons (reviewed in Keller et al. [7] and Faure et al. [3]) are capable of producing and releasing microvesicles called exosomes. Exosomes are small lipid membrane microvesicles (30–100 nm diameter) that are formed by fusion of multivesicular bodies (MVBs) with plasma membrane and the subsequent release of their cargo. Exosomes are biologically active entities which are important for a variety of pathways, and one of the biological functions of exosomal release is the secretion of membrane proteins meant to be discarded, or to be passed onto other cells (reviewed in Keller et al. [7] and van Niel et al. [21]). In neurodegenerative diseases, such as Alzheimer’s disease, β-amyloid peptides, that are intracellularly generated, were shown to be released to the extracellular space, in association with exosomes [15]. Similarly, in prion disease, infectious prion protein mediates its intercellular transfer...
via exosomes, bypassing cell–cell contact in the dissemination of prions [4].

In the present work human and endogenous mouse SOD1 were found to be secreted from NSC-34 cells via exosomes. These microvesicles might constitute a way of cell-to-cell communication and transfer of mutant SOD1 toxicity.

NSC-34/hSOD1wt and NSC-34/hSOD1G93A cells were lysed in RIPA buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% SDS, 1% DOC, 1% Triton X-100, 1% protease inhibitor cocktail Complete (Roche protease inhibitors)) for 10 min on ice. Cell lysates were cleared by centrifugation at 10,000 × g for 10 min at 4 °C. After ethanol precipitation protein samples were applied onto 15% acrylamide SDS-PAGE gels.

For supernatant analysis, NSC-34/hSOD1wt and NSC-34/hSOD1G93A cells were grown for 4 days in complete medium. A day before exosome preparation, culture medium was replaced with serum-free medium. Culture supernatants were collected and sequentially centrifuged at 500 × g for 10 min (to remove cells), 10,000 × g for 20 min (to remove cellular debris) and 100,000 × g for 2 h (to pellet membrane vesicles). Vesicles were directly solubilized in sample buffer, centrifuged at 150,000 × g for 2 h or further processed for gradient centrifugation.

Vesicles resuspended in 0.25 M sucrose were loaded on top of a step gradient comprising layers of 2, 1.3, 1.16, 0.8, 0.5 and 0.25 M sucrose. The gradients were centrifuged at 100,000 × g for 2.5 h using a Beckman SW 41 Ti rotor. Twelve 1 ml fractions were collected from the top of the gradient and precipitated with chloroform/methanol (1:4, v/v). Samples were analysed by SDS-PAGE and Western blot as described below.

Western blot was performed on polyvinylidene difluoride (PVDF) membranes that were blocked 1 h with phosphate buffered saline (PBS), pH 7.2, 0.1% Tween-20, 5% milk. They were incubated with the following primary antibodies: polyclonal anti-SOD1 (dilution 1:10,000, Santa Cruz); anti-C9 (dilution 1:1000, Santa Cruz), anti-calnexin (dilution 1:15,000) (kind gift from Prof. Helenius) and anti-p115 (dilution 1:1000, BD Biosciences). As secondary antibodies anti-rabbit (1:4000) and anti-rat (1:5000) coupled to horseradish peroxidase were used. Proteins were detected by the ECL Plus method following supplier’s protocol (Amersham Biosciences). All incubations were performed at RT.

To investigate if NSC-34/hSOD1wt and NSC-34/hSOD1G93A cells were capable of secreting wt and mutant hSOD1, cell culture supernatants were analysed by Western blot after sequential centrifugation at 500 and 10,000 × g to remove dead cells and cell debris (Fig. 1). Overexpressed hSOD1 (~22 kDa) as well as endogenous mouse SOD1 (mSOD1, 16 kDa) were detected. In addition, a degradation product was found in the cell debris fraction. Post-10,000 × g supernatants were further centrifuged at 100,000 × g to collect a fraction enriched in exosomes [5]. There was hSOD1wt and hSOD1G93A present in this fraction, which indicated that the cells secreted SOD1 to the extracellular medium associated to membrane vesicles. It appeared that the ratio between hSOD1 and endogenous mSOD1 was lower for the mutant than for the wild-type form of hSOD1.

The secreted hSOD1 was totally present in the 100,000 × g fraction, since there was no enzyme associated with the pellet of a subsequent centrifugation at 150,000 × g, nor in the corresponding supernatant. Since exosomes collected from the 100,000 × g pellet may be contaminated with apoptotic blebs [5], this pellet was further fractionated in a sucrose density gradient that ranged between 0.25 and 2.00 M. CD9 was used as a marker protein to identify exosome-containing fractions (reviewed in Keller et al. [7]), and revealed that exosomes were recovered in fractions between 0.50 and 1.16 M sucrose, corresponding to a density between 1.06 and 1.15 g/ml (Fig. 2). The density of these vesicles is compatible with those described for exosomes purified from other cells, such as ovarian carcinoma cell lines [5], dendritic cells [17], cortical neurons [3], and N2a cells [15].

Endogenous mouse and human SOD1 were found in the exosomes as revealed for its presence in the fractions corresponding to exosomes by comparison with the exosomal marker CD9 (Fig. 2, lanes 4–7). A peak corresponding to a molarity of 1.16 M was observed for both mouse and human SOD1 (Fig. 2, lane 6). These data demonstrated that the medium from NSC-34 cells had exosomes containing SOD1.

As negative control, calnexin, an ER resident protein, was used because it is known not to be included in exosomes, since exosomes have an endosomal origin and do not contain typically proteins of the nucleus, mitochondria, or ER, but all exosomal proteins are typically found in the cell cytosol or at the plasma membrane (reviewed in Keller et al. [7]). Indeed calnexin was not found in the exosome-containing fractions. However, it was associated with apoptotic membrane blebs. p115 a cytosolic GA vesicle tethering protein involved in the fusion of transport vesicles with acceptor compartments which allows maintenance of GA architecture [16], was also found associated with exosomes and apoptotic membrane blebs (Fig. 2).

A familial form of the neurodegenerative disease ALS is caused by dominant mutations in the ubiquitously expressed cytosolic SOD1. In the present work, we have shown that wild-type and mutant SOD1 were present in the supernatant medium from NSC-34 cells stably expressing hSOD1wtG93A, and that this protein was associated with exosomes.

Observations from several authors have also identified SOD1 present in the extracellular medium from different cell types [9,11,18,20]. It has also been reported that both wild-type and mutant SOD1 species are detected in CSF of both transgenic rats.
carrying human SOD1 [18] and ALS patients with the SOD1 mutation [6]. Nevertheless, the present work is the first report that describes SOD1 secretion in NSC-34 cells associated with exosomes.

The presence of hSOD1<sup>wt</sup> in the exosomes and hence in the extracellular medium of NSC-34 cells could be of biological relevance by protecting the cells against the physiological production of reactive oxygen intermediates present outside the plasma membrane surface. This is supported by studies using chimeric mice with mixed populations of cells expressing either endogenous or transgenic mutant SOD1<sup>G93A</sup> or SOD1<sup>G37R</sup>, where MN expressing transgenic SOD1 failed to degenerate if they were adjacent to large numbers of supporting cells (such as astrocytes and glia) without the mutant protein [2]. These findings sustain the protective role of a wild-type environment in the ALS pathology.

Mutant hSOD1, although present in the exosomes, seemed not to be incorporated to the same extent as hSOD1<sup>wt</sup>, and, consequently, might be entrapped inside the cell contributing possibly to deleterious effects. Turner et al. [18] have also observed that SOD1 was less abundant in the supernatant media from NSC-34/hSOD1<sup>G93A</sup> cells when compared with NSC-34/hSOD1<sup>wt</sup> cells. There has been evidence that the toxicity of SOD1 mutants is non-cell autonomous, that is, it requires mutant damage not just within MN but also to non-neuronal cells [2,8,13,14]. Concomitantly, in ALS there has been growing evidence of a role for inflammation, and the activation of microglia [13]. The presence of mutant SOD1, even though to a smaller extent, in the extracellular media via exosomes could be acting as an inflammation trigger. Urishtani et al. [20] have shown that extracellular SOD1 mutants cause microgliosis and neuron death, while wild-type SOD1 caused suppression of microglial activation in BV2 cells. The same authors [19] have observed therapeutic effects of immunization with mutant SOD1 in mice models of ALS with late onset and moderate levels of mutant SOD1. This result supports the notion of a role for extracellular mutant SOD1 in the pathogenesis of ALS. Exosomes could, in this sense, represent an additional mean of communication between cells. Besides, the capacity of exosomes to fuse with acceptor cells like neurons, astrocytes or microglia, has to be further investigated, so that one can be sure that they can act as a way to transfer cytosolic proteins like SOD1 between different cells [17]. Nonetheless, Mondola et al. [12] reported that extracellular SOD1 specifically interacts in a dose-dependent manner with the cell surface membrane of SK-N-BE cells and modulates intracellular calcium-dependent signalling pathways.

The role of the exosomes in neurodegenerative diseases has still to be further explored, but in Alzheimer’s disease β-amyloid peptides, that are intracellularly generated, were shown to be released to the extracellular space, where they accumulate, in association with exosomes [15]. These vesicles can act as a way to mediate intercellular transfer of Aβ. Similarly, infectious prion protein mediates its intercellular transfer via exosomes, bypassing cell–cell contact in the dissemination of prions [4].

This is, as far as we know, the first time that SOD1 was observed associated with exosomes in the NSC-34 ALS cell model. This evidence suggests a new cellular mechanism contributing for dissemination of mutant SOD1 toxicity in ALS pathogenesis.

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