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Exosomes as a potential mechanism of intercellular spread of prion protein

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SAMMANFATTNING

Prionsjukdomar kan drabba flera olika arter och orsakar utbredd nervvävnadsdegeneration med symtom som ataxi och demens. Sjukdomarna orsakas av prioner (PrP^{Sc}), omkonformerade varianter av ett i många cellslag vanligt protein, prionprotein (PrP^{c}). Prionproteinet är ett glycosyl-phosphatidylinositol (GPI)-förankrat glykoprotein i cellmembranet. Prionerna har en förmåga att förmå det normala prionproteinet att anta prionform, och är till skillnad från prionproteinet motståndskraftigt mot nedbrytning. Hur prioner sprids i kroppen är inte helt klarlagt, men prionprotein och prioner kan avges från celler med hjälp av små membranvesiklar, exosomer. Exosomer tros fungera som transportmekanism för olika molekyler mellan celler.

Projektets syfte var att undersöka om prionprotein är associerat till exosomer. Genom att isolera exosomer från celler som uttrycker prionprotein, märka in prionproteinet på exosomerna med guldmärkta antikroppar och sedan studera exosomerna i transmissionselektronmikroskop (TEM) kunde associering mellan prionprotein och exosomer påvisas.

ABSTRACT

Prion diseases affect several different species and cause extensive neural degeneration with symptom such as ataxia and dementia. They are caused by prions (PrP^{Sc}), deconformed variants of an in many cells common protein, the prion protein (PrP^{C}). The prion protein is a glycosylphosphatidylinositol (GPI) anchored glycoprotein in the cellmembrane. Prions have an ability to make the normal prion protein adapt prion conformation, and are in contrast to the prion protein resistent to degradation. How prions are spread within the body is not fully understood, but prion proteins and prions can be emitted from cells by small membrane vesicles called exosomes. Exosomes are thought to function as a transport mechanism of different molecules between cells.

The aim of the project was to investigate if the prion proteins are released from cells in association with exosomes. By isolating exosomes from cells expressing prion protein, label the prion protein on the exosomes with gold-labelled antibodies, and then examine the exosomes in an transmission electron microscope (TEM) an association between prion protein and exosomes could be observed.

INTRODUCTION

Prion diseases

Prion diseases or transmissible spongiform encephalopathies (TSE) affect humans (e.g. Creutzfeldt-Jakobs disease, Kuru), cattle (Bovine Spongiform Encephalopathy), cervids (Chronic Wasting Disease) and sheep (Scrapie) (Prusiner, 1998). They are characterized by extensive neural degeneration with symptoms of dementia and ataxia. The symptoms may differ, but are often very like other dementias such as Parkinson and Alzheimer's (Aguzzi et al, 2008). Some prion diseases, like Scrapie and CWD, can be horizontally transmitted in a manner similar to classically infectious diseases. All TSEs are progressive and fatal. The mechanism behind the neural degeneration is still unclear. Prion infection does not elicit an immune response.

The prion concept

Prion means *pro*teinaceous *in*fectious particle (Prusiner, 1998). It is a particle consisting of protein able to multiply and reproduce though it has no nucleic acid.

The normal prion protein and the abnormal prion protein

The cellular prion protein, PrP^c, is normally expressed on the surface of many different mammalian cells as a GPI -anchored glycoprotein, especially in nerve cells or in the lymphatic system (Prusiner, 1998). The function of the PrP^c is still unknown (Hu et al, 2007). It may be involved in cell adhesion and recognition, signaling, apoptosis, immunoregulation or other functions. The prion, PrP^{Sc}, is a modified isoform of PrP^c, where structural α-helices are irreversibly changed to β-sheets so that the prions accumulate as placks in the brain (Prusiner, 1998). The normal cellular PrP^c is easily digested by proteases, but the β-sheets makes the prion resistant to proteases.

In sheep there are several PrP genotypes (McIntyre et al, 2006). The VRQ allele is associated with the highest risk of scrapie (Goldman et al, 2005).

Cleavage of the prion protein

The PrP^C is subject to endoproteolytical cleavages, resulting in parts of the protein being shed from the cellsurface (Walmsley et al, 2008). Normally the cleavage occurs in a highly conserved hydrophobic region of the protein, resulting in a 9 kDa soluble N-terminal fragment (N1) and a 17 kDa C-terminal fragment (C1) still attached to the membrane. This cleavage is called the α-cleavage. The C1 fragment is then shed extracellularly in a second cleavage called β-cleavage, which is mediated by reactive oxygen species (ROS) (Klingeborn, 2006, Walmsley et al, 2008). The first cleavage can also occur within or adjacent to the octapeptide repeats of the PrP^C, resulting in a 19 kDa GPI-anchored C-terminal fragment (C2) and a 7 kDa N-terminal fragment (N2).

Transmission of prions

The prion differs from other infectious pathogens in that it is capable of replicating and transmitting infections without informational nucleic acids (Aguzzi et al, 2008). The infectious prion can be transmitted to another individual from an exogenous source or can be generated spontaneously in the individual. In

both cases the prion affects the normal cellular prion protein, and makes it assume a pathological prion conformation. The newly made prion will be transmitted to neighbouring cells. The prions can only infect cells expressing the normal PrP, and too large dissimilarities in the amino acid sequences of the prion and the normal PrP prevent conversion of the PrP (species boundaries, exceptions exist) (Prusiner, 1998).

When infectious prions are taken up orally, they must pass the epithelial barrier in the gut to reach the Peyer's patches (Aguzzi, 2001). The prions accumulate in the gut-associated lymphatic tissue (GALT) (Aguzzi et al, 2009). Exactly how the prions cross the mucosa is not known, M-cells are ports of entry cross the epithelia for enteric pathogens and prions could also be transported through enterocytes by different mechanisms. The prions then infect follicular dendritic cells (FDC) in Peyer's patches and the lymphoid organs (Aguzzi, 2001). B cells produce Lymphotoxin-β which induces maturation of FDCs and are therefore essential for the spread of prions. Sympathetic peripheral nerves in the spleen transport the prions to the CNS (Aguzzi et al, 2009). There are three different hypotheses on how the prions enter neurons: cell-cell contact, vesicle transport or "free-floating" extracellular prion infectivity. How the prions are transported within the neurons is also unclear, they could be transported within the axons like normal cargo proteins, or they could slowly convert PrP on the cell surface and thereby slowly spread along the cell surface. Studies indicate that spread of prions within the brain is dependent on an unbroken chain of cells expressing the normal PrP (Aguzzi et al, 2009). It cannot be excluded that prions are spread via blood to the brain.

Exosomes

Exosomes are small membrane vesicles (30-100 nm) secreted by several different cell types like immune defense cells, platelets, neurons and epithelial cells (Admyre, 2007). Small intraluminal vesicles form in cells by inward budding of the limiting membrane of endosomes, creating multivesicular bodies – MVBs. These MVBs can either transport proteins to degradation by fusing with lysosomes, or they can fuse with the cell membrane letting out the intraluminal vesicles extracellularly as exosomes. The function of exosomes depends on which type of cell that has released them. They can be used to remove unnecessary proteins, they have been shown to have functions in the immune system, and they can transport molecules between cells (Admyre, 2007).

Project background

Earlier investigations have shown that both the normal PrP and the PrP^{Sc} are actively released extracellularly from the cells, and that exosomes released from PrP^{Sc}-infected cells are able to transfer PrP^{Sc} to other cells in *in vitro* and in *in vivo* systems (Fevrier et al, 2004, Vella et al, 2007, Mattei et al, 2009). Exosomes may contribute to the transmission of prions between cells and thereby neural invasion.

Aim of project

The aim of the project was to investigate the association between exosomes and PrP^C as a way to release PrP^C from cells. This was done by using cell cultures and transmission electron microscopy (TEM).

DESIGN OF EXPERIMENTS

PrP^C was over-expressed in Baby Hamster Kidney-21 cells and used as a model for PrP^C release.

Semliki Forest Virus (SFV) vectors have a broad host range and a very high level of expression of proteins, which can be used to make cells produce large amounts of a certain protein (Liljeström et al, 1991). The SFV genome is a 5'-capped and 3'polyadenylated RNA molecule which has a positive polarity, i.e. it functions as a mRNA. The cells were transfected with pSFV1 mRNA or pSFV1-VRQ mRNA which induces expression of the sheep PrP with the VRQ genotype. The pSFV1 transfected samples were used as negative controls.

Medium and lysate from transfected cells were collected and analysed with enhanced chemilumiscence (ECL) Western Immunoblotting to check if PrP^c was expressed in the cells. Primary antibody anti-PrP mouseAb 6H4 was used. The secondary antibody was anti-mouse labelled with Horseradish peroxidase (anti-mouse HRP), which catalyses a reaction that luminate. This reaction can be detected on a photographic film. Western blots were performed on both glycosylated and deglycosylated samples.

For TEM analysis copper grids were prepared using the 100 000 x g exosome-containing pellets from the pSFV1 and pSFV1-VRQ samples, respectively. The grids from both pellets were either only stained with phosphotungstic acid (PTA), or immunolabelled with gold and stained with PTA.

MATERIALS AND METHODS

Cell lines

Baby hamster kidney cells (BHK-21 cells, ATCC) were grown to near confluence in BHK complete medium (Glasgow medium (Invitrogen) supplemented with 20 mM Hepes (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Invitrogen), 5 % Foetal Calf serum (Invitrogen), 10 % Tryptose phosphate broth (Invitrogen), 2 mM L-Glutamine (Invitrogen), 1% PEST (penicillum-streptomycin; Invitrogen)) and splitted 1:10 once before transfection.

mRNA preparation *in vitro*

The pSFV1 and pSFV1-VRQ plasmid-DNA were linearised with *Spe*I (New England BioLabs, 10 000 U/ml) using U *Spe*I per µg DNA. The mixtures were incubated at 37°C for 2 hours with NEBuffer2 (New England Biolabs) and 1% bovine serum albumin (BSA) in a total sample volume of 100 µl.

The *Spe*I-cleaved DNA was purified according to Strata prep Plasmid Miniprep Kit (Agilent Technologies, Stratagene Products Division) step 7 to 22. The samples were eluated with DEPC-H_2O. The concentration of the DNA was measured with a spectrophotometer (Nano Drop Technologies).

mRNA preparation was done according to SFV expression systems technical manual (Liljeström et al, 1991). Around 1.5 µg plasmid-DNA was mixed with 5 µl SP6 Buffert (GE Healthcare), 5 µl 10 mM m^7G(5')ppp(5')G (GE Healthcare), 5 µl 50 mM Dithiothreotol (DTT), 5 µl rNTP-mix (10 mM ATP, 10 mM CTP, 10 mM UTP, 5 mM GTP; Pharmacia), 2 µl 21.6 U/µl RNA-guard (GE Healthcare) and 1 µl 50U/µl SP6 RNA polymerase (GE Healthcare). DEPC-H_2O was added to the samples, making the total volume of each sample 50 µl. The samples were vortexed and then incubated at 40°C for 60 minutes.

Transfection of BHK-21 cells

Transfection was made according to SFV Expression systems protocol (Liljeström et al, 1991). The BHK-21 cells were grown to near confluence. The cells were washed with pre-warmed phosphate buffered saline (PBS^-, without Ca^{2+} and Mg^{2+}) and then trypsinised (0.025% trypsin in phosphate buffer) and incubated at 37° C until they detached. They were resuspended in BHK-complete medium and harvested by centrifugation at 1000 rpm for 5 minutes at room temperature. The cells were washed with 2x800 µl PBS^-, and then 780 µl each was mixed with 24 µl mRNA (pSFV1 and pSFV1-VRQ respectively) and pulsed twice at 850 V and 25 µF using a Bio Rad Gene Pulser. The time constant showed 0.4 after each pulse. The transfected cells were then resuspended and diluted 1:20 in complete BHK-medium. Six hours after transfection the cells were washed with PBS^- and 1 ml complete BHK-medium (ultracentrifugated 100 000 xg for 2 hours) was added.

Isolation of exosomes from cell culture supernatants

Medium from the transfected BHK-cells was collected and cleared from cells by centrifugation at 6000 rpm for 5 minutes at 10° C. The supernatant was then centrifuged at 10 000 xg for 5 minutes at 10° C and then ultra centrifuged at 100 000 xg for 2 hours. 2 ml of the supernatant was carefully pipetted from the top and saved. The rest of the supernatant was decanted, saved and analysed later. The exosome containing pellet was resuspended in PBS and kept in 4° C. All other fractions were kept in -20° C.

Cell lysate

The cells were washed twice with cold PBS^- and then lysed for 10 minutes in a lysis buffer (0.5% Triton X-100, 0.5% Sodium deoxycholate, 5 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA) while kept on ice. The samples were centrifuged at 6000 rpm for 5 minutes at 5° C and the supernatants were transferred to new tubes and kept in – 20 ° C.

Deglycosylation

Deglycosylation with Peptide-N-Glycosidase F (PNGaseF) was done according to the manufacturer's protocol (New England BioLabs). Five µl of the lysates (pSFV1-VRQ and pSFV1) were diluted with 10 µl PBS.

The lysates and supernatants were mixed with denaturing buffer (New England Biolabs), and incubated at 100° C for 10 minutes. 10xG7 buffer (New England Biolabs), NP-40 (New England Biolabs), 28x Complete Mini (Roche) and 50 000 U/ml PNGaseF (New England BioLabs) were added and the samples were deglycosylated for 2 hours at 37° C.

After the deglycosylation 4xLDS gel loading buffer (Invitrogen) was added to the lysates.

TCA precipitation

In order to reduce the volume before electrophoresis the supernatants were precipitated with ice cold Trichloroacetic acid (TCA) (final concentration, 10%) for 30 minutes, centrifuged at 14 000 rpm at 4° C for 10 minutes and then washed with ice cold 50%Aceton/50%EtOH. The pellets were resuspended in 1xLDS gel loading buffer (Invitrogen).

Western immunoblotting

Protein immunoblotting was performed according to NuPAGE Bis-Tris Electrophoresis System (Invitrogen). 100 mM Dithiothreotol (DTT) was added to the samples to reduce disulfide bonds. The samples were then denatured by heating at 100° C for 10 minutes. All samples (lysates, supernatants and 100 000 xg lower supernatants) were centrifuged at 14 000 rpm for 5 minutes, and then loaded on NuPage 12 % Bis-Tris Gel (Invitrogen) with NuPAGE MOPS SDS Running Buffer (Invitrogen) at 110 V for 2 hours. NuPAGE antioxidant (Invitrogen) was added in the upper (cathode) buffer chamber. The proteins were transferred from the gel to a PVDF-membrane (Hybond-P polyvinylidene diflouride membrane filter; GE Healthcare) using a Hoefer TE 22 Mighty Small Transphor wet transfer apparatus with transfer buffer (24 mM Tris base, 192 mM glycin, 20% v/v methanol, H_2O) at 60 v for 2 hours.

The membrane was blocked with 5% non-fat milk (5g Semper + 100 ml PBS-Tween) and washed in PBS-Tween (Medicago) and probed with the primary antibody 6H4 (Prionics AG) diluted 1:14000, for one hour. The membrane was washed with PBS-Tween 3x5 minutes and then probed with secondary antibodies (horseradish peroxidase conjugated antimouse IgG) (GE Healthcare) diluted 1:5000, for one hour. The membrane was then washed 5x10 minutes with PBS-Tween. This was all done on a shaker. The membrane was flooded with detection fluid (Immobilon Western Chemiluminescent HRP Substrate; Millipore), and films (Hyperfilm; GE Healthcare) were developed in darkroom.

Electron microscopy

The exosome containing pellets (pSFV1-VRQ and pSFV1) were resuspended in PBS^-, and transferred to two Formvar carbon coated electron microscopy grids (Analytical Standards AB) each, resulting in four grids, two with pSFV1 exosomes, and two with pSFV1-VRQ exosomes. One of each pSFV1 and pSFV1-VRQ grids were labeled with primary (6H4) (Prionics AG) and secondary (anti mouse IgG-gold) (Novakemi AB) antibodies. The four grids were then stained with 2% Phosphotungstic Acid (PTA), air dried and then analysed in TEM.

RESULTS

The pSFV1 and pSFV1-VRQ mRNA were transfected to the Baby Hamster Kidney cells and PrP^c was expressed in the pSFV1-VRQ transfected cells (fig 1, lanes 3 and 5) and could be seen in the supernatant as C1 segments (Fig. 1, lane 9) showing that PrP^c was released into the medium.

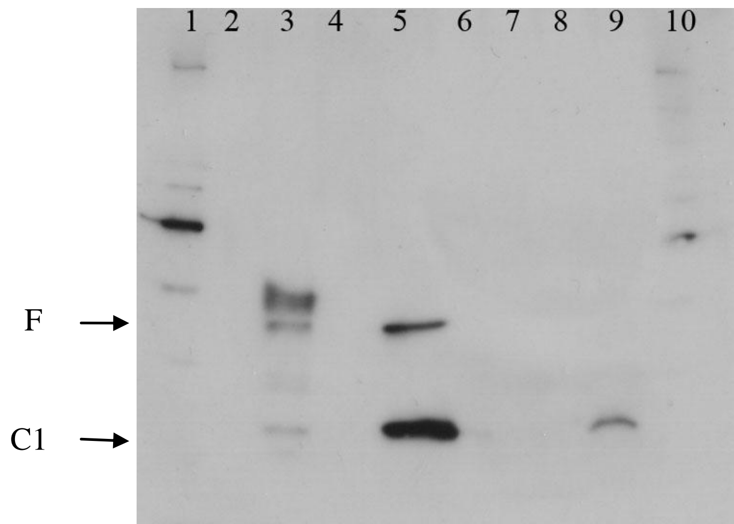

Figure 1: Western blot.
F = full length PrP^c, C1 = C1 fragment
Lanes 1 and 10 markers.
Lanes 2-5 are lysates from transfected cells, lane 2 pSFV1, lane 3 pSFV1-VRQ, lane 4 pSFV1 deglycosylated , lane 5 pSFV1-VRQ deglycosylated.
Lanes 6-9 are 100 000 xg supernatant, lane 6 pSFV1, lane 7 pSFV1-VRQ, lane 8 pSFV1 deglycosylated , lane 9 pSFV1-VRQ deglycosylated.

In order to analyse if PrP^c was associated with exosomes, the exosomal pellets obtained after ultracentrifugation were transferred to copper grids and stained for analysis by TEM. A few membrane vesicles of similar size as exosomes (30-100 nm) could be seen on the grids. In the gold labeled grids there were exosome-like vesicles with gold particles attached to them (Fig. 2). Free unattached gold particles and vesicles without gold attached could also be seen.

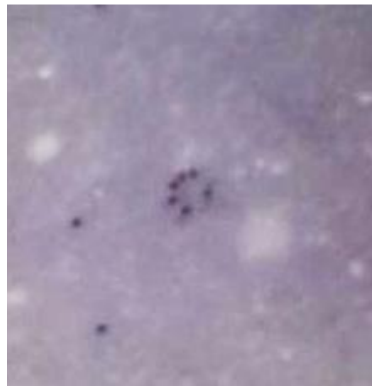

Figure 2: Exosome like vesicle with immunogold stained PrP. Grid contrasted with PTA.

DISCUSSION

Prions (PrP^{Sc}) are distortered variants of a common protein, the prion protein (PrP^c). The PrP^c is a glycosylphosphatidylinositol (GPI) anchored glycoprotein in the cell membrane of many cell types, mainly in the neural and immune systems. Prions cause diseases mainly affecting the neural system. Prions have an ability to make the normal prion protein adapt prion conformation, and are in contrast to the prion protein very resilient to degradation. How prions spread between cells is not fully understood. When the prions are taken up orally, they pass through the

epithelial barrier in the gut in a similar manner as other alimentary infectious agents and enter the bloodstream and reach the Peyer's patches. There they infect immune cells, rendering it possible for the prion infection to spread to other lymphoid organs and peripheral nerves, and in time to the CNS, the primary site of their morbid influence. There are three different hypotheses on how the prions spread from cell to cell: cell-cell contact, vesicle transport between cells or as “free-floating” extracellular agents.

Exosomes are small membrane vesicles (30-100 nm) secreted by several different cell types, like immune defense cells, platelets, neurons and epithelial cells. They have several different functions, and transport molecules between cells.

Baby Hamster Kidney cells were transfected with plasmid Semliki Forest Virus mRNA coding for the VRQ genotype (pSFV1-VRQ mRNA), and pSFV mRNA used as a negative control. Medium and lysate from both glycolysated and deglycolysated samples from the transfected cells were analysed with enhanced chemilumiscence (ECL) Western Immunoblotting. Four copper grids were prepared for transmission electron microscopy analysis using exosome-containing pSFV1 and pSFV1-VRQ samples. The grids were either only stained with phosphotungstic acid (PTA), or stained with PTA and labelled with goldcomplex.

The Western Blots showed that the cells expressed PrP^c. After PNGase F treatment, two main bands were detected, the full-length PrP and the C-terminal fragment C1.

In three grids that were analysed by TEM the sample was in thick layers seen as solid black areas. The thickness of the sample was probably due to an impure preparation. There were few exosomes and a lot of debris. This made it very difficult to see whether there were any exosomes in them or not. One sample contained an exosome with gold-labelled PrP along its membrane, which indicates PrP being attached to the exosomal membrane. Exosomes are known to transport proteins between cells, and an eventual transfer of PrP^c to another cell could be mediated by fusion of membranes.

The results show that PrP occurs extracellularly both as a soluble protein and membrane bound. The soluble proteins are shed PrP fragments, whereas the membrane bound still has GPI-anchors. It is possible that this difference is important regarding the uptake and spread of the PrP to new cells. It is possible that the GPI-anchor is essential for uptake of PrP.

Although PrP were localised to exosome like vesicles, it is not possible to conclude by this experiment alone that they indeed are associated with exosomes. There were also vesicles with no gold attached on them.

This was a first attempt to analyse exosomes as a potential mechanism of intercellular spread of PrP. Further work remains to elucidate the role of exosomal transport of the PrP.

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