CJD Support Group Network - Report 2016

Modulation of neuron-derived, prion-positive membrane microparticles

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Preamble

Prion accumulation in neurones leads to the clinical manifestations of prion disease by inducing neuronal apoptosis or dysfunction. Clinical variation in prion strain phenotype results from regional differences in prion accumulation. Experimental models indicate that infectivity spreads from one brain region to another often in a very well defined manner. The spread of infectious prions presumably occurs via direct cell contact or via near or far field spread of prions in brain extracellular fluid. It is now well established that culture supernates of cells permitting prion replication contain the infectious agent or prion. Some years ago, we showed this for cellular fragments <100nm called exosomes (Vella *et al,* 2007) and Mattei et al (2009) showed that plasma membrane MP also contain infectivity. Understanding how prions spread in culture models may suggest avenues for therapeutic intervention.

Extracellular vesicles including microparticles (MP) and exosomes are released from all known cell types particularly in the context of infection (Combes *et al,* 2010). We have recently not only confirmed that microparticle (MP) numbers are increased in the plasma of untreated patients with multiple sclerosis (MS), but also that fingolimod treated MS patients have very low plasma microparticle numbers. Phenotypically, these plasma MP are derived from diverse cell types suggesting to us that fingolimod has a general suppressive effect on MP release (Zinger *et al,* 2016); an effect that we showed was potent in human brain endothelial cell lines.

We have previously shown that anti-prion antibodies suppress replication *in vitro* (Beringue *et al,* 2004) and even *in vivo* (White *et al,* 2003). Given that prions are proven constituents of these subcellular particles, and our antibodies bound both cellular prion protein, PrP^{C} - also recently shown to be constituents of MP (Ritchie *et al,* 2013) - , an attractive hypothesis is that the antibodies are working by binding to MP/exosomes and targeting them for destruction prior to their interaction with adjacent cells. MP are known to influence diverse functions in adjacent or far-flung cells. Proving that subcellular fragments have a role in the pathogenesis of prion disease in humans and in animal models has not been possible without the availability of drugs proven to inhibit subcellular particle release. It should be noted that in many other neurodegenerative diseases, accumulated and misfolded proteins are thought to slowly spread throughout the CNS in a similar fashion.

Research plan proposed in 2015

- Part I – We planned to answer the following: Does fingolimod - or other agents known to inhibit prion replication in vitro - inhibit the production/release of subcellular fragments? We initially sought to determine if MP release from cell lines permissive for prion replication such as N2a and GT1 was inhibited by fingolimod. In parallel experiments we also planned to look for similar inhibition using known inhibitors of prion replication including anti-prion antibodies and dextran sulphate thought to inhibit prion replication by binding to cell surface glycosaminoglycans (Beringue *et al,* 2004). Over the last five years the Hawke and Hill laboratories have collaborated to produce a number of new anti-prion monoclonal antibodies that we planned to use in these experiments.

Part II - If we identified inhibitors of MP/exosomal release we planned to determine if they suppressed prion replication in established cell culture models of prion replication; using the mouse adapted Fukuoka strain M1000 that infects mouse neuronal GT-1 and N2a cells with defined kinetics. The readout of infectivity is based on the Prion Infected Cell Assay (PICA; developed in the Hill Lab) and standard high resolution immunoblotting.

Results for 2016

In the last 12 months we have started characterising extracellular vesiscles from the mouse neuronal cell line, GT-1, permissive for prion replication. Binding cell surface PrP^C with mAbs or disrupting cell surface glycosaminoglycans with dextran sulphate potently inhibits *in vitro* prion transmission. It remains uncertain whether these treatments modulate the release of infectious prions within exosomes and /or MPs. As discussed, having found the S1P modulator, fingolimod, potently downmodulated MP release from human brain endothelial cells (Zinger *et al, Mult Scler J* 2016) we performed a number of similar experiments using GT-1 cells. A considerable number of experiments were required to optimise the quantitation of MP released by this neuronal cell line after which we tested all the known active concentrations of the molecule and allowed for the widest kinetics of action. Unfortunately, even using phosphorylated fingolimod, the active drug (obtained from Novartis AG, Basel), we were unable to show any effects on this neuronal cell line similar to those we demonstrated on brain endothelial cells. Neurones are known to express S1P receptors, but we have not yet determined if GT-1 similarly express the S1P receptor family though fingolimod has been used to modulate murine experimental allergic encephalomyelitis (EAE).

We then focussed our attention on other inhibitors of MP release. Earlier this year we have disclosed the substantial modulating effect of Diannexin on MP production by various cell types, including monocytes, platelets and endothelial cells. Diannexin, donated by Dr A.C. Allison (Alavita Inc., Mountain View CA), is a recombinant homodimer of human annexin V that binds phosphatidyl-serine (PS) with a higher affinity than that of annexin V and displays a longer half-life than its monomer. By shielding PS exposed at the surface of activated cells, Diannexin reduced the interaction with procoagulant proteins such as factor Va. Using flow cytometry, confocal and scanning electronic microscopies we established that Diannexin can

inhibit endothelial vesiculation by binding PS present either at the cell surface or at the level of the inner leaflet of the plasma membrane (Combes *et al, Int J Innov Med Health Sci* 2016).

In contrast to what we obtained with fingolimod, Diannexin treatment of GT1 cells appeared to significantly downregulate MP release, using our standard microanalytical methods. Not only total MP numbers were reduced, in a dose-dependent manner, by Diannexin, but also the numbers of PrP^C positive MPs (Figure 1).

Furthermore, using nanoparticle tracking analysis (NTA), we found that Diannexin unexpectedly also reduced exosome release by GT-1 cells (Figure 2). As Diannexin has been previously used in phase I/II clinical trials in stroke, it will be important now to assess its therapeutic efficacy on prion transmission in vitro (using the Prion Infected Cell Assay developed in the Hill Lab and standard high resolution immunoblotting).

Future directions

We also now plan to assess MP release by cells treated by DS500 and anti-prion mAbs using the NTA (NanoSight) technology. If these agents proven to be inhibitory also affect MP release, then this technology will be useful to screen for other therapeutic agents on a large scale.

It has been our aim for a number of years to better understand the molecular machinery of MP release. Inhibiting enzymes or signalling pathways involved in membrane vesiculation potentially represents a novel way to inhibit prion transmission. In our recent review (Latham *et al, Trends Cell Mol Biol* 2014) we list a sizeable number of agents that alter membrane vesiculation and therefore MP release. Interestingly, some of these molecules, including chlorpromazine, have already been identified by Prusiner and colleagues as agents that inhibit prion replication.

We previously showed that *abca1* gene deletion is associated with a dramatically reduced MP release and a full protection against cerebral malaria in a murine model (Combes *et al, Am J Pathol* 2005). Similar knockdown of this molecule in GT-1 cells is achievable, by either siRNA or lentiviral vectors. The full profile of EV release in such mutant cell lines could be readily assessed by NTA and its effect on prion transmission using the scrapie cell assay. We would also hope to identify other targets involved in the production and release of extracellular vesicles that could be similarly modulated.

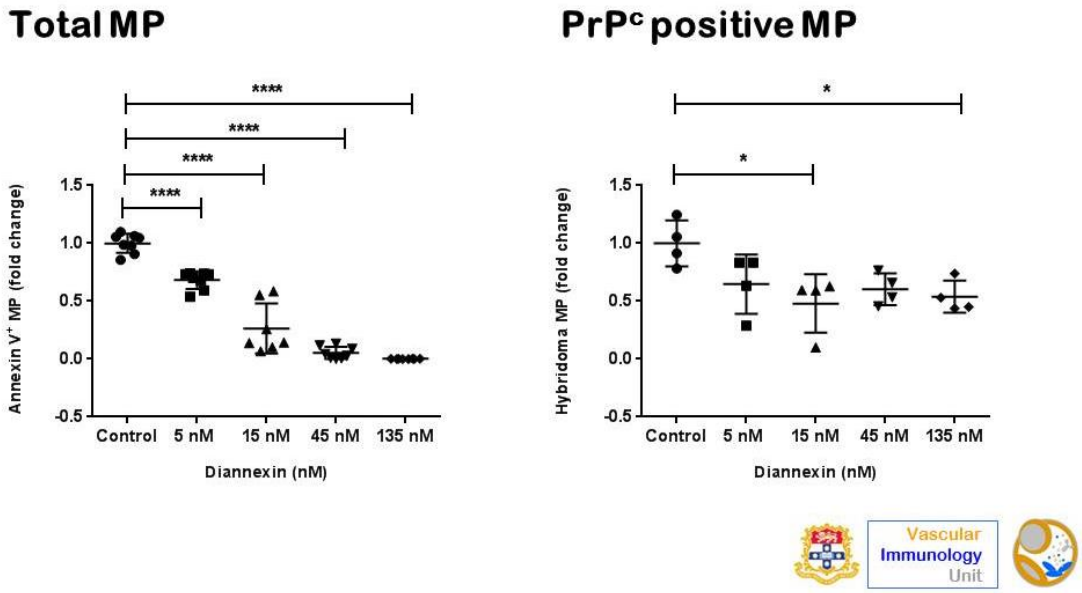

Figure 1. Effect of Diannexin on microparticle (MP) release by GT-1 cells

Left: total MP numbers, as assessed by FITC-annexin V + events. Right: PrP^C positive MP, as assessed by staining with anti-PrP^C ICSM18 hybridoma. MPs were quantitated in the first pellet of supernates from GT-1 cells, either resting or Diannexin treated, after centrifugation at 18,000 x g for 45 min.

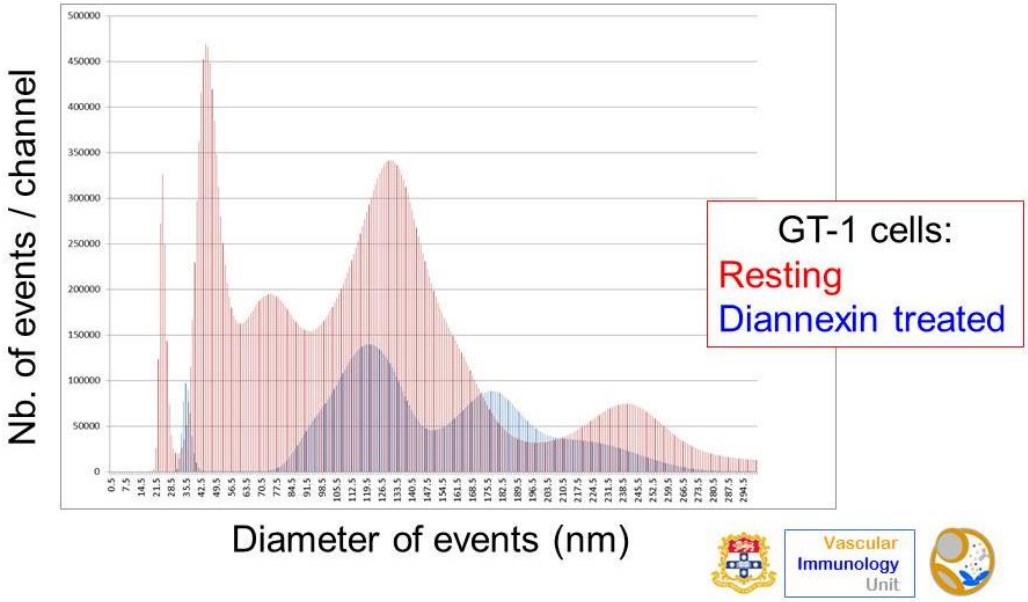

Figure 2. Effect of Diannexin on events smaller than 300 nm in diameter

The MP-free supernate from GT-1 cells, either resting or treated with Diannexin, was investigated by using nanoparticle tracking analysis (NanoSight NS300). This device exploits the properties of both light scattering and Brownian motion in order to obtain the particle size distribution of samples in liquid suspension. Results are expressed as numbers of event per size channel.

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