Scientific report - **Clusterin as a new therapeutic target to improve clearance of vascular amyloid (PN-III-P4-ID-PCE2020-1622)**

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Alzheimer's disease (AD) is the comonest form of dementia, for which there is no cure. The key element of the pathological feature of AD is the deposition of amyloid-β (Aβ) in the walls of capillaries and arteries as cerebral amyloid-angiopathy (CAA). Clinical observations and experimental studies demonatrate that CAA results from the failure of clearance of Aβ along the walls of capillaries and arteries. The drainage of interstitial fluid and Aβ occurs along the basement membranes of capillaries and arteries as Intramural Periarterial Drainage (IPAD), failing with increasing age and posession of Apolipoprotein E4 genotype and resulting in CAA.

Clusterin (Apolipoprotein J) is a heterodimeric glycoprotein and genetic studies have demonstrated its association with both cortical plaques and sporadic age-related CAA. Experimental studies have demonstrted that Clusterin is implicated in the clearance of Aβ42 by low density lipoprotein receptor 2. Clusterin is sequestered with Aβ in CAA and in white matter lesions associated with cerebral autosomal dominant arteriopathy with leukoenkephaly (CADASIL). Experimental studies show that in the absence of clusterin, CAA worsens in a mouse model of AD. The data point to a possible involvement of Clusterin in IPAD nd the pathogenesis of CAA.

AIm of the study

Here we aim to test the hypothesis that Clusterin facilitates IPAD and can delay/prevent CAA and AD.

Objectives

1. Project implementation. Setting up the colonies of mice. Synthesis of Clusterin
2. Pilot studies of intracerebral injections
3. Injecting clusterin and Aβ intrahippocampally in order to test IPAD.

Specific Activities for each objective

1. **Project implementation was set up in the work package** *Analysis of the research*:

1.1. A thorough analysis of AD pathogenesis, IPAD and what is known about the role of Clusterin in AD

Result: 1 review of literature

1.2. Research into the best methods for the study

Result: 1 study

1.3. Work protocols and risk assessments for the experimental study

Result: 1 protocol of work, risk assessmet for mouse work and sectioning of brains

2. **Pilot studies:** within the work pachage: *Setting up the experimental model*:

2.1. Setting up, breeding and ageing of experimental mouse models

Result: Transgenic Clusterin knock-out mice and APP/PS1 model of Alzheimer's disease of 6 months old and 12 months old

2.2. Synthesis of Clusterin

Result: Recombinant Clusterin ready for treatment of mice

3. **Intrahippocampal injections of Aβ in Clusterin knock-out (*Clu-/-*) mice** will be performed as part of the work package *Experimental in vivo research*:

3.1 . Understanding how to use the stereotactic frame

Result: gaining skills on using the stereotactic frame.

3.2. Intrahippocampal injections of Aβ in Clu-/- mice

Result: 6 month old Clu-/- mice injected with fluorescently labelled Aβ40 in the hippocampus and brains postfixed for analysis

3.3. Study of IPAD in the presence/absence of Clusterin

Result: Immunocytochemistry for collagen IV and smooth muscle actin on sections of brains from mice with fluorescent Aβ injected. Confocal microscopy and image analysis.

3.4. Treatment of APP/PS1 mice of young (6month old) and old (12 month old) with Clusterin

Rezultat: APP/PS1 mice of 6month old and 12 month old treated with Clusterin. Brains postfixed ready for immunohistochemical analysis of CAA burden.

3.5. Analysis of CAA burden in APP/PS1 mice treated/untreated with clusterin

Result: Immunohistochemistry for Aβ and markers of vascular walls (collagen IV, smooth muscle actin) in young and old treated/untreated APP/PS1 mice

Pilot studies performed for the use of stereotactic frame

I. Aparatul stereotaxic - folosit pentru injectarea șoarecilor - tehnică

This technique allows the precise delivery of minute quantities of tracer in a specific location of the mouse brain (0,5 µL/2,5 min). In our pilot studies for refining the technique of intracerebral injection, we have used n=20 adult 6 month old C57Bl6 mice injected intrahippocampally with Evans Blue. After identification of the place for drill and injection, the capillary was left *in situ* for 2 minutes allowing bolus diffusion. 5 minutes were allowed for IPAD. The stereotactic Induction of anaesthesia for surgery

1. Set oxygen flow meter to 1.7 L/min.
2. Switch airflow valve to direct isoflurane and oxygen to the induction chamber.
3. Weigh mouse and place in the induction chamber.
4. Set isoflurane vaporizer to level four to induce anaesthesia.
5. Monitor the level of anaesthesia by using the toe pinch response; surgical plane is reached when the mouse does not respond to toe pinch.
6. Switch airflow valve to redirect isoflurane and oxygen to the stereotaxic frame.
7. Transfer mouse from induction chamber to the stereotaxic frame isoflurane mask.

3.2 Stereotaxic injection of tracers into mouse hippocampus

This technique allows for the injection of small amounts of tracers into mouse brain parenchyma at a controlled and physiologically relevant rate (0.5 µL / 2.5 min). After injection, the capillary tip is left in situ for 2 min to allow for bolus diffusion. A further 5 min is then given for perivascular drainage. All procedures are performed using a dissection microscope.

1. Position anesthetized mouse on to a heated pad on the stereotaxic frame using mouthpiece and ear bars. The head needs to be secure and not move when pressure is applied to the scalp.
2. Cover rectal probe in Vaseline and insert into mouse. Regulate temperature at 37 °C.
3. Apply Lacrilube to mouse eyes.
4. Make an incision down the midline of the scalp to expose the skull.
5. Clean the surface of the skull using PBS and cotton buds. Air-dry with Dust Off.
6. Front load the microcapillary pipette with tracer using a 1 mL Termo syringe attached to the pipette by narrow gauge tubing. Attach on to stereotaxic frame using the injection pipette holder

7. Use the stereotaxic XYZ controls to manoeuvre the microcapillary pipette so that the tip is located over Bregma.
8. Adjust XY so that the pipette tip is positioned at the hippocampus (ML = -1.5 mm, AP = -2.0 mm)
9. Mark the top of the skull underneath the pipette tip with a fine marker pen.
10. Adjust Z-axis to lift the tip away from the skull.
11. Use a fine drill at medium to high speed to gently shave away the skull previously marked by the pen. Stop when the dura mater becomes visible.
12. Clean the surgery area using PBS and cotton buds.
13. Gently adjust the Z axis to lower the pipette tip until it touches the dura and then lower into the hippocampus (DV = 1.7 mm).
14. Slowly press the plunger of the syringe to inject 0.5 µL of tracer over 2.5 min.
15. Leave pipette tip in situ for 2 min to prevent reflux and then slowly remove it from the brain using the Z axis stereotaxic control.
16. After another 3 min inject pentobarbitone intraperitoneally (200 mg/kg), using a 1 mL syringe and a 26G ½" x 0.45 x 13 mm needle.
17. Wait for 1 min and then transfer mouse on to perfusion mat. Prepare mouse for perfusion and start the flushing procedure. This should be timed to start when the 5 min perivascular drainage time has been reached.

Pilot studies of immunocytochemistry and immunofluorescence

For these studies, Evans Blue was used to detect if the intracerebral injection reached the desire target (withi the body of the hipocampus). The brains were postfixed in 4% paraformaldehyde, embedded in paraffin and and sectioned in 10µm thick sections.

The histological analyses as well as immunocytochemistry and immunofluorescence are performed for the whole duration of the study in the Microscopy Laboratory of the Advanced Centre of Research - Centrul de Cercetare Avansată Medico-Farmaceutică (CCAMF) as well as in the Laboratory of Histology and Molecular Biology of UMFST GE Palade Târgu Mureș:

1. For microscopy an Olympus BX46 where 3 people can observe the slides simultaneously and equipped with a camera is used: https://erris.gov.ro/Laboratory-of-Immunohistoche-1.

2. Immunocytochemistry is performed using a Bond-MAX Fully Automated Immunostainer – Leica Biosystems, http://erris.gov.ro/CCAMF.

3. Imaging will be done on a Confocal microscope Leica TCS SP8 http://erris.gov.ro/CCAMF

All protocols for fixation are now optimised:

- 4% în 0.01 M PBS, at a pH of 7,2 is used for perfusion-fixation of mice and post-fixation of brains.

- Embedding in wax and sectioning has been optimised

- Din fiecare bloc de parafină se va face o secțiune, ce se va colora cu o colorație standard (Hematoxilină Eozină - HE).

- The optimisation of antibodies for detection of vascular structures is scheduled for 2022

Summary

In this initial stage of the project we have gained all the knowledge and skills for the optimal delivery of this international project. All equipments and reagents have been purchased. The stereotactic injections have been practised and optimised using the tracer Evans Blue. Clusterin has been synthesised by Prof Mark Wilson, University of Wollongong and has been delivered safely to UMF Târgu Mureș. Both knock-outClusterin transgenic mice as well as APP/PSq1 transgenic model of Alzheimer's disease micehave bred and are awaiting transport from Mayo Clinic to Târgu Mureș. The background of the project has been presented during the research days of UMFST Târgu Mureș 6-10 Dec 2021.

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