Neuroprotection and angiogenesis: new molecular factors and delivery systems with potential therapeutic application

Project number PN-II-ID-PCE-2012-4-0133, contract 58/02.09.2013

Intermediate report for the period: September 2nd 2013 to December 31st 2014

Requirements:

1) Vector conception and production
2) Conception of nanovectors (pH sensitive not HIF sensitive)
3) MSC preparation and loading/drug testing
4) Additional; studies
Stem cell preparation:

Stem cells were isolated characterised and cultured throughout the study as described within [http://www.ncbi.nlm.nih.gov/pubmed/25084218](http://www.ncbi.nlm.nih.gov/pubmed/25084218) and associated references.

Drug-loading of MSCs: Here we have characterised uptake and release of a) a potent anti-inflammatory drug IL-1R-alpha-antagonist; and b) biotinylated CIP. So in our combinational therapeutic approach directed to hyper-acute phase we have:

inhibition of inflammation + neuroprotection

The standard uptake of IL1Ra looks to be a passive process, so we tried a time course. We saw that cells were capable to load half a nanogram (14000 cells, that would be around 35fgram/cell) in one hour. 90% of this amount is loaded in the first five minutes. See images below.

We tried to permeabilize the cell before the treatment, trying to increase the amount of IL1Ra uptake. We have tried different concentrations of triton (0.1nm-10nM), at different times (10-30-60min), just before adding the IL1Ra for two hours (image LDH triton). IL-1-R –antagonist ELISAS demonstrated a significantly enhanced uptake of the molecule.

**Standard hu IL-1ra and Kineret curve**  
(DuoSet ELISA kit, exp n°1)

- Standard hu IL-1ra dilutions in BSA 1%  
- Kineret dilutions in culture medium (DMEM+10%FBS)
Kineret uptake release with human BM-MSCs
(DuoSet ELISA kit, exp n°1)

[Graph showing CM 48h BM-MSCs/Kineret values]

<table>
<thead>
<tr>
<th>CM 48h dilution</th>
<th>Log 10 CM dilution</th>
<th>Log 10 (OD pm)</th>
<th>Kineret curve</th>
<th>Kineret (pg/ml)</th>
<th>Kineret (ng/ml)</th>
<th>Kineret (ng/ml/mm²)</th>
<th>Mean of linear slope ± s.d.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/2</td>
<td>0.36</td>
<td>0.61</td>
<td>0.83</td>
<td>18.25</td>
<td>26.20</td>
<td>10.80</td>
<td>0.22 ± 0.51</td>
</tr>
<tr>
<td>1/4</td>
<td>-</td>
<td>0.56</td>
<td>0.80</td>
<td>17.00</td>
<td>26.20</td>
<td>10.50</td>
<td>0.29 ± 0.55</td>
</tr>
<tr>
<td>1/8</td>
<td>-</td>
<td>0.56</td>
<td>0.80</td>
<td>17.00</td>
<td>26.20</td>
<td>10.50</td>
<td>0.29 ± 0.55</td>
</tr>
<tr>
<td>1</td>
<td>-</td>
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<td>17.00</td>
<td>26.20</td>
<td>10.50</td>
<td>0.29 ± 0.55</td>
</tr>
</tbody>
</table>

Mean of linear slope ± s.d.

Standard hu IL-1ra and Kineret curve
(DuoSet ELISA kit, exp n°2)

[Graphs showing Standard hu IL-1ra and Kineret curves]

- Standard hu IL-1ra dilutions in BSA 1%
- Kineret dilutions in culture medium (DMEM+10% FBS)
Methodology for the above experiments was as follows:

BM-MSCs: human bone marrow mesenchymal stromal cells

hSDFs: human skin derived fibroblast (also tested in parallel)

Kin: Kineret®

O.D.: Optical density

CM= conditioned media

**Kineret priming of hBM-MSCs and hSDFs**

Sub confluent culture of BM- MSCs or hSDFs (4*10^5 cells/flask 25 cm^2 ; 7.35 *10^5 cells/flask 25 cm^2 respectively) were exposed to 200 µg/ml Kineret and after 24 hours the cells were washed twice with PBS and seeded in a new flask. After 48 hours of culture the cell conditioned media (CM, 4 ml/flask) were collected and test with Duo Set ELISA Kit in 96-well microplate. CM from untreated cells were used as negative controls. hSDFs were also detached and sonicated (Labsonic U Apparatus, Braun). The cell lysate was centrifuged (2500 x g, 10 min) to remove the debris and is resuspended in complete medium for further experiments.

A standard IL-1ra curve in BSA 1% from 2500 to 39 pg/ml and a Kineret curve in culture medium (dmem+10% FBS) from 5000 to 78 pg/ml were also made. A well with BSA 1% and a well with culture medium respectively were used as negative controls. The O.D. of each well were established using a microplate reader set to 450 nm and 545 nm. The readings at 545 nm were subtracted from which of 450 nm as indicated in protocol, and the relatives blanks were also subtracted.

The release of Kineret from BM-MSCs in CM was evaluated through the straight line equation: y= 1.26x-1.02 of Kineret curve (exp n°1).
The release of Kineret from hSDFs in CM and lysate was evaluated through the straight line equation \( y = 0.96x - 0.17 \) of Kineret curve (exp n°2).

**Second series experiments:**

**Preliminary results on ILR1a experiments**

**Experiment 1: can we load MSC with ILR1a just by adding it to the media?**

In the first experiment, cells were treated with increasing amounts of kineret for 24h, and after that time the media was changed by new fresh media. After 24h, supernatants and cell lysates were collected to further analysis.  

![Figure 1. ELISA results from supernatants and lysates collected at 48h (24h of treatment + 24h fresh medium).](image)

ELISAs to detect ILR1a in our samples were performed, and they show a trend to uptake more ILR1a if it is present in the media. It looks that despite ILR1a is secreted, it can be also stored in the cytoplasm (lysate content). We also tried to increase the secretion time from 24h to 48h, and we didn’t see in increase in the amount released/stored. These data suggest that uptake might be a passive process.
*NOTE: in these first experiments we were using all the components that came with the ELISA kit, also the standards. After having some problems with them we started using a kineret standard in all our experiments, and the concentrations associated to each optical density are increased. This could lead to big changes in the final concentrations, since we were probably underestimating the amount of kineret in the first experiments.

Experiment 2: Confirm that the uptake and release are passive processes.

To address this question, we incubated MSC cells with ILR1a (50ng/ml), for different periods of time (5min-24h). Most of the ILR1a is loaded into the cells in the first 5 minutes. After 30 minutes of incubation, there is no difference between the different times. In one hour, 14000 cells can uptake around half a nanogram, which would correspond to 35-40fg/cell.

NOTE: using our own kineret standard, the amount of ILR1a in untreated cells is increased 1500 times! The real value should be confirmed for other more quantitative methods.

This resulted in an uptake-release of the equivalent of 2-5μg/1,000,000 cells which could have therapeutic potential.

![Figure 2: amount of ILR1a in the lysates, collected just after finishing the treatment with ILR1a.](image)

Experiment 3: increasing the permeability of cells (ongoing).

Since time and concentration variations were not inducing a big increase in the uptake, we tried to increase the amount of ILR1a that can be uptaken by increasing the permeability of cell membranes. We used increasing concentrations of Triton, applied during different times, just before adding the treatment with kineret (50ug/ml, 2hours).

We chose triton concentrations ranging from 0.1nM to 10nM, applied for 10, 30 and 60 minutes, just before adding the IL1Ra. These experiments are still ongoing, but we can say that any of the times and concentrations are toxic for the cells, which was our main concern (measured by LDH release). This cell death measurement was performed just after the triton treatment and after 24h with fresh media, just in case the cell death was a delayed process.
CONCLUSIONS:

- MSC can uptake a certain amount of ILR1a when it is added to the media. Nevertheless, the amount uploaded is small considering the amount added externally: treatments are in ug/ml, while uptake needs to be measured in pg/ml, and individual content are in the range of femtograms.

- The mean amount they can load is around half a nanogram (50pg/cell). Despite most of the ILR1a is secreted, some is still stored inside the cells, so new strategies need to be design to force this secretion.

LDH result above shows no significant cellular cytotoxicity from the process.

In addition, we have primed (activated) the cells to secrete anti-inflammatory cytokines. MSCs stimulated by hypoxia were shown to increase secretion of anti-inflammatory interleukins several fold. This work requires optimisation and work-up in consideration of it as an avenue to ‘prime’ cells prior to delivery in vitro (2015 work).

Small molecule inhibitor as an IL-1R-alpha antagonist: (work to be carried out during 2015)
There is a paucity of literature describing the design and evaluation of non-peptidic small molecule inhibitors of IL-1β. Nevertheless, a single report detailing aromatic-based scaffolds with equipotent IL-1β and IL-1β activity does provide a molecular blueprint for the initiation of studies directed towards the design of novel interleukin-1 receptor antagonists. These studies reveal that molecules based on a trisubstituted benzene core, specifically N,N-disubstituted amides with hydrophobic substituents 1 are active at <10 μM. These molecules do have the drawback of high cLogP which may make them promiscuous against other targets.

![Chemical structure of lead 1](image)

It is proposed to prepare a series of compounds resembling lead 1 and also pursue the synthesis of molecules with less planarity and conservative chemical space. A series of molecules that have developed synthetic routes satisfy these criteria can be rapidly evaluated, in silico, using a computer model under development at MMU. Those earmarked can then be prepared for biological evaluation.

For instance, analogues with a cyclohexane-based core 2, derived from alkenes will be readily available by Diels-Alder cycloaddition of alkenes 4 with 1,3-dienes 5.

Furthermore, simple changes around the CONR2 group in 1 may also afford novel molecules with similar bioactivity profiles.
We also plan to investigate a series of isoquinolines 2 - mimetics of the phenylalanine-type core found in compounds 1. These are readily available via Pictet-Spengler reaction of phenylalanine derivatives 7.

\[
\begin{align*}
\text{6} & \quad \text{Pictet-Spengler} \quad \text{7} \\
\text{O} & \quad \text{H} & \quad \text{NH}_2 \\
\text{R}_1 & \quad \text{O} & \quad \text{O} & \quad \text{R}_2 \\
\end{align*}
\]

Work Plan (2015)

1. Develop an in-silico model (at MMU, in house development underway)
2. Test putative compound structures 1, 2, 6 against in silico model.
3. Synthesise compounds highlighted by modelling process.
4. Source potential summer students and incentivisation/pump prime funding for preparation of compounds highlighted in stage 2.
MSC-pH sensitive production.

Briefly, Cells (20,000) were allowed to adhere to micro wells (n=4). Peptide was added and incubated for 30 minutes. The wells were then washed 3x with PBS and buffer added, along with 10μl of Calcein liposomes. The plate was then incubated for a further 30 minutes. Then fluorescence intensity read (Ex 485nm Em 520nm).

The data seems to suggest, that stem cells can be sensitised with a pore forming peptide. This peptide can then rupture liposomes while associated with the stem cells. However, in this preliminary the rupture of liposomes is not highly dependent on pH. It is encouraging to note that such stem cells may have the potential to be developed into bio-responsive delivery vehicles. However the risk usually is whether the cells remain viable once associated with the bio-responsive peptide. This was tested by a viability assay. Data shown below.
The stem cells remain viable when associated with the pore forming peptide. Taken all the data together, it may be possible to release drug payload from liposomes or drug loaded stem cells as a delivery vehicle.

Conclusions: We are able to show increased tendency of the stem cells to move towards hypoxic-acidic environments by incorporation of calcein liposomes. Within 2015 we will investigate this further to optimise potential targeting towards stroked tissue.
Biotinylation of p5 from CIP-performed and produced by 21st Century BIO Pharma.

**CIP-p5 peptide loading:** 1-5μg/ml of peptide was incubated with cells according to the protocol below:

hMSCs (3×10^5) were treated 24 hours with p5. At the end of the incubation, the cells were washed twice with PBS, then trypsinized, washed twice in HBSS and seeded in a new flask. After 24 h of culture, the cell conditioned medium (CM) was collected and replaced by repeating this procedure at 48, 72, 96 and 144 hours. The CM was tested for its p5 content and associated activity in vitro by using CM from untreated MSCs as negative controls. The passive membrane drug adsorption and release by fixed cells were also verified.

**Cell Culture** has been used to create viable stores of Human Mesenchymal Stem Cells (hMSCs) for all experiments. Basic cell culture protocol was used (provided by Ria Weston).

**Quantitation and Optimisation of Drug Uptake and Release (p5),** was initially conducted using the HABA reagent. This however was unsuccessful as the colour metric results were affected by the phenol red colour of the stem cell media. A biotin quantitation kit was then purchased from Pierce (Thermo Scientific), which provided an accurate standard curve using fluorescence, from which biotin/P5 concentrations could be accurately derived. This initial work was all undertaken with known concentrations of P5 and conducted in accordance with the protocols provided by Thermo Scientific.

The next stage was to conduct the quantitation of P5 uptake and release, using the in vitro model shown in Pessina, A et al. 2011. HMSCs (2.8 x10^6) were treated for 24 hours with P5 in two separate doses of 100 μg/ml and 10 μg/ml. At the end of the incubation period 100 μl of each loaded media was taken and the cells were washed 3 times in PBS, trypsinized and seeded into a new flask. After a further 24 hours of culture, the conditioned medium was collected and replaced. This was then repeated again at 48 hours.

**Pilot results**
Table 1. Biotin Fluorescent Standard Curve Optimisation

<table>
<thead>
<tr>
<th>Provided Standards (pmol/10µl)</th>
<th>Emission</th>
<th>P5 Biotin Standards (mg/ml)</th>
<th>Emission</th>
</tr>
</thead>
<tbody>
<tr>
<td>A 100</td>
<td>108</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B 80</td>
<td>66</td>
<td>2000</td>
<td>149</td>
</tr>
<tr>
<td>C 60</td>
<td>62</td>
<td>1600</td>
<td>98</td>
</tr>
<tr>
<td>D 40</td>
<td>52</td>
<td>1200</td>
<td>338</td>
</tr>
<tr>
<td>E 20</td>
<td>47</td>
<td>800</td>
<td>143</td>
</tr>
<tr>
<td>F 10</td>
<td>35</td>
<td>400</td>
<td>94</td>
</tr>
<tr>
<td>G 5</td>
<td>43</td>
<td>200</td>
<td>70</td>
</tr>
<tr>
<td>H BLANK</td>
<td>87</td>
<td>BLANK</td>
<td>47</td>
</tr>
</tbody>
</table>

Table 1 shows the initial optimisation of the standard curve, conducted with both the Pierce Kit provided standards and P5 Biotin standards, made up in human mesenchymal stem cell basal media (HMSCBM).

Table 2. Biotin Uptake and Release in vitro

<table>
<thead>
<tr>
<th>Dose (µg/ml)</th>
<th>HMSCBM Only</th>
<th>Conditioned Medium at 24 hours</th>
<th>Conditioned Medium at 48 hours</th>
<th>Conditioned Medium at 72 hours</th>
<th>P5 Positive Control in HMSCBM</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>267</td>
<td>252</td>
<td>268</td>
<td>268</td>
<td>N/A</td>
</tr>
<tr>
<td>100</td>
<td>N/A</td>
<td>237</td>
<td>243</td>
<td>238</td>
<td>308</td>
</tr>
</tbody>
</table>

Table 2 shows the quantitation results of uptake and release of P5 by hMSCs with a 72 hour period. The results however were inconclusive.

Table 3. Further Optimisation of Assay Kit

<table>
<thead>
<tr>
<th>Biotin (µg/ml)</th>
<th>Biotin Emission</th>
<th>DMEM</th>
<th>P5 (100 µg/ml)</th>
<th>DMEM Only</th>
<th>HMSCBM Only</th>
</tr>
</thead>
<tbody>
<tr>
<td>A 1000</td>
<td>2777</td>
<td>441</td>
<td>1005</td>
<td>11</td>
<td>1</td>
</tr>
<tr>
<td>B 800</td>
<td>2526</td>
<td>6</td>
<td>4</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>C 400</td>
<td>2527</td>
<td>6</td>
<td>2</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>D 200</td>
<td>2212</td>
<td>6</td>
<td>2</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

Table 3. Lanes 1 and 2 show the emissions of just biotin at known concentrations. Lane 3 is blank.
Lane 4 shows standard DMEM with the reagent to investigate the background florescence of the naturally occurring biotin in the media. Lane 5 shows the P5 (biotin) protein at a known concentration. Lanes 6 and 7 show DMEM and HMSCBM with no reagent as a negative control for the cell media.

Estimated uptake per 1,000,000 cells is 205 micrograms and this is being used in the in vivo pilot MCAO experiments (determined by ELISA/Mass spectrometry) data not shown here.
CDK5 vector gene transfection studies: see also appendix 1 for details

Based on mapping of the amino-acid sequence of p35 protein through CDK5-inhibitory peptide (CIP), the peptide sequence was identified as shown below.

A

Mapping of p25, p16 and CIP to p35 (human).

p25

1  NTVL6LPS YRKA1FLFDQ AATGQHTV AQQEKNKDD EKRS1ISVL
51  NMRIVAVSA EKKNNKEAY QNYQNL1T0LE LK1BNKRS1L SCAN5STFAP
101  PPPAQPPAPP ASQGSGTQG GSSVOKPARK PAVTSAGTPK KVIVQASTSHE
151  LLRCLOQFCL RCYRKLHLS VTDPPV1MLRS VDR2LLQGQ QOQFITDPAN
201  VVFLNLCRD VISSEVOSDCL ELOAVLTTCL YLSYSMHE ISTYKPLFLV
251  ESCKEAFMR CLSVINLAV EMLQINADPK YFTQVPSDLK NESQKEDEKR
301  LLLG1DR

p16

CIP

p35

1

154

279

307

Peptide (CIP)

154

279

B

C

![Graph showing Cdk5 activity (normalized) vs. CIP concentration (µM)]
Figure 1. Identification of the Cdk5/p25 inhibitory peptide (CIP) derived from p35. (A) Mapping of p25, p16 and CIP to p35 (human sequence). Red segments are the alpha helices in p25 as determined by Tarricone et al. [21]. The sequences comprising p25, p16 and CIP are indicated by the labelled arrows. (B) A combination of N-terminal and C-terminal truncations of p35 produces a nonactivating fragment (154–279, CIP) that inhibits Cdk5 activity and binds with high affinity. (C) Inhibition of Cdk5 activity by CIP. Cdk5 kinase activity was determined by preincubating various amount of CIP with Cdk5/p25 for 2 h at 30 °C followed by incubation in the kinase reaction for an additional hour in the presence of [γ-32P]ATP and histone H1 (protocol can be included if required).

Cloning of vector:

Step 1

PCR was carried out on the CIP gene resulting in production of different products, depending on primers used. Electrophoresis of products from different combinations of primers are shown below: DNA was cut from the gels, extracted and the following elements incorporated into the CIP vector:

- L1 A + pHSV IRES1 GFP
- L2 B + pHSV IRES1 GFP
- L3 C + pHSVeGFP
- L4 D + pHSVeGFP
- L5 pHSV IRES1 GFP
- L6 pHSVeGFP

L5, L6: Negative controls

1. Calculate Insert and Vector amounts

- Ratio insert:vector = 3:1 > calculate after formula

\[
X = \text{Insert (ng)} = \frac{\text{Vector (ng)} \times \text{Insert (bp)}}{\text{Vector (bp)}} \times \text{ratio insert:vector}
\]

\[
\text{Insert (µl)} = \frac{X}{\text{insert (ng/µl)}} \times Y (\text{Vector(µl)})
\]

pHSV IRES1-GFP (B) and pHSVeGFP were purified and cultured up (ecoli transfection) to produce stocks of the viral peptide-containing vector. This is now ready for incorporation into our in vitro and in vivo model studies to be carried out during 2015.
In vitro studies will: confirm CIP expression and provide physiological assays for CIP functions. E.g. by exposing cells to calpain/hypoxia, normally we get cleavage of p35 protein to p25 so we can measure CIP bound to p25 this will indicate the potency of inhibition provided by CIP (should not bind to p35)- also the same CDK5 cell free assay using p25 and cell culture medium from infected cells can be used to identify release concentrations (GFP fluorescence).

The 4 different HSV-1 amplicon vectors (see appendix 1 for details):
Primer 1 (fw) + Primer 2 (rev)  > product A (for CIP-NLS-IRES-GFP)
Primer 3 (fw) + Primer 2 (rev)  > product B (for CIP-GFP-IRES-GFP)
Primer 1 (fw) + Primer 4 (rev)  > product C (for CIP-NLS-GFP)
Primer 3 (fw) + Primer 4 (rev)  > product D (for CIP-GFP)

Summary: We now have stocks of CIP as a fusion protein with GFP and CIP alone with and without a nuclear localization signal. The vectors that express CIP separately express also GFP, but not as a fusion with CIP.
**Magnetised targeted therapy:** We have the VSIONps and we have partially characterized their uptake by MSCs and are in the process of investigating their paramagnetic properties.


Imaging of VSOP labeled stem cells in agarose phantoms with susceptibility weighted and T2* weighted MR Imaging at 3T: determination of the detection limit.

Lobsien D1, Dreyer AY, Stroh A, Boltze J, Hoffmann KT.

Using this pilot data we will be able to evaluate the detectability of stem cells labeled with very small iron oxide particles (VSOP) at 3T with susceptibility weighted (SWI) and T2* weighted imaging as a methodological basis for subsequent examinations in small/large animal stroke model (sheep).

500 VSOP labelled stem cells can be detected with SWI imaging at 3 Tesla using an experimental design suitable for large animal models.

**Our work during 2015 will:** detail the magnetic capability to focus nanoparticle containing MSCs within matrigel 3D complex in vitro and identify any issues relating to co-labelling with our pharmaceutical drugs.

- attached cells
- cytospins

Above: Prussian blue staining of attached stem cells labelled with 3mM SPIO by following stereotactic transplantation
Above: MRI imaging showing localised and targeted transplanted magnetic MSCs and below, magnetised MSCs localised within medium sized blood vessel.
**CDK5 further characterization studies:** It is important to fully characterise the mechanisms and activity of the CIP/p5 peptides to ensure optimal effectiveness as a therapeutic after stroke. Below, we demonstrated that CIP-released from transfected brain endothelial cells reduced the effects of hypoxia by reducing HSP70 expression and activation of caspase-3 a marker of cell apoptosis.

The relative expression of hsp70 in EC and CIP under normoxic and hypoxic condition

![Graph showing relative expression of HSP70](image)

The result shown in Fig 3.2 indicated that the expressions of HSF70 in ECs and CIP under normoxic and hypoxic conditions. The graph represented the average of three experiments.

Expression of caspase3 in ECs and CIP under normoxic and hypoxic conditions

In control cells incubated for 24 hours in hypoxia, the expression of caspase3 was significantly higher (2.8 fold) compared to control cells in normal conditions. In CIP treated cells, the level of caspase3 expression was significantly higher (2.2 fold) when exposed to 24 hours hypoxia conditions compared to CIP treated cells in normal conditions.
MCAO ongoing studies: Study 1 is in process now and results will be analysed over the next 6 weeks.

3 experiment in progress: data being generated over November-December for protocol-pilot study (can be supplied in 1 month)

Drugs for stem cell loading:

Neuroprotective drugs:


In addition BOTH molecules are strongly pro-angiogenic but do not induce vascular leakage so we have DUAL action!

Anti-Inflammation drugs: Inflammation is the other key event in the acute and hyper-acute phase and here we have

1) IL-1R-alpha (primary)


Single and cocktail mixtures of drugs will be tested during 2015-2016-within 1-4 h of MCAO

1) MCAO model in progress now (2014)

Transient (temporary) Stroke will be induced by the hook method. The right middle cerebral artery (MCAO) was slowly lifted with a tungsten hook attached to a micromanipulator (Maerzhaeuser Precision Micro-manipulator Systems, Fine Science Tools) and electrocoagulated. Both common carotid arteries were then occluded by tightening pre-positioned thread loops for 90 min. Throughout surgery, anesthesia was maintained by spontaneous inhalation of 1-1.5% halothane in a mixture of 75% nitrous oxide and 25% oxygen. Body temperature was controlled at 37°C by a Homeothermic Blanket System (Harvard Apparatus). The local changes in blood flow were monitored using a laser Doppler device (Perimed, Stockholm, Sweden), and blood gases were measured at several time points
during ischemia. A decrease in laser Doppler signals to <20% of control values was considered to be successful MCA occlusion. After 90 minutes, the common carotid arteries were re-opened. Subsequent to survival times of 14 or 28 days, the rats were deeply anesthetized with 2.5% halothane in 75% nitrous oxide and 25% oxygen, and perfused with neutral buffered saline followed by buffered 4% freshly depolymerized paraformaldehyde. The brain was removed, post-fixed in 4% buffered paraformaldehyde for 24 hr., cryoprotected in 15% glycerol prepared in 10 mmol/l phosphate buffered saline, flash-frozen in isopentane and stored at -70°C until sectioning.

Transplantation:

For transplantation at 14 days after MCAO, animals will be anaesthetized with isoflurane and placed in a stereotaxic frame. Using a glass cannula attached to a Hamilton syringe, cell suspension (here we need details) will be injected (i) directly into the perilesional cortex (3 location = 200,000 cells/location); and for comparison, (ii) intrathecally. Control animals will receive just vehicle.

Below shows our pilot results implanting 1 million human MSCs into stroked aged rat brain tissue: This demonstrates a significant number of cells can be retained in the hypoxic brain regions after stroke following IV or intrathecal delivery.

Top left image, intravascular injection with TRITC red staining showing MSCs in peri-infarcted region; top right image, the intrathecally delivered samples double labelled with a rat neuronal marker (FITC-
green) and TRITC red. Bottom left image, iv-injected cells labelled with CD166 marker of human MSCs (FITC green) and bottom right-co-labelling with human neuronal marker and CD105.

3. Behaviour

Testing procedure involves two persons, one person who does the surgery and is in charge of handling the animals according to group assignment and another one who has tested the animals and was not aware of groups’ identity.

3a. Morris water maze

Day 1: Visible platform 5 trails (90 sec each)

Day 2-5: Hidden platform 5 trails

Day 6: Probe trail- 1 trail with no platform

The Morris water-maze task was used to assess spatial learning and memory. One week before surgery aged rats were trained to find a submerged platform in a large (180 cm-diameter) pool filled to within 20 cm of the upper edge with water maintained at 26°C. The pool was divided into four compass quadrants (north, south, east, and west). Several visual stimuli were placed in each of the four quadrants. For the acquisition of spatial learning, each animal underwent a block of four trials per day for seven days. Before the first trial, the rat was placed on the hidden platform for 30s by the investigator. Each trial consisted of placing the rat in the water at one of the randomly selected four starting locations around the pool perimeter. Each rat was allowed a maximum of 60s to find the hidden platform and remain on it for 30s. If a rat failed to find the platform within 60s, the rat was placed on the platform for 30s by the investigator. The time and distance required to find the hidden platform during these four acquisition trials were averaged. The swim path was recorded by an image analysis system (VideoMot2, TSE, Bad Homburg, Germany) that computed path length and percentage of time spent in each of 4 quadrants. Single path length and escape latency for each test subjects each day will be recorded. For day 6 — path length, escape latency and time spent in the platform quadrant will be recorded.

4. Rotating Pole

Will be performed weekly after surgery i.e on 7, 14, 21, 30 days. The beam-walking, or Rotarod, task assesses fine vestibulomotor function in the MCAO model. Each rat was tested for its ability to negotiate a rotating (6 rpm) horizontal rod. The time taken for the rat to traverse the rotating cylinder and join a group of rats visible at the finish line was measured. The score assessment was two-fold: (i) time (seconds) required to traverse the rotating cylinder and, (ii) the score as follows: 0, rat falls immediately (onto a soft surface); 1- rat does not walk forward, but stays on the Rotarod; 2 - rat walks, but falls before reaching the goal; 3 - rat traverses the rod successfully, but the limbs are used asymmetrically; 4 - the left hind-limb is used less than 50% of the time taken to traverse the rod; 5 - the rat successfully traverses the rod, but with some difficulties; 6 - no mistakes, symmetric movements. Results will be presented graphically by plotting Score vs Post-stroke time Limb-placing
Cylinder test: The cylinder test will be used to assess imbalance between impaired and non-impaired forelimbs before cortical photothrombosis and on post-operative day 14 and 28 (Mitkari et al., 2014). For the test, the rat is placed in a transparent cylinder (ø 20 cm). A mirror is placed at 45° angle beneath the cylinder so that their behaviors could be filmed from below the cylinder. Exploratory activity for 1 to 3 min is analyzed using a video recorder with slow motion capabilities. Number of contacts by both forelimb and by either impaired or non-impaired forelimb is counted. Cylinder score for impaired forelimb is calculated as: [(use of impaired forelimb+0.5×both forelimbs uses)/(total contacts)] x 100%.

5. Immunohistochemistry

Sections (25 µm-thick) were cut on a freezing microtome and processed for immunohistochemistry as previously described [3]. For DAB staining, sections were blocked in 3% donkey serum/10 mmol/l PBS/0.3% Tween 20, overnight at 4°C. Secondary biotinylated antibodies were raised in the donkey (Jackson ImmunoResearch Laboratories, West Grove, PA). Sections were stained using the ABC Elite reagents (Vectastain Elite Kit, Vector) using 0.025% 3’,3’ dianminobenzidine (DAB) and 0.005% hydrogen peroxide as the chromogen. For BrdU detection by dianminobenzidine-(DAB) staining, free-floating sections were pre-treated with 50% formamide, 0.3 M NaCl, 10 mM sodium citrate at 65°C for 2 h, incubated in 2 M HCl at 40°C for 1h, and rinsed in 0.1 M borate buffer (pH 8.5) at room temperature for 10 min. Sections were incubated with the mouse monoclonal anti-BrdU antibody (1:300, Roche, Mannheim, Germany) at 4°C for 24 hr. Secondary antibodies and DAB staining were performed as described above.

6. Determination of Infarct Volume

To assess the size of the infarct induced by permanent focal ischemia, every twentieth section was stained with NeuN, a marker of neuronal viability (see below). Images of the stained sections were taken to cover the entire infarcted area, which was then calculated as the sum of partial areas using the Scion image analysis software. Integration of the resulting partial volumes gave the total volume of the ipsilateral hemisphere along with the total volume of the infarct; lesion volume was then expressed as percent of the hemispheric volume.

2) Proposal for pilot experiments for Co[St]2 (2015 June)

Series 1: In vitro assessment of viability of SPIO-labeled drug-loaded MSC
Human drug-loaded (pro-angiogenic, anti-inflammatory) MSC and control-MSC will be labeled with 3.0 mM SPIO. Cell viability / proliferation capacity will be assessed 24h, 2d, 4d and 7d post labeling (n=3 independent experiments).

In case 3mM labeling decreases viability in drug-loaded versus control MSC, lower SPIO molarities will be tested, i.e. 2.0 mM, 1.0 mM and 0.5 mM will be tested. The best protocol will be selected for subsequent in vivo experiments.

Series 2: In vivo assessment of cell detectability, survival, and basic therapeutic impact

Experimental stroke will be induced in atherosclerotic mice by cortical photothrombosis (n=8). Lesion size and location will be assessed 24 hours and 3 days after stroke by 7T MRI (T2). Immunosuppression will be initiated by cyclosporine A treatment (10 mg/kg/d), starting right after photothrombosis.

1x10^5 drug- and SPIO-loaded MSC will be stereotactically transplanted through the lesion center 3 days after photothrombosis (n=6). T2* or SWI signal extinction will be used to detect local SPIO-concentrations 24h, 3d and 7d after MSC transplantation.

Primary endpoint: detection of T2* signal or SWI extinctions (yes/no). Potential discrimination from focal hemorrhages (blooming effect, i.e. signal extinction size etc.), n=6.

Animals will be sacrificed and brains will be harvested.

N=3 brains each will be selected for histological/immunohistochemical investigations or FACS-based description of cerebral cell populations. For FACS-based assessments, brains will be homogenized, depleted from myelin. Obtained cell suspensions will be analyzed.

Histology/immunohistochemistry (spatial information): Evidence for local MSC depots will be assessed by Prussian Blue staining and antibodies against human nuclei. Brains of untreated mice (n=2) will serve as negative control while internal validity will be assessed by omitting the primary antibody. Procedural endpoints comprise: exclusion of persistent focal hemorrhages, detection intracellular iron, attempt to assess MSC survival (and if so, numbers of surviving cells). Therapeutic endpoints comprise: vascular density.

FACS-based analysis of cerebral cell populations (non-spatial information): presence neutrophil granulocytes, monocytes, T-, B- and NK-cells, dendritic cells, and humans MSCs will be assessed by FACS and described quantitatively.

3) Cortical photothrombosis: (2015 January)

Cortical photothrombosis will be produced by using Rose Bengal (Zhao et al., 2005). Briefly, the rats will be anesthetized with 5% isoflurane in 30% O2/70% N2O and placed in a stereotaxic frame. The anesthesia is maintained through the operation with 1-2% isoflurane delivered by a nose mask, and the body temperature is maintained at 37 oC using a rectal probe and heating pad. The skull will be exposed and a cold white light (Olympus, Denmark) with a 4 mm aperture is positioned onto the skull.
0.5 mm anterior to the bregma and 3.7 mm lateral to the midline over the right motor cortex. The photochemical dye Rose Bengal (Sigma) will be infused into the femoral vein via a microinjection pump within 2 minutes (20 mg/kg), after which the light is turned on for 10 minutes. Skull surface temperature is monitored with a probe placed between the skull and the light source, and kept constant by cool air flow. Sham-operated animals will be treated similarly but the light is not in use. The rats are removed from the frame, sutured, and allowed to wake up in an incubator (32 °C) before being returned to their home cages. Size and location of lesion will be confirmed by MRI 24 h later.

Transplantation:

For transplantation 3 days after cortical photothrombosis, animals will be anaesthetized with isoflurane and placed in a stereotaxic frame. Using a glass cannula attached to a Hamilton syringe, cell suspension will be injected into the perilesional cortex (3 location = 200,000 cells/location) (Hicks et al., 2009). Vehicle animals will receive identical injections of PBS. All rats will receive the immunosuppressant cyclosporine A (5 mg ⁄kg, i.p.) starting a day prior to the transplantation and thereafter until the end of the 28 day follow-up.

Functional outcome measures:

Limb-placing test: The limb-placing test will be used to assess the sensorimotor integration of fore- and hindlimb responses to tactile and proprioceptive stimulation (Jolkkonen et al., 2000). The test has seven limb placing tasks, which are scored as follows: 2 points, the rat performs normally; 1 point, the rat performs with a delay (>2 s) and/or incompletely, and 0 point, the rat does not perform normally.

Cylinder test: The cylinder test will be used to assess imbalance between impaired and non-impaired forelimbs before cortical photothrombosis and on post-operative day 14 and 28 (Mitkari et al., 2014). For the test, the rat is placed in a transparent cylinder (ø 20 cm). A mirror is placed at 45o angle beneath the cylinder so that their behaviors could be filmed from below the cylinder. Exploratory activity for 1 to 3 min is analyzed using a video recorder with slow motion capabilities. Number of contacts by both forelimb and by either impaired or non-impaired forelimb is counted. Cylinder score for impaired forelimb is calculated as: [(use of impaired forelimb+0.5×both forelimbs uses)/(total contacts)] x 100%.

Beam-walking test: A beam-walking test will be used to assess deficits in hind/forelimb function before cortical photothrombosis and on post-operative day 14 and 28 (Zhao et al., 2005). The beam-walking apparatus consists of a narrow beam with a “crutch” ledge connected to a black box with a platform at the other end. The performance will be videotaped and later the use of the ledge by the impaired hind/forelimb will be quantified. A full error is stepping onto the ledge, and a half error is if the limb is on the side of the beam. Slip%= Limb errors/total steps X 100%.

Histology:
After follow-up, the rats will be perfused and frozen brain sections (35 μm) will be cut with a microtome. Every eight section will be stained for anti-human nuclei (MAB-1248) to assess cell survival (Hicks et al., 2009). Lesion size will be measured from Nissl-stained sections.

References:


Zhao C-s, Puurunen K, Schallert T, Sivenius J, Jolkkonen J. Effect of cholinergic medication, before and after focal photothrombotic ischemic cortical injury, on histological and functional outcome in aged and young adult rats. Behav Brain Res 156:85-94, 2005


Mitkari B, Nitzsche F, Kerkelä E, Kuptsova K, Huttunen J, Nystedt J, Korhonen M, Jolkkonen J. Human bone marrow mesenchymal cells produce efficient localization in the brain and enhanced angiogenesis after intra-arterial delivery in rats with cerebral ischemia, but this is not translated to behavioral recovery. Behav Brain Res 259: 50-9, 2014

Final summary:

We have completed objectives 1 2013 and activities 1.1 and 1.2

We have completed objectives 1 and 2 from 2014 incorporating activities 1.3, 2.1 (with limitations) and 2.2

In progress: we have not finalised nanoparticle-vector linkages at this point but anticipate completion of this by April 2015

However, we have also in addition:

Completed objective 2 from 2015 and activity 2.3 and 2.4 and are in the middle of testing for 3.1, 3.2 and 3.3

In addition, we have our first pilot in vivo test in progress as stated earlier (objective 4-2016). Hence we are overall AHEAD of schedule

Project Director
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