主論文の要旨

Dedifferentiated fat cells, as a novel source for cell therapy to target neonatal hypoxic ischemic encephalopathy

（新規細胞源として脱分化脂肪細胞を用いた
新生児低酸素性虚血性脳症に対しての幹細胞療法）

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Introduction

Neonatal hypoxic ischemic encephalopathy (HIE) is a major cause of mortality and morbidity during the perinatal period. With hypothermia being the only legitimate treatment option, scientific interest has shifted towards cell based therapy. Adipose tissue meets the criteria of an ideal stem cell source, such as abundance, easy accessibility and low immunogenicity. In particularly, mature adipocytes, being the predominant cell type in the adipose tissue, can be exploited with ceiling culture technique to differentiate into fibroblast like cells exhibiting multipotency and a strong proliferative potential, that have been named as dedifferentiated fat (DFAT) cells. Our main objective with the present study was to determine whether the outcome of HIE can be improved by DFAT cell treatment.

Materials and Methods

Hypoxic Ischemic insult and administration of treatment

The Rice-Vannucci adaptation of the Levine procedure was used to produce HIE in 7 days old Sprague Dawley (SD) rat pups. After an 1 h recovery period with the dam, the pups were induced to 8% hypoxia for 60 min. Following 18-20 h after reperfusion, diffusion weighted MRI was performed as a criterion of injury (Fig 1). At 24 h after the insult, the treatment and control groups received an intravenous injection of 10^5 cells/ pup diluted in 0.1ml of Ringer's bicarbonate solution or vehicle. Intraperitoneal injections of 5-bromo-2′-deoxyuridine (BrdU) were given daily on days 3,4 and 5 after the inflicted HI injury. Animals were sacrificed on postnatal day 9 or 50, with the purpose of studying both the acute and chronic effects of DFAT cell treatment (Fig 2).

Immunohistochemistry

Paraffin coronal sections of the hippocampus of animals sacrificed on P9, were stained for the active caspase-3, ED-1 and 4-hydroxyl-2-nonenal (4-HNE) antibodies in order to quantify neuronal apoptosis, microglial activation and lipid peroxidation respectively. For cell proliferation and survival analysis, anti-BrdU immunostaining was performed on sections of the hippocampus of animals sacrificed on P50. Cells positive for active caspase-3, ED-1 and 4HNE were counted using Stereo Investigator version 10 stereology software throughout the hippocampus as well as in two square fields in the parietal and temporal cortex. In a similar manner, BrdU-labeled cells were counted in the granular cell layer and subgranular zone of the contralateral dentate gyrus.

Behavioral tests

From the 21st day of age, animals were subjected to behavioral tests that evaluate both the motor and cognitive functions as follows: i) the Rotarod treadmill, with which we measured the length of time an animal could remain on an accelerating rotating rod as a measurement of motor learning and coordination, ii) the cylinder test, which assesses forelimb use preference, while animals are rearing against the wall of a transparent cylinder and recorded from above, and

finally iii) the novel object recognition test (NOR), a working memory test, where the time spent exploring a familiar and novel object is recorded and a discrimination index is calculated.

Brain volume measurement

To compare the loss of tissue between the vehicle and DFAT groups in the chronic stage of damage, every 100th section of the brain of animals sacrificed at P50, was stained with a marker for microtubule associated protein 2 (MAP-2). The stained areas were outlined and measured with the Stereo Investigator software and brain volume was determined using the Cavalieri principle.

Excitotoxicity experiments

Primary cortical neuron cultures were obtained from the brain of SD rat embryos at embryonic day 16 (E16). DFAT conditioned medium (DFAT-CM) was collected from DFAT cell cultures at 80% confluency, rinsed and replenished with neurobasal medium supplemented appropriately for usage in the cortical neuron cultures. At 8 days in vitro (div) excitotoxic damage was induced to the neurons by oxygen-glucose deprivation (OGD). To investigate the potential neuroprotective effects of DFAT-CM, it was added to the neuronal cultures at various concentrations (2.5, 5, 7.5 and 10%) at 48 h and 24 h prior to OGD as well as immediately post-OGD. Controls received normal culture medium (CM-). Neuronal death was quantitatively calculated at 24 h after OGD, by measuring the absorbance of the released lactate dehydrogenase (LDH).

Enzyme linked immunosorbent assay (ELISA)

Brain homogenates from pups injected with DFAT or vehicle and aliquots from DFAT-CM or neuronal culture medium (CM-) were analyzed with ELISA for nerve growth factor (NGF), neurotrophin-3 (NT-3), brain derived neurotrophic factor (BDNF), glial derived neurotrophic factor (GDNF) and insulin-like growth factor-1 (IGF-1).

Results

Effects of DFAT treatment on the expression of acute injury markers

Photomicrographs of the representative hippocampi are shown in Fig. 3. Apoptotic cells at 48 h after the HI injury were visualized by the immunostaining of active caspase-3 and quantitative analysis indicated lower apoptotic activity in the hippocampus and temporal cortex of the DFAT group than that in the vehicle group (Fig. 4a; 193.9 ± 124.0 cells/mm^2 *versus* 729.3 ± 192.0 cells/mm^2; $p < 0.05$ and Fig. 4b; 0.50 ± 0.17 cells/mm^2 *versus* 1.03 ± 0.18 cells/mm^2; $p < 0.05$ respectively). Additionally, the number of activated microglia (i.e. ED-1 positive cells) was significantly lower (vehicle *versus* DFAT; $p < 0.05$) in the counted hippocampal and temporal-cortical areas (Fig. 4d and Fig. 4e; 66% and 44% lower, respectively) in the DFAT treated animals. The immunoreactivity of 4-HNE increased in the brains of the vehicle-treated pups with significant reductions of 68% and 50% in the hippocampus and parietal cortex, respectively (Fig. 4g and Fig. 4i; $p < 0.05$).

Effects of HI injury and DFAT treatment on the sensorimotor and cognitive functions

The rotarod performance, tested on P25, showed that the motor function was significantly impaired in the vehicle-treated animals as compared with the sham operated group (Fig. 5a; 117.0 ± 9.2 s *versus* 170.0 ± 12.8 s; $p < 0.01$), whereas there was no difference between the DFAT and sham groups. Similarly, in the cylinder rearing test performed on P42 and P43, there was a significant difference between the vehicle-treated and sham operated animals (Fig. 5b; 17.45% ± 3.19% *versus* 3.14% ± 2.845; $p < 0.01$) which manifested as a marked increase in the use of the left (ipsilateral) forepaw in the vehicle group. A significant preference for the left forepaw was not evident in the DFAT-treated rats as compared with the sham group. Working memory, examined with the NOR test seemed to be unaffected by the HI injury in our model, as all groups were able to distinguish the memorized and novel objects (Fig. 5c).

Effect of DFAT treatment on proliferation

Cells positive for BrdU were counted in the granular and subgranular layer of the contralateral dentate gyrus without any detected significant difference between the vehicle and DFAT groups, reflecting a lack of efficacy by the DFAT treatment in promoting cell proliferation (Fig. 6).

Volumetric analysis

Sections throughout the whole cerebrum of pups sacrificed on P50 were stained with MAP2 and evaluated with the Stereo Investigator version 10 stereology software. At the time point of sacrifice, there were no significant differences between the two groups in terms of preservation of brain volume (Fig. 7).

Reduction of OGD-induced cell death by DFAT-CM

OGD experiments were performed to replicate the pathophysiological conditions of HI *in vitro.* When DFAT-CM was added to neuron cultures at 48 h prior to OGD, there were significant reductions in LDH release of 27%–49% at all the concentrations tested (2.5%–10%) (Fig. 8a; $p < 0.001$). The addition of DFAT-CM at concentrations of 5%–10% only 24 h prior to OGD reduced the LDH release by 30%–45% (Fig. 8b; $p < 0.01$ for CM 5%; $p < 0.001$ for CM 7.5 and CM 10%). Addition of DFAT-CM to the cultures immediately after the end of OGD did not confer any protective effects (Fig. 8c).

Effects of DFAT treatment on the expression and secretion of IGF-1 and NGF in cerebral cortex

Administration of DFAT cells intravenously resulted in increased levels of IGF-1 and NGF ($p < 0.05$) in the ipsilateral to the lesion cortex in comparison with the contralateral, as was detected by ELISA.

Neurotrophic factors in DFAT conditioned medium

To find out which factors are responsible for the neuroprotective effect of DFAT-CM, we examined its active components, which may exert a trophic influence on cortical neurons. ELISA confirmed the presence of IGF-1 (Fig. 8d; $p < 0.001$), NGF (Fig. 8e; $p < 0.001$) and NT3 (Fig. 8f; $p < 0.05$), which were not detected or were at borderline detection levels in the normal culture medium we used as control.

Discussion

In the present study, we showed the possibility that an intravenous injection of DFAT cells after HI facilitated functional recovery by interrupting endogenous apoptotic mechanisms and reducing inflammation and we obtained evidence that DFAT-CM is neuroprotective against OGD at a range of concentrations *in vitro.*

The present study made three main findings. First, we detected decreases in the immunoreactivity of the acute injury markers, involved in apoptosis, activation of microglia and lipid peroxidation, ultimately interrupting the acute response after the HI insult. Second, there was a tendency for improved motor function that persisted for at least 6 weeks after the HI event, as was confirmed by the rotarod and cylinder test performances. Finally, we showed that the soluble products of cultured DFAT cells, requiring no direct cell-to-cell contact, can confer protection to neurons exposed to excitotoxic injury and we further identified IGF-1, NGF and NT3 in DFAT-CM. These findings support the hypothesis that the main effect of the cells is trophic and does not require differentiation or migration to the injury site.

There were several limitations concerning the results of the present study. A major drawback was that we could not provide clear evidence for the long-term behavioral benefit of DFAT cell treatment, difficulty that can be attributed to the high inter-animal variability associated with the Rice-Vannucci model. It is also possible that extension of the study period to longer than 43 days, might have yielded more definite results. Another limitation could be that there were no memory deficits in the NOR test, which can be explained by the fact that rodents exhibit lateralization of their cognitive functions, with right hemisphere lesions leading to memory dysfunction where left hemisphere lesions do not. Furthermore, while DFAT treatment *in vivo* is added after HI, we were not able to demonstrate a treatment effect with the addition of DFAT-CM post-OGD *in vitro.* Differences in the time course of injury between the two models can be incriminated for the failure to replicate the actual response *in vitro.* Finally, DFAT cell treatment failed to reduce the infarct size in the chronic stage of damage.

In conclusion, DFAT cells provide direct (acute phase) and indirect (chronic phase, *in vitro*) neurological benefits in neonatal HIE, and the major mechanism underlying their treatment effect is the secretion of neurotrophic/ neuroprotective factors.