Scientific Report Dr. Martina Monti – half period.

Role of TAT-BH4 in preventing apoptosis of neuronal and endothelial cells after spinal cord injury

Background: The prevention of neuronal and vascular cell death allows damage reduction and tissue recovery from oxidative stress injury, that plays an important role in many chronic CNS diseases, such as Alzheimer and Parkinson's diseases, traumatic brain injury, as well as spinal cord injury (SCI).

SCI is characterized by apoptotic cell death and inflammation, and by a protracted neuronal loss driven by changes in oxygen, glucose, neuroactive lipids, and eicosanoid production and through the release of free radicals, biogenic amines, endogenous opioids, and excitatory amino acids (Yip PK 2012). Several molecular pathways become activated during the first 48 h after SCI. Apoptotic and proinflammatory responses are likely to be detrimental to cell survival, while other more protracted growth signals facilitate tissue repair (Malaspina A 2008).

Apoptosis is a finely regulated process activated by tissue injury and ischemia. Among the proteins involved in this process, the Bcl-2 family members include both pro- and anti-apoptotic proteins. The proteins of this family are characterized by four different homology domains, of which only BH4 (Bcl-2 homology domain 4) has anti-apoptotic properties (Sugioka R 2003).

BH4 domain of the anti-apoptotic protein, Bcl-xL, attached to TAT, a membrane transport peptide (TAT-BH4, a 20 aminoacid peptide), protects endothelial cells exposed to oxidative stress from cell death by inhibiting caspase activation (Cantara S 2004) and triggering a survival program, Akt/eNOS dependent (Cantara S 2007). Endothelium and neurons are close related and their relationship is fundamental to recover cerebral damaged tissue. Previously, I contributed to demonstrate that TAT-BH4 administration protects against acute hypoxia/ischemia injury in the brain by preventing endothelial and neuron cell apoptosis and by inducing neuronal plasticity (Donnini S 2009).

AIM: the project aims to investigate the protective role of TAT-BH4 in SCI, with particular attention to damage reduction in the vasculature which may contribute to recover spinal and motor functions. The project has been divided in two task: 1. in vitro studies to investigate whether TAT-BH4 exerts a protective effect on neurons and endothelial cells; 2. in an in vivo model of SCI, in collaboration with Prof. S. Cuzzocrea, to evaluate the protective effect of TAT-BH4.

Methods

Animals

The experiments were done on adult male C57BL76, E16/6 mice (6 weeks old). All procedures were carried out in accordance with the Italian law (Legislative Decree no.26, 4 March 2014) and the European Directive 2010/63/UE.

Isolation of murine cortical cells

Primary murine cortical cells (CNs) were isolated as described by Messer with some modifications (Messer A 1981). Briefly, CNs were obtained from cortex of mice embryos (C57BL76, E16). Cortex isolation was carried out in cold HIB solution (120 mM NaCl, 25 mM Hepes, 5 mM KCl, 9.1 mM D-(+)-glucose, pH 7,4). Cells were trypsinized and after centrifugation, the pellet was re-suspended in Neuronal Basal Medium (NBM), (containing 10% FCS and 2% B27). After filtration with a 100 µm filter, viable cells were counted using trypan blue exclusion technique and plated at density of $8x10^5$/ml on polylisine (250 µg/ml) coated wells. After 3 days, 50% of the medium was replaced with fresh medium containing 10 µM cytosine arabinoside (ARAC) to block glia replication.

Microvascular endothelial cells

Bovine coronary venular endothelial cells (CVEC) were obtained from bovine heart and cultured as described (Schelling ME 1988). Passages between 14 and 18 retaining endothelial markers were used in these experiments.

Proliferation

CVEC or Neuronal Cells ($1.5x10^3$ cells/well in 96 multiplates), after adherence, were treated with TAT-BH4 (50 µM, 24 h) in low serum or in presence of H_2O_2 (increased doses, 24 h). Cells were then fixed, stained and randomly counted at 20 x original magnification in 5 fields as previously reported (Donnini S 2009).

Immunofluorescence

To evaluate neuron maturation, neurons were analysed for β-3 tubulin. Neuronal cells were plated on glass cover-slips coated with polylisine and after 3 days were co-cultured with endothelium for 7 days as described below. Cells were fixed in 4% paraformaldehyde/PBS solution, incubated with 3% BSA/PBS solution and then incubated with β-3 tubulin in the presence of 0.1% Triton X to allow intracellular antibody entering. Cells were then washed, incubated for 1 h with a rodhamine-conjugated secondary antibody (Sigma-Aldrich, St. Louis, MO) diluted 1:50 in PBS with 0.5% BSA. Coverslips were mounted in Moviol 4-88 (Calbiochem, La Jolla, CA) and pictures of stained cells were taken using a confocal microscope (Zeiss LSM500)

Co-culture test

To investigate the endothelium effects on neuronal cells, endothelial and neuron cells were co-cultured using Transwell technique (Monti M 2013). Cell co-cultured were treated with TAT-BH4 (50 µM)

with or without H_2O_2 (100 µM). The co-culture was maintained for 7 days and then cell were analysed for β3-tubulin expression (neuronal cells) and cell number (endothelial cells) as describe above.

Results: in these first months, I focused my attention to the in vitro studies, to mimic the in vivo conditions of damaged tissue occurred during SCI. In particular I investigated: i. the role of TAT-BH4 on endothelial and neuronal cell apoptosis; ii. the role of endothelium in TAT-BH4 anti-apoptotic activity on damaged neuronal tissue, setting up an in vitro models of co-culture (task 1).

As, endothelial dysfunction represents the first indicator of vascular damage during ischemia/reperfusion injury, and it has been shown to be, at least in part, dependent on the production of ROS and apoptotic cell death, I selected two different conditions: nutrient deprivation (0.1% serum), or H_2O_2 (Monti M 2010). I selected these two conditions, as low serum increase ROS production and activate apoptotic cell death cascade, reducing cell survival (mild endothelial dysfunction), while H_2O_2 (100 µM) treatment represent a strong condition characterized by cell loss, as occurred during ischemic damage. In these conditions I tested the protective effect of TAT-BH4 on microvascular endothelial cells. TAT-BH4 exerted a protective effect on endothelium both in low serum and H_2O_2, promoting cell survival and reducing cell death.

Also neuronal cells were subjected to the same damaged treatments. First of all, I set up the method to isolate neuronal cells from mice embryo, as described by Messer with some modifications (Messer A 1981). After isolation cells were maintained in culture until they acquired organized structure before to use them. Cell organization was monitored by beta-3 tubulin immunofluorescence expression. At day ten, cells started to show both neuritic connections and cell bodies, organized in neurospheres which appeared mature and functional. This organization was maintained for 13 days, when cells began to degenerate and die. Before to start all the experiments, staining with GFAP was carried out to exclude glia contamination (<1%). Once I set up the neuronal cell isolation method, I evaluated their response to serum deprivation and H_2O_2 treatment. As expected, in both condition cells were damaged (loss of neuritic connections). Treatment with TAT-BH4 failed to protect neurons from apoptosis either when exposed to low serum or H_2O_2.

These unexpected data suggested us to recreate the in vivo condition where endothelium and neuronal cells are in contact, as we previously reported that TAT-BH4 reduced ischemia/reperfusion damage in brain (Donnini S 2009). In view to understand the in vivo protective effect of TAT-BH4, a co-culture model was set up, supposing that the rescue exerted on the endothelium was responsible of the peptide activity on damaged neuronal tissue.

Preliminary, I assayed the favorable co-culture condition to have the optimal endothelium/neuronal cells interaction. I performed experiments using optimal serum concentration to growth both cell lines. Endothelium was seeded in the upper chamber of a transwell system for co-culture,

and the neuronal cells in the lower well. The time line of the co-culturing was particularly studied: it was necessary to individuate the right moment to put in contact the two cell lines to avoid the influence of endothelium on neuronal cell morphological characteristic. At the end of these preliminary tests, the optimal co-culture condition was individuated: neuronal cells, seeded in the transwell, were isolated and after 3 days (just before starting to show both neuritic connections and cell bodies) were inserted on the well of microvascular endothelium. The co-culture experiment was carried out for 7 days. Once individuated the right co-culture condition, the experiments with low serum and H_2O_2 were performed. These treatments blocked the formation of neuritic connections, effect which was prevented by the treatment with TAT-BH4. In this co-culture system, TAT-BH4 rescued neurons from cell death maintaining their morphological characteristic, suggesting that endothelium mediated TAT-BH4 effect on neurons.

Conclusion: In conclusion these data demonstrate that TAT-BH4 exerts a protective effect on endothelium but not on neuronal cells per se. The neuronal protective effect is obtained in presence of endothelium, probably by the release of neurotrophic growth factors-mediated by TAT-BH4.

Future work: To conclude the task 1 of the project, I will investigate, at mechanistic level, the way of action of TAT-BH4 on endothelium, in particular, the expression of growth factors as Vascular Endothelial Growth Factor (VEGF) or the neurotrophic growth factor Fibroblast Growth Factor (FGF2), responsible of neuron protection and differentiation, will be performed.

In mean time, the task 2 will be started. Currently, TAT-BH4 has been administered in rats subjected to spinal cord trauma induced by the application of vascular clips. Rats were sacrificed after 24 h (acute damage) or after 7 d (recovery) (Genovese T 2006). These experiments was carried out to the laboratory of Prof. Cuzzocrea. Spinal cord tissue were removed and, in next months, will be assessed for histological and western blot analysis with particular attention to the investigation of morphological changes and expression of apoptotic and inflammatory markers. Moreover, following the indication of in vitro studies, the expression of growth factors (FGF-2 and VEGF) will be evaluated.

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