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Thymosin β 4: A candidate for treatment of stroke?

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Abstract

Neurorestorative therapy is the next frontier in the treatment of stroke. An expanding body of evidence supports the theory that after stroke, certain cellular changes occur which resemble early stages of development. Increased expression of developmental proteins in the area bordering the infarct suggest an active repair or reconditioning response to ischemic injury. Neurorestorative therapy targets parenchymal cells (neurons, oligodendrocytes, astrocytes and endothelial cells) to enhance endogenous neurogenesis, angiogenesis, axonal sprouting and synaptogenesis to promote functional recovery. Pharmacological treatments include statins, phosphodiesterase 5 inhibitors, erythropoietin, and nitric oxide donors which have all improved functional outcome after stroke in the preclinical arena. Thymosin β 4 is expressed in both the developing and adult brain and it has been shown to stimulate vasculogenesis, angiogenesis and arteriogenesis in the post-natal and adult murine cardiac myocardium. In this manuscript, we describe our rationale and techniques to test our hypothesis that Thymosin β 4 may be a candidate neurorestorative agent.

Keywords

Thymosin β 4; Neurorestorative; stroke; neurogenesis

Introduction

Stroke is a major cause of morbidity and mortality worldwide [1]. Most survivors exhibit some form of disability and functional recovery is slow and unpredictable. Over the past two decades, research on treatment of stroke has focused on neuroprotection and revascularization strategies. The only successful clinical trials that has resulted from this research is the use of tissue plasminogen activator (rt-PA) administered within 4.5 hours of symptom onset [2, 3]. Use of t-PA has been limited because of its narrow time dependent treatment window as most stroke patients present to the Emergency Department well beyond 4.5 hours of symptom onset [4, 5]. Moreover, use of rt-PA is complicated by a 6.4% symptomatic intracerebral hemorrhage (ICH) rate which has caused considerable controversy regarding its practical use, particularly amongst Emergency physicians. The restriction on time and potential adverse effects have limited the use of rt-PA to approximately 3% of stroke patients [6, 7]. New strategies need to be designed to produce a treatment for stroke patients that are not limited to a symptom onset time window and produce improved outcomes with minimal side effects. Therapeutic advances would be to initiate treatment many hours or even days after stroke onset with the intent of improving neurological function rather than just simply reducing the area of infarction.

Pharmacologically based therapies designed to enhance endogenous neurogenesis to promote functional recovery is the next frontier in stroke research.

Neurorestorative therapy

Recovery recapitulates ontogeny [8]. Genes and proteins that are present and active during development are reactivated after ischemic brain injury. Expression of these developmental genes and proteins after ischemia, especially with limited cerebral metabolic resources, demonstrates a conservation of plasticity that is capable of activation even in an aged adult organism. Examples of increased cerebral protein expression during development and ischemia are structural proteins (nestin and MAP-2), growth factors (VEGF and BDNF) and differentiation factors (NeuroD) [9, 10]. In a similar fashion, the number of dendritic spines reach a maximum several years after birth and subsequently decline. Likewise after stroke, the number of dendritic spines and synapses are increased during recovery and pruned [11, 12]. Neurorestorative therapy capitalizes on these fundamental observations to enhance these brain repair processes

Neurorestorative therapies using cell-based or pharmacological treatments are designed to be administered 24 hours or later after stroke onset to remove the time restraints of thrombolytics thereby enabling most strokes to be treated. Neurorestorative therapy treats the intact tissue and targets specific parenchymal cells (neural progenitor cells, astrocytes, oligodendrocytes or endothelial cells) to activate endogenous neurogenesis, oligogenesis, or angiogenesis [13]. Cell based therapies currently under investigation are bone-marrow mesenchymal cell, cord blood cells, fetal embryonic cells. Pharmacological treatments include statins, phosphodiesterase 5 inhibitors erythropoietin, and nitric oxide donors [14-18]. These treatments have all demonstrated improved functional outcome in the preclinical arena.

Stroke induced neurogenesis

In the adult rat brain, neurogenesis occurs primarily in the subventricular zone (SVZ) lining the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus (DG) [19]. The SVZ contains a heterogeneous group of progenitor or adult stem cells which have the potential of differentiating into neurons, astrocytes or oligodendrocytes. Type A cells are neuroblasts, while a subpopulation of type B cells (astrocytes) are neural stem cells. Slow dividing type B cells generate rapidly dividing type C (progenitor cells) cells that differentiate into type A cells (migrating neuroblasts) [20]. Type A cells ultimately travel the rostral migratory stream to the olfactory bulb where they differentiate into interneurons throughout rodent life [21]. A small subpopulation (5%) of type C cells in the SVZ become oligoprecursor cells (OPC) [22].

Stroke induces neurogenesis in the SVZ and dentate gyrus [23, 24]. Ischemia induces neurogenesis by activating quiescent stem cells in the SVZ into a proliferative state by a symmetric mode of cellular (blast) division. Doublecortin (DCX) is a microtubular associated protein required for neuroblast migration [25]. Stroke significantly increases the number of DCX expressing neuroblasts in the ischemic SVZ and ischemic boundary region (Figure 1A and 1B) [24].

Thymosin β 4 a potential neurorestorative agent?

Thymosin β 4 (T β 4) is a developmentally expressed 43-amino acid peptide that was originally isolated from thymic extract and exists in numerous tissues and isoforms [26]. T β 4's fundamental action is to inhibit organization of the actin-cytoskeleton by sequestration of G-actin monomers and thereby enabling cells to migrate [27]. T β 4 has been shown to promote cardiomyocyte survival and to improve cardiac function after myocardial infarction

(MI) in experimental adult mice [28]. T β 4 has been shown to regulate vasculogenesis, angiogenesis and arteriogenesis in the post-natal and adult murine cardiac myocardium. Vascular progenitor cells isolated from the adult epicardium differentiated into smooth muscle and endothelial cells when cultured in the presence of T β 4 [29]. It is postulated that T β 4 may improve cardiac function by acting in conjunction with other angiogenic factors (ie VEGF) to revascularize damage myocardium through stimulation of adult vascular progenitor cells. Additionally, T β 4 was found to be an essential paracrine factor of embryonic endothelial progenitor cell cardiac protection after acute MI [30].

T β 4 has been shown to be expressed in both the developing and adult brain [31, 32]. Specifically, T β 4 has been to be localized in growing neurites of cerebellar granule cells [33, 34]. In many disease states such as focal ischemia, hippocampal denervation and Huntington's disease, gene expression of T β 4 is upregulated [35-37]. Since actin dynamics contribute a critical role in cell migration and synaptogenesis, speculation exists that upregulation of T β 4 could be vital to recovery. Given these observations and the robust neurogenesis observed after stroke in the SVZ, T β 4 could potentially act as a neurorestorative agent by promoting the differentiation and migration of stem cells from the SVZ to the ischemic area of injury.

Experimental Techniques

Emboic stroke rat model

Our embolic stroke rat model has been used to test a variety of thrombolytic, antiplatelet, neuroprotective and neurorestorative therapies [17, 38-43]. The model is reproducible and predicted the FDA approved three hour treatment window of rt-PA. The robust neuroprogenitor proliferation and upregulation of DCX after ischemia provides an avenue for studying potential neurorestorative treatments.

Procedure—Male Wistar rats are anesthetized with 3.5% halothane and anesthesia maintained with 1.0% halothane in 70% N₂O and 30% O₂ using a face mask. Rectal temperature is maintained at 37 ± 0.5 °C throughout the surgical procedure using a feedback regulated water heating system. The right femoral artery is cannulated with a PE-50 catheter (Becton Dickinson and Company, Sparks, MD) for continuous monitoring of blood pressure and measurement of blood gases and the right femoral vein is cannulated with a PE-50 catheter for drug administration.

Preparation of the emboli—Before occlusion of the middle cerebral artery (MCA), the clot must be prepared the previous day from arterial blood from a donor rat. Femoral arterial blood from a donor rat is withdrawn into 20 cm of PE-50 tubing and retained in the tube for 2 hours to clot at room temperature and subsequently maintained at 4 °C for 22 hours. A total of 5 cm of the PE-50 tube containing the clot is cut and attached at each end to a 20-cm PE-10 tube interconnected by a syringe filled with saline. The clot is shifted by continuous alternating movement from one syringe to the other for 5 minutes. A single clot (1.0 μ L) is transferred to a modified PE-50 catheter with a 0.3-mm outer diameter filled with saline.

Emboli placement—The MCA is occluded by placement of the embolus at the origin of the MCA. Using an operating microscope (Carl Zeiss, Inc., Thornwood, NY) the right common carotid arteries (CCAs), the right external carotid artery (ECA) and the internal carotid artery (ICA) are isolated via a midline incision. A modified PE-50 catheter with a 0.3-mm outer diameter filled with a 1.0 μ L clot, which is attached to a 100- μ L Hamilton syringe filled with 0.9% saline, is introduced into the ECA lumen through a small puncture (figure 2A) [40]. A 15-to 16-mm length of catheter is then gently advanced from the ECA into the lumen of the ICA. The clot in the catheter is injected into the ICA along with 2 to 3

μL of 0.9% saline. Finally, the catheter is withdrawn from the right ECA 5 minutes after injection (figure 2B) and the right ECA is ligated

Functional tests

After neurological injury, functional outcome is the most important indicator of drug efficacy. In our rat model, neurological functional testing is routinely performed to access treatment effect. Specific behavioral tests have been developed to test motor and sensory functions [44]. These tests are performed before MCA occlusion and at specific days after MCA occlusions by an investigator who is blinded to the experimental groups. The battery of tests consists of the adhesive-removal test and the modified Neurological Severity Score (mNSS). The adhesive removal test, which measures somatosensory deficit, is performed according to the method developed by Schallert and Whishaw [45]. Briefly, the rats are removed from their home cages so that the adhesive paper dots are firmly and accurately attached. Two small pieces of adhesive-backed paper dots (of equal size, 113.1 mm²) are used as bilateral tactile stimuli occupying the distal-radial region on the wrist of each forelimb. The rats are returned to their cages and typically remove each stimulus one at a time using their teeth. The time required to remove both stimuli from each limb is recorded in 5 trials per day. Before surgery, the rats are trained 5 times a day for 3 days and all rats are able to remove the dots within 10 sec at the end of training.

Measurement of lesion volume

After completion of the functional testing the rats are sacrificed and the brains removed. Rat brains are fixed by transcardial perfusion with saline and embedded in paraffin. Seven brain sections are traced using a microcomputer imaging device (MCID) analysis system (Imaging Research, St. Catharines, Canada). The indirect lesion area, in which the intact area of the ipsilateral hemisphere is subtracted from the area of the contralateral hemisphere, is calculated. Lesion volume is represented as a volume percentage of the lesion compared with the contralateral hemisphere. Measurement of lesion volumes is correlated to functional outcome. However, reduction of lesion volume is not always correlated to improved functional outcome.

Immunohistochemical staining

Standard paraffin blocks are obtained from the center of the lesion, corresponding to coronal coordinates for bregma -1-1 mm. A series of 6 μm thick sections at various levels (100 μm interval) are cut and stained with hematoxylin and eosin (H&E). Tissue sections are incubated in commercially prepared antibodies used to identify specific cellular and vascular cell types. The sections are treated with an ABC kit (Vector laboratories, INC) and diaminobenzidine (DAB) is used as a sensitive chromogen for light microscopy. Coronal sections are digitized using a 40x objective using a three-CCD color video camera (Sony DXC-970MD) interfaced with a MCID system for quantification.

Summary

Tβ4 is a potential neurorestorative agent because of its actin binding properties that may enhance neuroprogenitor cellular migration and differentiation in the area of ischemic injury promoting a brain repair or regenerative process. Tβ4 is presently being tested in our rat model of embolic stroke Tβ4. In future publications, we hope to provide evidence that Tβ4 promotes restorative mechanisms and recovery after stroke and that recovery will not depend on a narrow time window of treatment.

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Figure 1.

Figure 1 (left) demonstrates neuroblasts (green, DCX positive cells) along with activated astrocytes (red, GFAP (glial fibrillary acidic protein)), in the ischemic SVZ and striatum 14 days after stroke. **Figure 1 (right)** represents the non-ischemic SVZ hemisphere [24].

Figure 2.
Figure 2A is a schematic drawing of the embolic stroke rat model. **Figure 2B** demonstrates the clot placed in the MCA [40].