Neuroscience Letters 659 (2017) 7–13

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Research article

Thymosin β4 for the treatment of acute stroke in aged rats

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ARTICLE INFO

Keywords:
Thymosin β4
Neurorestorative
Stroke
Gliosis

ABSTRACT

Thymosin β4 (Tβ4) is a 5 K peptide which influences cellular migration by inhibiting organization of the actin-cytoskeleton. Tβ4 has neurorestorative properties and is a potential candidate for the treatment of sub-acute stroke. Previous research demonstrated that Tβ4 improved neurological outcome in a young (3 months) rat model of embolic stroke. We hypothesized that Tβ4 would improve neurological outcome in an aged rat model of embolic stroke when administered 24 h after embolic stroke. Aged Male Wistar rats (Charles River, France 18–21 months) were subjected to embolic middle cerebral artery occlusion (MCAo). Rats were randomized to receive Tβ4 (12 mg/kg, RegeneRx Biopharmaceuticals, Inc.) or control 24 h after MCAo and then every 3 days for 4 additional doses. The dose of 12 mg/kg was the maximal dose of Tβ4 that showed functional improvement in a young rat model of embolic stroke. Functional tests (adhesive-removal test (ART), foot fault test (FFT) and the modified Neurological Severity Score (mNSS)) were performed weekly. The rats were sacrificed 56 days after MCAo and lesion volumes were measured. Immunohistochemical analysis for oligodendrogenesis, myelination and gliosis was also performed. Twenty-three rats were included in the study: control group (n = 12) and Tβ4 group (n = 11). After randomization, there were three deaths in both the control and Tβ4 groups. The Tβ4 treatment reduced infarct volume by more than 50% (12.8% ± 9.3%, mean ± SE, $p < 0.05$) compared to the control group (26.0% ± 4.3%). However, Tβ4 did not show improvement in functional outcome compared to control. There was no significant increase in oligodendrogenesis, myelination and gliosis between control and treatment with Tβ4, however, we unexpectedly observed that overall (control and Tβ4 groups) astrocytic gliosis as measured by GFAP immunoreactivity was significantly inversely correlated with neurological outcome measured using the modified Neurological Severity Score (mNSS) ($p < 0.01$), suggesting that greater gliosis may be related to improvement of neurological outcome in aged rats. In summary, Tβ4 treatment of stroke aged rats significantly reduces infarct volume compared to vehicle treated stroke, however, Tβ4 treatment did not show improvement in functional outcome, myelination or gliosis when compared to control. GFAP staining was significantly inversely correlated to improvement in the mNSS, suggesting that gliosis in the aged rat may be of benefit in improvement of functional outcome.

1. Introduction

Age is a well-known risk factor for stroke with 75% of all strokes occurring in people over the age of 65 years old [1]. The risk of having a stroke more than doubles each decade after the age of 55 years old. Furthermore, recovery from stroke is also influenced by age; youth fare better after stroke than the aged given the same degree of ischemic insult [2]. In experimental models of stroke, aged rats recover more slowly, less completely and have higher mortality rates when compared to younger rats [2,3]. Based on these facts, the aged post-acute stroke rat is the most clinically relevant model to investigate therapies hypothesized to improve stroke outcome.

Neurorestorative agents treat the surrounding intact healthy neural tissue and promote repair of damaged neural tissues from neurological injury [4]. Neurorestorative agents act on intact parenchymal cells including neuroprogenitor (adult neural stem cells), oligodendrocyte progenitor cells, astroglial cells, and cerebral endothelial cells, to promote neurogenesis, oligodendrogenesis, axonal sprouting,

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http://dx.doi.org/10.1016/j.neulet.2017.08.064
Received 15 March 2017; Received in revised form 10 August 2017; Accepted 28 August 2017
Available online 31 August 2017
0304-3940/

synaptogenesis and angiogenesis, in the injured brain. These restorative processes are associated with improvement in neurological functional outcome [5].

Thymosin β4 (Tβ4) is a developmentally expressed 43-amino acid peptide that sequesters G-actin monomers [6–8]. Based on our observations that Tβ4 improves recovery in four different models of neurological injury: stroke, multiple sclerosis, traumatic brain injury and peripheral neuropathy, we proposed that Tβ4 is a neurorestorative agent [9–12]. A common observation in all four models is oligodendrogenesis and/or the production of mature myelin-secreting oligodendrocytes (OL) from oligoprogenitor (OPC) cells, or in the case of peripheral neuropathy, production of myelin from Schwann cells. In order to develop Tβ4 for use in clinical trials, we performed a dose-response study in our rat model of embolic stroke in young rats and determined that a Tβ4 dose of 2 and 12 mg/kg improved neurological outcome [13]. Specific observations showed that Tβ4 increased proliferating OPCs in the subventricular zone (SVZ) and in the white matter of the corpus callosum, as well as increased myelination in the ischemic vulnerable striatal boundary in the 12 mg/kg treated group.

The next logical step for clinical translation was to test Tβ4 in an aged rat model of embolic stroke. We hypothesized that Tβ4 would show functional improvement in aged Wistar rats (Charles River) treated with 12 mg/kg Tβ4, the maximal dose that showed improvement in young rats. However, our results differed from those obtained in the young rat model of embolic stroke, suggesting an aged related effect on the treatment of acute embolic stroke with Tβ4.

2. Material and methods

All experimental procedures were approved by the Institutional Animal Care and Use Committee of Henry Ford Hospital. Aged (18–20 months) male Wistar rats imported from France were obtained from Charles River Laboratories. These aged rats (n = 29) were acquired at age 15 months and kept at Henry Ford Health Systems animal care facilities for 3 months before initiation of the study. This group of rats was used to test the therapeutic efficacy of Tβ4 at 12 mg/kg, the highest dose that showed efficacy in the dose-response study using young rats.

2.1. Embolic stroke rat model

The middle cerebral artery (MCA) of the aged male Wistar rats was occluded by placement of an embolus at the origin of the MCA, as described in previous publications [13,14]. Briefly, under an operating microscope (Carl Zeiss, Inc., Thornwood, NY, USA), the right common carotid artery (CCA), the right external carotid artery (ECA) and the internal carotid artery (ICA) were isolated via a 3 cm ventral neck midline incision. A 6-0 silk suture was loosely tied at the origin of the ECA and ligated at the distal end of the ECA. The right CCA and ICA were temporarily clamped using a curved microvascular clip (Codman & Shurtleff, Inc., Randolph, MA, USA). A modified PE-50 catheter filled with a fibrin rich clot from a donor rat, was attached to a 100- μl Hamilton syringe, and introduced into the ECA lumen through a small puncture. The suture around the origin of the ECA was tightened around the intraluminal catheter to prevent bleeding, and the microvascular clip removed. The catheter was then gently advanced from the ECA into the lumen of the ICA to reach the origin of the MCA. The clot along with 5 μl of saline in the catheter was injected into the ICA over 10 s. The catheter was withdrawn from the right ECA 5 min after injection and ligated. Buprenex was administered intraperitoneally (IP) post-surgery at a dose of 0.01 mg/kg if the rat showed signs of distress.

2.2. Experimental design

Tβ4 (RegeneRx Biopharmaceuticals Inc) was administered IP 24 h after MCA occlusion (MCAO) and then every 3 days for 4 additional doses. Randomization was performed using a computer generated list. Since this study was designed to observe functional outcome, we only included those rats which completed the functional tests until time of sacrifice (56 days). For labeling proliferating cells, 5-bromo-2′-deoxyuridine (BrdU, 100 mg/kg; Sigma, St. Louis, MO) was injected IP into rats 24 h after MCAo and then daily for 7 days. Fifty-six days after MCAO, rats were anesthetized with ketamine (80 mg/kg) and xylazine (13 mg/kg) and transcardially perfused with saline followed by 4% paraformaldehyde. The rat was then decapitated and brain removed and fixed in 4% paraformaldehyde.

2.3. Neurological functional tests

In the embolic stroke rat model, a battery of behavioral tests was performed at 1, 7, 14, 21, 28, 35, 42, 49 and 56 days after MCAO by an investigator who was blinded to the experimental groups. The battery of tests consisted of the adhesive-removal test (ART), the modified Neurological Severity Score (mNSS) and foot fault test (FFT) [15–17]. Briefly, the ART measures both motor and sensory function and it involves placing two small pieces of adhesive-backed paper dots on the wrist of each forelimb (of equal size, 113.1 mm^2) to act as bilateral tactile stimuli occupying the distal-radial region. The time required for the rat to remove both stimuli from each limb was recorded in 5 trials per day. Each animal received 5 trials on all testing days after MCA occlusion and the mean time required to remove both stimuli from limbs was recorded. In order to increase sensitivity of the test, the adhesive-backed paper dots were reduced in size by one-half at day 35. The mNSS test is a composite score in which motor, sensory, balance and reflex measures are used to calculate a value ranging from 1 to 18, with the higher score implying greater neurological injury. Points are awarded for the inability to perform the tasks or for the lack of a tested reflex (normal score 1, maximal deficit score 18). Rats with a mNSS less than 8 were excluded from the study. The FFT tests cerebellum function by measuring the ability of the rat to walk along an elevated hexagonal wire grid and the number of times the paw slips (foot fault) between the wires. The total number of foot faults is divided by the total number of steps the rat uses to cross the grid. The average number of foot faults of a normal rat is 2–3%. As anticipated, rats which died due to the MCAo procedure (at day 1 or 2 before and after randomization) were excluded from the analysis.

2.4. Histological and immunohistochemical assessment

After fixed in paraformaldehyde, the brains were embedded in paraffin and cut into seven equally spaced (2 mm) coronal blocks. A series of adjacent 6 μm-thick sections was cut from each block in the coronal plane and was stained with hematoxylin and eosin. Seven brain sections were traced using a microcomputer imaging device (MCID) image analysis system (Imaging Research, St. Catharines, Canada) by laboratory associates who were blinded to the dose and functional testing results. The indirect lesion area, in which the intact area of the ipsilateral hemisphere was subtracted from the area of the contralateral hemisphere, was calculated. Lesion volume is represented as a volume percentage of the lesion compared with the contralateral hemisphere.

Standard paraffin blocks were 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) were cut. Immunostaining was performed on these sections. Antibodies used for identification of OPCs and OLs were NG-2 (chondroitin sulfate proteoglycan) (1:800, polyclonal rabbit, incubated overnight at 4 °C, Chemicon, CA) and APC (CC1) (adenomatous polyposis coli) (1:20, monoclonal mouse, incubated 60 min at room temperature, Genway, CA), respectively. To identify proliferating OPCs and OLs, double immunostaining of NG2/BrdU and APC/BrdU was performed, respectively. Antibodies used for identification of Aquaporin4 (AQ4) and endothelial cells were AQ4 (1:1500, polyclonal rabbit, incubated overnight at 4 °C, Millipore, Germany) and Endothelium Barrier antigen