Transforming growth factor beta proteins and receptors following focal ischemia in the rat brain

Ph.D. thesis

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I. INTRODUCTION

1. Ischemic stroke

Stroke is the third leading cause of death worldwide, and the major cause of severe and chronic adult disability. About 80% of acute stroke are of ischemic origin. Today, this disease is a major problem around the world due to mortality rates, limited treatment options, and high medical expenses. Focal ischemia occurs when a cerebral artery is occluded and the related area is affected. Ischemia activates various programs of cell death. The mechanism of tissue damage includes both cellular and molecular processes.

Thrombolysis is now commonly used to help restore cerebral blood flow in order to prevent damage of the penumbra, but it can only be used within a narrow time window. The ischemic penumbra is the area, which can be still saved, and therefore, it is the main target of additional neuroprotective treatments. Hopefully, the future therapy of ischemic stroke will be based on a combination of neuroprotection, thrombolysis, antithrombotics and neurorepair.

2. TGF-β proteins and their receptors

The transforming growth factor betas (TGF-βs) influence differentiation of various cell types, usually inhibit cell proliferation, but can stimulate the growth of some stromal cells and affect extracellular matrix formation in a variety of tissues. The normal distribution pattern of the TGF-βs was described at the protein level using immunohistochemistry and at the mRNA level by means of in situ hybridization histochemistry. Widespread expression of TGF-β1

mRNA was described including intense labeling in some cortical and hippocampal cells, the medial preoptic area, the paraventricular hypothalamic nucleus, the central amygdaloid nucleus, and the superior olive. In the cerebral cortex, TGF-β2 expression was very intense in layer V. Layers III and IV also contained TGF-β2 and -β3, respectively, while TGF-βs were absent in the caudate putamen All three TGF-β proteins were induced after focal ischemia, but the expression pattern and the time course were different.

Receptors of TGF-β superfamily are receptor serine kinases. Their canonical signal transduction pathway includes SMAD proteins, which need to be phosphorylated to exert their effects within the nucleus. Based on their role and sequence homology, the receptors of the TGF- β superfamily can be divided as type I receptors, which can phosphorylate SMAD proteins, type II receptors required for the action of type I receptors, and accessory receptors, wich may play a role in recruiting the ligands. TGF-β receptor I (TGF-β RI), or activin-like receptor kinase 5, was initially recognized as the receptor of all 3 types of TGF-βs, which are all dimeric ligands. The ligands bind to TGF-β receptor II (TGF-β RII), which in turn forms the functional receptor with TGF-β RI and phosphorylates it. A functional receptor is a heterotetramer consisting of 2 TGF-β RI, and 2 TGF-β RII. In addition, TGF-β receptor III (TGF-β RIII), or betaglycan, has the ability to influence the TGF-β receptor complex formed by TGF-β RI and RII. Although TGF-β RIII has a short intracellular domain, it presents TGF-βs to TGF-β RII. The TGF-β receptor III is particularly important for the recognition of TGF-β2, which binds poorly to the TGF-β receptor. More recently, another type I receptor, activin-like receptor kinase 1 (Alk1) was shown to signal TGF-β1, and -β3 in addition to bone

morphogenic protein 9. It has also been demonstrated that the actions through Alk1 are often different from, sometimes even antagonistic to those exerted by TGF-β RI. In most cells, TGF-βs signal via the type I receptor TGF-β RI/Alk5. In endothelial cells and neurons, the signal can go through the TGF-β RI/Alk1 receptor complex. TGF-β receptor mRNA was detected by RT-PCR in rats at different stages of development and similar levels were found in several regions of the CNS, including cortex, midbrain, cerebellum, brain stem and hippocampus.

3. The role of the TGF-β system in cerebral ischemia

An increase in the level of TGF-β has been reported in different animal models of experimental cerebral ischemia. After focal ischemia, all three TGF-β proteins were induced but with a different temporal and spatial pattern in the rat brain. TGF-β1 was induced in the ischemic penumbra, which is surrounding the ischemic core. An elevation in the level of TGF-β1 also has been reported after ischemic stroke in human brain tissue. The involvement of the other TGF-β subtypes (TGF-β2 and -β3) after focal ischemia is less well studied. TGF-β was suggested to be neuroprotective because injection of TGF-β1 decreased, while antagonizing the endogenous action of TGF-β1 with injection of an antagonist, TGF-β type II receptor, increased the infarct size following middle cerebral artery occlusion (MCAO) in rats. TGF-βs protect from the overactivity of the immune system by inhibiting proliferation, differentiation, activation and effectivity of the immune cells following focal ischemia.

4. The answer of the different cell types of the central nervous system after focal ischemia

Ischemic stress in the brain causes acute and massive cell death in the targeted core area followed by a second phase of damage in the neighboring penumbra. In the core of the lesioned area, severe damage mediated by glutamate excitotoxicity and oxygen and glucose deprivation leads to a rapid halt of cellular functions resulting in necrotic or acute cell death. The maintenance of essential cellular functions within the penumbra is an opportunity for neuronal rescue and survival, and this is critical for minimizing long-term damage. Astrocytes respond to injury and disease in the central nervous system with a process referred to as reactive astrogliosis. The astrocytic scar in the penumbra serves a primary role in confining inflammation to the lesion epicenter and protecting intact neural networks from uncontrolled damage. Microglia are resident immune cells of the central nervous system. They are active sensors in healthy brain and versatile effectors under pathological conditions. Cerebral ischemia evokes a strong neuroinflammatory response, which can induce significant changes in the gene expression profiles and phenotype of different cell types in the nervous system. During brain injury, microglia rapidly undergo a shift in their effector program by transforming their morphology, proliferating, releasing proinflammatory compounds and increasing expression of immunomodulatory surface antigens.

II. OBJECTIVES

The purpose of the dissertation was to describe the temporal and spatial distribution of the TGF-β receptors following MCAO and to identify the types of cells, which express TGF-β proteins and receptors and also to reveal the mechanism of their induction. Therefore, the following objectives were adressed:

1. What is the time course of induction of mRNA of TGF-β RI, RII, RIII, and Alk1 following MCAO? We performed in situ hybridization histochemistry to describe the distribution of the TGF-β receptor mRNAs at 24, 72 hours and 1 month after MCAO. The changes in the mRNA levels were confirmed by densitometry and quantitative analysis.

2. Which cells express the different subtypes of TGF-βs after MCAO? The combination of in situ hybridization histochemistry with immunolabeling for neuronal (NeuN), astrocyte (GFAP), and microglial (Iba1) markers were applied to identify cells that expressed the 3 different subtypes of TGF- β s in the ischemic rat brain.

3. Which cell types express the different TGF-β receptors following MCAO? The combination of in situ hybridization histochemistry and immunolabeling for neuronal (NeuN), astrocyte (S100), microglial (Iba1), endothelial cells (vWF), and smooth muscle cells (αSMA) markers were applied to identify the cells that express the different TGF-β receptors in the ischemic rat brain.

4. What mechanisms activate TGF-βs following ischemia? TGF-βs were double labeled with the immediate early genes Fos and activating transcription factor-3 (ATF-3), markers of neuronal activation and axonal damage, respectively.

III. METHODS

A total of 58 adult, male Wistar rats (300–450 g body weight; Charles Rivers Laboratories, Hungary) were used in this study. The Animal Examination Ethical Council of the Animal Protection Advisory Board at Semmelweis University, Budapest approved this study, which is in accordance with EU Directive 2010/63/EU for animal experiments.

Focal ischemia was induced using a modified intraluminal suture method. After electrocoagulation of the external and common carotid arteries, a silicon rubber-coated monofilament was inserted through the common carotid artery into the internal carotid artery to the base of the middle cerebral artery. The monofilament was removed 1 h later, except for 24 h permanent ichemia. The rats were sacrificed at different time points after the beginning of the reperfusion and their brains were dissected and frozen for in situ hybridization histochemistry or perfused transcardially for immunohistochemistry. Alternative series of sections from the MCAO treated rats were TTC and Nissl stained to identify the lesion.

We performed in situ hybridization histochemistry to describe the distribution of the TGF-β RI, RII, RIII and Alk1 mRNAs at 24, 72 hours and 1 month after MCAO. The changes in the mRNA levels were confirmed by densitometry, and quantitative analysis.

We combined in situ hybridization histochemistry with immunohistochemistry to identify cells that expressed TGF-βs and their receptors after focal ischemia. The following markers were applied:

neuronal (NeuN), astrocyte (GFAP, S100), microglial (Iba1), endothelial cells (vWF), and smooth muscle cells (alpha-SMA) markers. TGF-βs were double stained with the immediate early genes Fos and activating transcription factor-3 (ATF-3), markers of neuronal activation and axonal damage, respectively.

IV. RESULTS

We examined TGF-β RI, RII, RIII and Alk1 among TGF-β receptors. We showed that each receptor mRNA has a specific spatial and temporal expression pattern after focal ischemia. The cell types expressing the different types of TGF-β receptors were identified by co-localization with known cellular markers. The TGF-β expressing cells were also identified. The TGF-β1, TGF-β RI and RII are induced in microglial cells within the lesioned area at 72 h after MCAO. We have shown that TGF-β2 appears in neurons, in which Fos activity is also detected in the ipsilateral cerebral cortex.

1. Time course and distribution of TGF-β receptors following focal ischemia in the rat brain

TGF-β receptors were not present within the infarct area, in fact, even their very low level of expression disappeared at 24 h after transient ischemia. The expression did not change in the intact brain. A high level of TGF-β RI expression was observed in layer IV of the intact cortex while the basal expression level of TGF-β RII, RIII, and ALK1 mRNA was very low. The peri-infarct area represented a transition between the lesion and the intact tissue without specific induction in the mRNA level of any TGF-β receptor.

The most significant change at 72 h after MCAO was that TGF-β RI mRNA appeared within the lesion. Some cells were strongly labeled with TGF-β RI mRNA. Their distribution was uneven, but they were present generally all over in the infarct area. The density of TGF-β RI mRNA was higher in the penumbra than in the intact brain tissue. The

TGF-β RII mRNA was also induced at 72 h following MCAO. In fact, the distribution of TGF-β RII expression was similar to that of TGF-β RI except in the intact cerebral cortex, where TGF-β RII showed the same, very low level of expression as in the intact brain. TGF-β RIII mRNA showed an elevated level within the lesion and in the penumbra. The increase was less pronounced than for other TGF-β receptors but still clearly visible. Alk1 mRNA was also apparent at 72 h after MCAO. The distribution of induced Alk1 mRNA was similar to that of TGF-β RIII. Alk 1 mRNA expression was found more intense along the blood vessels than in the case of TGF-β RIII.

The remaining, not shrunken tissue showed an even higher expression level of TGF-β RI and RII mRNA at 1 month than at 72 h following MCAO. Interestingly, TGF-β RIII mRNA was further induced within the lesion and its distribution became similar to that of TGF-β RI and RII. In contrast, the distribution and labeling intensity of Alk1 remained similar to that at 72 h.

2. The identification of TGF-β expressing cell types following MCAO

To identify TGF-β1 expressing cells, a combination of Iba1 immunohistochemistry and TGF-β1 in situ hybridization histochemistry was used, which indicated colocalization of Iba1 and TGF-β1 within the ischemic core as well as around the lesion. Approximately 95% of the TGF-β1 cells contained Iba1 immunoreactivity. In addition, a small number of TGF-β1-expressing cells around the lesion were also labeled by GFAP. TGF-β1 mRNA did not appear in NeuN positive neurons.

Over 80% of TGF-β2 and -β3 mRNA-expressing cells co-localized with the NeuN positive cells suggesting that these subtypes are expressed in neurons. In turn, TGF-β2 and -β3 were not present in microglial cells as concluded from their absence in Iba1 immunoreactive cells.

Heat shock protein 70 immunoreactivity, indicative of the penumbra, was distributed in cell bodies around the lesion 24 h following transient MCAO but was absent within the lesion and the intact brain tissue. TGF-β1-expressing cells had a similar distribution at this time point. The distribution of TGF-β2 and Hsp70 overlapped within the penumbra 24 h following MCAO. However, additional TGF-β2-expressing cells were present in the intact brain tissue in layers II, III, and V of the cerebral cortex. Interestingly, approximately 75% of the cells expressing TGF-β2 within the penumbra also contained Hsp70. A glial scar was present around the lesion 1 month following MCAO. The scar tissue was visualized by intense GFAP immunolabeling. TGF-β1-expressing cells were abundant within the glial scar and the infarct area but were virtually absent in the intact tissue.

3. The cell types expressing TGF-β receptors after MCAO

The distribution of Iba1 immunoreactive cells was similar to that of TGF-β RI and RII-expressing cells. Furthermore, a combination of Iba1 immunohistochemistry and in situ hybridization for TGF-β RI and RII indicated co-localization of Iba1 and these two receptor types within the ischemic core as well as around the lesion. However, the

fairly large number of TGF-β RI-expressing cells in the cerebral cortex away from the lesion was neurons.

The distribution of TGF-β RIII and Alk1-expressing cells had different distributions but neither showed co-localization with Iba1. Thus, apart from the above described Iba1-positive microglia, blood vessels were present within the infarct. In fact, the labeling intensity of immunolabeling with vWF and alpha-SMA was higher within the infarct than outside of it. Both S100 and NeuN immunoreactive cells were essentially absent in the ischemic core. The Alk1 and TGF-β RIII are expressed in endothelial cells labeled with vWF but not in smooth muscle cells and microglia. Furthermore, TGF- β RII was also expressed in endothelial cells.

4. Possible mechanisms of induction of TGF-βs following focal ischemia

Fos-expressing neurons were present throughout the cerebral cortex ipsilateral to the lesion 24 h after MCAO. The highest density of Fos immunoreactive cells was observed in layer II. In contrast, intensely labeled ATF-3 immunoreactive cells were present only around the lesion. We did not observe Fos or ATF-3 immunoreactivity in TGF-β1 mRNA expressing cells. In contrast, almost all of the examined TGF-β2-expressing neurons contained Fos immunoreactivity, while other Fos-ir cells did not express TGF-β2. The area containing ATF-3-ir cells only slightly overlapped with the area containing TGF-β2-expressing cells. In these regions, ATF-3 immunoreactivity did not substantially co-localize with TGF-β2.

V. CONCLUSIONS

The inductions of TGF-β proteins and their receptors have distinct spatial and temporal resolutions following a local ischemic lesion suggesting their involvement in different functions. Our results significantly extended the knowledge of the induction of the TGF-β system following focal ischemia.

1. In the intact cerebral cortex a high level of TGF-β RI expression was observed in neurons in layer IV. At 24 h after occlusion, none of the TGF-β receptors were induced. At 72 h following MCAO, all four TGF-β receptors induced within the ischemic core, and TGF-β RI and RII also appeared in the penumbra. Furthermore, all four receptors were induced within the lesion 1 month after MCAO, the TGF-β RIII was particularly significantly induced at this time point relative to 72 h.

2. Combination of immunohistochemistry and in situ hybridization histochemistry indicated co-localization of TGF-β1 mRNA and microglial marker Iba1, while only small portion co-localized with astroglial markers with no neuronal expression at 72 h after MCAO. In contrast, TGF-β2 was linked to the neuronal marker NeuN labeled positive cells.

3. In the penumbra and within the infarct area, a profound induction of TGF-β RI and RII were detected in microglial cells. In turn, Alk1 is induced in epithelial cells within the infarcts, which implies the role of this receptor type in angiogenesis followed by MCAO. The TGF-

β RIII is expressed mainly in endothel cells at early time points but also in other cells within the infarct at 1 month after MCAO.

4. The co-localization of TGF-β2 with Fos but not with ATF-3 suggests that cortical spreading depolarization, but not damage to neural processes, might be the mechanism of its induction away from the lesion.

The alterations in TGF-β receptor expression following MCAO suggests that similar changes take place in and around the lesions in stroke patients, too. These data suggest that TGF-β proteins and their receptors are involved in distinct neuroprotective processes.

VI. BIBLIOGRAPY OF THE CANDIDATE'S PUBLICATIONS

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