The transforming growth factor betas in the central nervous system

Ph.D. thesis

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I. INTRODUCTION

Transforming growth factors were originally named based on their abilities to induce a transformed phenotype in non-neoplastic rat kidney fibroblasts. Transforming growth factor-βs (TGF-βs) belong to a superfamily of cytokines that includes TGF-β1, -β2, and -β3, the bone morphogenetic proteins (BMPs), the growth/differentiation factors (GDF), activins and inhibins, anti Müllerian hormone, in Drosophila the decapentaplegic proteins and in Xenopus the vegetal 1 proteins. There are three separate genes encoding TGF-β1, -β2, and -β3. TGF-βs are synthesized as homodimeric proproteins (pro-TGF-βs) and are cleaved in the trans-Golgi to provide TGFβ and latency-associated protein (LAP), which remain noncovalently associated as small latent complex. The complex is covalently bound to latent TGF-β binding proteins (LTBPs) in the extracellular space and forms the large latent complex of TGF-βs. LTBPs are large multidomain proteins, which are needed for secretion, correct folding, and matrix deposition of TGF-βs and also have been suggested to play a role in the extracellular activation of TGF-βs. Many potential activators of the extracellular βs have been proposed, including proteases, thrombospondin-1, integrins, reactive oxygen species, and pH. There are 4 LTBPs (and different splice variants for each of them identified), however, only limited information is available on their selectivity to bind TGF-βs. The potential selective binding of different LTBPs to different pro-TGF-β types is not well

characterized yet. In vitro, LTBP-1 and LTBP-4 bind all three types of TGF-βs, LTBP-3 has some selectivity for TGF-β1 while LTBP-2 does not seem to bind any TGF-βs. Although the precise mechanisms remain to be elucidated, LTBPs may mediate signals toward TGF-β activation. After activation, the free TGF-βs can travel as dimers to receptors at the extracellular surface of the target cell, and bind to heteromeric complexes of type I and II receptors, which belong to the serine/threonine kinase family of receptors. In most cells, TGF-βs signal via the canonical type I receptor TGF-β receptor I/activins-like kinase receptor 5 (Alk5). In endothelial cells and in neurons, TGF-βs may also signal via the type I receptor Alk1. The type II receptor then phosphorylates the type I receptor, which relays the signal by binding and phosphorylating a receptor-regulated Smad protein. Alk5 induces phosphorylation of Smads 2 and 3 while Alk1 mediates phosphorylation of Smads 1, 5 and 8. The activated receptor-regulated Smad proteins form complexes with Smad4. Active Smad complexes translocate into the nucleus to exert their actions on gene expression. TGF-βs may also use non-Smad signaling pathways including the phosphoinositide 3-kinase-Akt-mTOR pathway, the small GTPases Rho, Rac, and Cdc42, and the Ras-Erk-MAPK pathway.

The TGF-β are involved in the regulation of proliferation, differentiation, and survival of various cell types. The physiological roles of TGF-βs in the nervous system are not fully understood. Their diverse roles in the development of the CNS have been

established. During the development of the CNS, TGF-β immunolabeling was most prominent in zones where neuronal differentiation occurs and less intense in zones of active proliferation. TGF-β had an anti-mitotic effect on progenitors and increased expression of neuronal markers in hippocampal and cortical primary cell cultures of developing mouse. TGF-β may also be involved in the differentiation of selected neuronal subtypes at the expense of other subtypes. The survival of motoneurons may also depend on TGF-βs as a potentially continuous trophic support factor from muscle fibers or other cell types. Endogenous TGF-βs are available to affect the survival of dopaminergic neurons in the substantia nigra. TGF-β2 was demonstrated to influence synaptic transmission, rather than synaptogenesis, at some central synapses. TGF-β2 was found to be essential for proper synaptic function in the pre-Botzinger complex, a central rhythm organizer located in the brainstem while it was not crucial for the morphology and function of the neuromuscular junction of the diaphragm muscle. TGF-βs have also neuroendocrine functions. Gonadotropin-releasing hormone (GnRH) neurons in the preoptic area contain also TGF-β receptors as well, suggesting that they are fully capable of responding directly to TGF-β1 stimulation. TGF-β1 and -β3 also co-localize with arginine vasopressin in magnocellular neurons of the supraoptic and paraventricular nuclei of the hypothalamus suggesting that TGF-β secreted by the neurohypophysis might regulate the proliferation and secretion of certain anterior pituitary cells.

Furthermore, a diurnal pattern of expression of TGF-β was found in the suprachiasmatic and paraventricular nuclei of young animals, a rhythm that was not observed in older mice suggesting a diurnal and age-dependent function of the TGF-β system in these nuclei. Among the potential neural functions of TGF-βs, their involvement in neuroprotection is the best established. TGF-β1 administered into the brain reduces the infarct size in experimental models of ischemia, whereas antagonizing the endogenous action of TGF-β1 with the injection of a soluble TGF-β type II receptor, which binds TGF-β1 and prevents its biological actions, resulted in a dramatic increase in infarct area. Since some of the experiments did not differentiate between the subtypes, a role of TGF-β2 and -β3 is also conceivable. The possible mechanisms include anti-inflammatory actions, promotion of scar formation, anti-apoptotic actions, protection against excitotoxicity, and the promotion of angiogenesis and neuroregeneration. In addition, although the evidence is scarce, TGF-βs, as endogenous neuroprotective proteins, could participate in ischemic tolerance or preconditioning, too. The role of TGF-βs are also investigated in several pathologic conditions. High-grade human gliomas secrete TGF-β and can activate latent TGF-β. Yet, they are resistant to its growth inhibitory effects as they develop mechanisms that change the anti-proliferative influence of TGF-β into oncogenic cues. It is likely, that TGF-βs are involved in several neurodegenerative diseases, e.g. Alzheimer`s and Lewy body

dementia, Huntington`disease, amyotrophic lateral sclerosis and Pick`s dementia.

II. OBJECTIVES

- The distribution of TGF-βs in the central nervous system are not investigated on the mRNA level as yet, although they significance in brain functions are from growing evidence. Up to the present the distribution of TGF-βs were studied on protein level by immunocytochemistry. Therefore, the first objective of our studies was to examine the TGF-β1, -β2 and -β3 mRNA expression in the normal rat brain by radioactive in situ hybridization histochemistry. Furthermore, to compare the distribution pattern of the mRNAs
    - with the distribution of TGF-β immunoreactivity known from the literature.
    - with the distribution of LTBP1, LTBP2, LTBP3 and LTBP4 mRNA, which were previously examined by our research group.
- To understand the neuroprotective functions of TGF-βs, we examined the induction of TGF-β1, -β2 and -β3 mRNA expression after middle cerebral artery occlusion (MCAO). Our questions were the following:
    - What is the time course of induction of TGF-β1, -β2 and -β3 mRNA following MCAO? In situ hybridization

histochemistry was used at 3h, 24h, 72h, and 1 month after MCAO to address this question.

- Ischemia itself or the reperfusion evokes the induction of TGF-ßs after MCAO? To answer this question we examine the induction of TGF-βs after 1h transient and permanent (24h) MCAO in the rat brain.

III. METHODS

All procedures involving rats were carried out according to experimental protocols approved by the Animal Examination Ethical Council of the Animal Protection Advisory Board at the Semmelweis University, Budapest, and meet the guidelines of the Animal Hygiene and Food Control Department, Ministry of Agriculture, Hungary. Focal ischemia was induced by using a modification of the intraluminal suture method of the described previously (Longa et al., 1989). Briefly, left common, internal, and external carotid arteries were exposed. A silicon rubber-coated monofilament was inserted through the common carotid artery into the internal carotid artery 18–20 mm beyond the carotid bifurcation to the base of the middle cerebral artery. The clip and the monofilament were removed 1h later for transient ischemia and left in place for 24h for permanent ischemia. After the required survival period (3, 24, 72 h and 1 month after transient MCAO or 24 h permanent ischemia), the brains of the rats intended for single in situ hybridization histochemistry were dissected and cut coronally at 1 mm rostral to bregma. The anterior

parts of the brains were stained with 2,3,5- triphenyltetrazolium chloride (TTC) and the posterior parts were frozen for in situ hybridization histochemistry. To investigate the distribution pattern of TGF-βs we performed a radioactive in situ hybridization histochemistry. For the probe preparation total mRNA was isolated and cDNA was synthesized by reverse transcription. The cDNA was used as template in PCRs which were performed for each TGF-β with 2 specific primer pairs. Then, the PCR products were multiply in cloning vectors. [35S]UTP-labeled riboprobes were generated from the above-described DNA probes by in vitro transcription. After hybridization and washes, slides were dipped in NTB nuclear track emulsion, then, the slides were developed counterstained with Giemsa.

IV. RESULTS

1. Probe preparation

RT-PCR demonstrated the expression of all three types of TGF-βs in the cerebral cortex of rats using two primer pairs for each TGF-β The resulting PCR products were used to develop two non-overlapping hybridization probes for each TGF-β. The primers were chosen to generate probes that recognize all known RNA species for the particular gene. The two antisense probes for each TGF-β resulted in identical hybridization patterns. Therefore, they will not be separately described. The resulting PCR products are intron-

spanning to allow us to recognize potential genomic DNA contamination by its larger size. PCR products were run on gel to control, that the calculated length of the probes are corresponding to the calculated values. At the end, the identities of the cDNA probes were verified by sequencing them with T7 primers.

2. Topographic distribution of the expression of TGF-βs in the normal rat brain

In situ hybridization histochemistry revealed the distribution of mRNA of each TGF-β. The distribution patterns of the three TGF-βs overlapped to some degree but were generally different from each other. In the cerebral cortex, mRNAs of all three types of TGF-β were found. A cortical layer-specific expression was observed for each TGF-β. Layer I contained no TGF-βs. TGF-β1 was most abundant in layer V, and it was also present in layers II and VI. TGF-β2 showed a very-high-intensity signal in the inner part of layer V and layer VI and much less intense labeling in other layers of the cerebral cortex. In contrast, the expression of TGF-β3 was high in the outer part of layer V. In addition, TGF-β3 showed a marked expression in layer VI immediately above the cerebral commissure. All three types of TGF-βs were also expressed in the hippocampus, but their intensity and distribution showed great variations. TGF-β1 was abundantly expressed throughout the hippocampus. TGF-β2 had high level of mRNA in the dentate gyrus, but it was present in only some scattered cells in other parts of the hippocampus. TGF-β3 was

abundantly expressed in the CA2 regions of the hippocampus and was also present other regions in the pyramidal cell layer. TGF-βs were also expressed in other parts of the forebrain but demonstrated distinct expression patterns. TGF-β1 expression was present throughout the amygdala, with a particularly intense signal in the central amygdaloid nucleus, especially its medial subdivision. TGF-β3 mRNA-containing neurons were also present in several amygdaloid nuclei; however, the most abundant expression was observed in the basal and lateral amygdaloid nuclei. In contrast, TGF-β2 mRNA had a relatively high expression level in the anterior amygdaloid area. The caudate-putamen, the globus pallidus, and the claustrum were devoid of the mRNA of TGF-βs. The thalamus was a rich source of all TGF-βs, with major differences in their distributions. TGF-β1 expression was relatively even throughout the thalamus at low to moderate levels, except for the lateral geniculate body, where it was absent. In contrast, TGF-β2 was very highly expressed in the parafascicular nucleus, and its mRNA was also abundant in the midline thalamic nuclei. However, it showed only a low level of expression in most other thalamic nuclei and was absent in the ventral nuclei. In general, TGF-β3 had the highest level of expression in the thalamus; this subtype was abundant in several thalamic nuclei, including the anterior, midline, reticular, and ventral nuclei. In the hypothalamus, the expressions of all three types of TGF-βs were highly localized. TGF-β1 mRNA was present in the medial preoptic area, with a very high abundance in the medial

preoptic nucleus. This subtype was also highly expressed in the paraventricular nucleus, especially in its parvicellular subdivision, but showed only low expression levels or was absent in other hypothalamic nuclei. In contrast, TGF-β2 mRNA expression was the highest in the posterior hypothalamic area and the medial subdivision of the medial nucleus of the mamillary body. Apart from these regions, only the medial preoptic area and the supraoptic nucleus contained considerable amounts of TGF-β2 mRNA, whereas the arcuate nucleus, the lateral hypothalamic area, and the ventromedial and dorsomedial nuclei were devoid of the mRNA of TGF-β2. A particularly intense labeling of TGF-β3 was present in the supramamillary nucleus, and the TGF-β3 signal was also high in the arcuate nucleus .Apart from these regions, TGF-β3 expression was also found in the preoptic, anterior, lateral, and posterior hypothalamic areas and the paraventricular nucleus but was absent in the supraoptic, dorsomedial, and ventromedial nuclei. In the midbrain, mRNAs of all three types of TGF-βs were present with characteristic distributional patterns. TGF-β1 was the dominant form of TGF-βs in the substantia nigra, the red nucleus, the ventral tegmental area, and the interpeduncular nucleus, where its expression level was considerably high, whereas TGF-β2- and -3-expressing cells were very scarce. A lower intensity of TGF-β1 signal was found in the zona incerta, the pretectal area, the periaqueductal gray, the raphe nuclei, the inferior colliculus, and the cuneiform nucleus. In contrast, TGF-β2 expression was very high in the dorsal and median

raphe nuclei; moderate in the periaqueductal gray, oculomotor nuclei, inferior colliculus, and cuneiform nucleus; and low in the zona incerta, pretectal area, and superior colliculus. TGF-β3 was also present in several other midbrain regions, but only with low to moderate intensities. In the pons and medulla oblongata, TGF-βs were relatively widely expressed, and their expression patterns showed similarities in these brain regions. Differences were found in the locus coeruleus, the gigantocellular reticular nucleus, and the nucleus of the solitary tract had high levels of expression of TGF-β2 but only moderate amounts of mRNA of TGF-β1 and -β3. In contrast, the pontine nuclei were devoid of TGF-β2 but contained a moderate amount of TGF-β1 and -β3. The most conspicuous finding about TGF-β expression in the medulla oblongata was the very-high-intensity expression of TGF-β3 in the inferior olive, which contained only low levels of mRNA of TGF-β1 and no TGF-β2. In the cerebellum, the highest expression level was found for TGF-β, the Purkinje cell layer. Apart from TGF-β2, the cerebellum contained only a few scattered cells labeled with TGF-β1 in all layers, and TGF-β3 mRNA was absent in the cerebellum. The choroid plexus contained a very high density of cells expressing mRNA of TGF-β2 A much less intense signal was found for TGF-β1 and -β3. Labeling for TGF-βs in the choroid plexus had the same intensity in all ventricles.

3. Comparison the distribution pattern of the mRNAs with the distribution of TGF-β immunoreactivity known from the literature

The distribution pattern of the mRNA of TGF-βs resembles that of TGF-β immunoreactivities in several brain regions. TGF-β2 and -β3 immunoreactivities were present constitutively in cerebral cortical layers II, III, and V, and their expression depended on the cortical layer rather than the areas within the cerebral cortex, as we found for TGF-β mRNAs. Furthermore, different regions of hippocampus, as well as widely distributed cells in the hypothalamus and amygdala, contained TGF-β2 and -β3 immunoreactivities, which correlate well with the presence of TGF-β mRNAs in these regions. Intense immunolabeling of TGF-β was also found in cerebellar Purkinje cells, brainstem monoaminergic neurons, and motor nuclei. All these regions contained particularly high level of mRNA of TGF-β2. In turn, the striatum, most thalamic nuclei, and the superior colliculus were almost devoid of TGF-β2 mRNA, which is consistent with the lack of TGF-β2 immunoreactivities in these regions. Apart from these similarities, we also found considerable differences between the distribution of mRNAs and immunoreactivities of TGF-βs. Most importantly, TGF-β1 immunoreactivity was reported to be present constitutively only in meninges and the choroid plexus in the brain, but we found a more widespread expression of the mRNA of this subtype. Furthermore, TGF-β2 and -β3 immunoreactivities entirely overlapped and, in general, were found in large multipolar

neurons, the level of TGF-β2 being considerably higher. In contrast, we found significantly different expression patterns of TGF-β2 and -β3 mRNAs. Insofar as the immunoreactivities were found in cell bodies rather than fibers, it is not likely that the differences stem from the transport of TGF-βs from the cell bodies. In turn, the different sensitivity and selectivity of in situ hybridization histochemistry and immunocytochemistry could be the underlying reason of the reported differences.

4. Distribution of TGF-β expression in relation to LTBPs in the brain

We previously described the localization of mRNA of the four subtypes of LTBPs in the rat brain. The four subtypes of LTBPs had distinct distribution patterns, which allow the comparison of the distribution of TGF-β and LTBP subtypes. Brain areas that express one of the subtypes predominantly provide the best opportunity for suggesting the possibility of subtype-specific co-expressions. In conclusion, our comparisons suggest that all three subtypes of TGF-βs can bind to LTBP3 in the brain. In addition, TGF-βs can also bind to other types of LTBPs in certain brain regions.

5. Time course of induction of TGF-β1, -β2 and -β3 mRNA following middle cerebral artery occlusion in rat

A 1h occlusion of the middle cerebral artery resulted in a lesion that was histologically visible 3h after the onset of the

occlusion. TGF-βs were not present within the infarct area but TGF-β1 was induced in the area around the lesion. The induction of TGF-β1 was striking in the non-lesioned area of the caudate putamen. In contrast, the distribution of TGF-β2 and -β3 mRNA did not change compared with the distribution in the intact brain. A lesion that includes a large part of the caudate putamen as well as a large part of the ipsilateral cerebral cortex was found 24h after MCAO. TGF-β1 mRNA was detected in the peri-infarct area around the lesion in all cerebral layers as well as in the caudate putamen. TGF-β2 was also induced around the infarct area in response to MCAO in the cerebral cortex but not in the caudate putamen. The induction of TGF-β2 were most intensively in the II and V cortical layer, some positive cells appeared also in layer III. Induction of TGF-β3 was visible only in a few cells in layer II of the cerebral cortex in the vicinity of the lesion. The induction of TGF-βs became more pronounced than following transient MCAO. TGF-β2 induction remained restricted to layers II, III, and V of the cerebral cortex. but it was enhanced up to the midline in the ipsilateral cerebral cortex. A similar induction pattern of TGF-β3 was observed in layer II of the ipsilateral cerebral cortex. At 72h after MCAO, TGF-β1 expression remained elevated around the lesion. In addition, a number of TGF-β1 mRNA expressing cells appeared within the infarct area and in the corpus callosum. In parallel, the levels of TGF-β2 and -β3 mRNA showed a tendency to return to the intact levels of expression in the cerebral cortex. 1 month after transient MCAO TGF-β1 expression remained

very high within the infarct area. TGF-β2 and -β3 were no longer induced in the cerebral cortex but their pre-lesion level of expression was retained.

V. CONCLUSIONS

1. We demonstrated that TGF-βs are expressed in the intact rat brain.
2. The TGF-β1, -β2 und -β3 mRNA expression shows an individually topographically distribution in the rat brain, which suggest a spatially regulated expression.
3. We found significantly different expression patterns of TGF-β mRNA expression and previously reported TGF-β immunoreactivities. Insofar as the immunoreactivities were found in cell bodies rather than fibers, it is not likely that the differences stem from the transport of TGF-βs from the cell bodies. In turn, the different sensitivity and selectivity of in situ hybridization histochemistry and immunocytochemistry could be the underlying reason of the reported differences.
4. The comparisons of distribution of TGF-βs expression in relation to LTBPs in the brain suggest that all three subtypes of TGF-βs can bind to LTBP3 in the brain. In addition, TGF-βs can also bind to other types of LTBPs in certain brain regions.
5. Endogenous TGF-β1, -β2 and -β3 are expressed in brain tissue following a focal ischemic lesion caused by MCAO in a temporally and spatially regulated manner. This suggests that their inductions

requires distinct mechanisms and they have different roles in neuroprotection.

6. The induction of all 3 types of TGF-ßs became more pronounced by permanent occlusion, as opposed to 1h occlusion followed by reperfusion. It is likely that ischemia itself rather than reperfusion evokes the induction of TGF-ßs.

VI. PUBLICATIONS RELATED TO THE THESIS

1. **Vincze C**., Pál G., Wappler E., Szabó E. R., Nagy Z., Lovas G., Dobolyi A. (2010). "Distribution of mRNAs encoding transforming growth factors-β1,-2, and-3 in the intact rat brain and after experimentally induced focal ischemia." *J. Comp. Neurol.* 518(18): 3752-3770. IF: **3,774**

2. Dobolyi A., **Vincze C.**, Pál G, Lovas G. (2012). "The neuroprotective functions of transforming growth factor Beta proteins." *Int. J. Mol. Sci.* 13(7): 8219-8258. IF: **2,464**

3. Pál G., **Vincze C.**, Renner É., Wappler E., Nagy Z., Lovas G., Dobolyi A. (2012). "Time course, distribution and cell types of induction of transforming growth factor betas following middle cerebral artery occlusion in the rat brain." *PLoS One* 7(10): e46731. IF: **3,730**

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