Connections of asztroglia and connective tissue

PhD Thesis

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1. Introduction

To form tissues in multicellular organisms the cells have to recognize the adjacent cells and the surrounding extracellular matrix but they have to form contacts with them. The cell contacts are dynamical since they may dissolve and reorganize upon request.

In the central nervous system the connections to the connective tissues have a great importance, along the meninges and the vessels penetrating the brain. The difference between the relations of the connective tissue and the central nervous system on one hand and the peripheric nervous system on the other hand underlies at least in part their different regenerative capabilities. In the central nervous system the astroglia take the task to form contact the surrounding connective tissue.

2. Scientific background

The term astroglia comprises the major elements of the central nervous tissue which are capable of GFAP (glial fibrillary acidic protein) production under proper influence, or ancestors of cells like this. GFAP is the typical intermediate filament protein and immunohistochemical marker of astroglia. During development and in reaction to lesions astroglia also produces vimentin and nestin.

Astroglia are multifunctional. Beside others, astroglia promote and guide vascularization, induce and support the formation of the blood-brain barrier, has a decisive role in the reactions to lesions, the formation of the post-lesion scar. The central nervous system is almost completely covered by astroglial endfeet, which separate it from both the meninges (membrana limitans gliae superficialis), and from the cerebral vessels (membrana limitans gliae perivascularis). The two systems are actually continuous with each other where the vessels enter the brain (spaces of Virchow-Robin).

The extracellular matrix in the central nervous system has a decisive role in the cell migration, axon growth, vascularisation and post-lesional reactions. It forms a thin layer between the cells. The extracellular matrix as well as the basal lamina adjacent to the pia mater and the cerebral vessels is produced by the astroglia. The two main adhesive glycoproteins are the laminin and the fibronectin.

Laminin consist of three polypeptide chains (α,β,γ). The chains have isoforms, therefore by their combinations multiple types of laminins can be formed, until now 15 types have been described, being specific for localisation and function. The fibronectin interconnects the fibers to the other elements of the connective tissue.

In the cell membrane there are laminin receptors: integrin and non-integrin type receptors. Integrin is a transmembrane protein which anchors cytoskeletal elements by its intracellular part. It consists of two subunit, α and β, and both have several isoforms, their combinations results multiple types of integrins, being specific for localization and function The most important non-integrin laminin receptor is the dystroglycan. There is a β-dystroglycan, a transmembrane protein which binds the α-dystroglycan, the laminin-binding element, to the cell membrane. The intracellular part of the β-dystroglycan binds dystrophin. This so-called dystrophin-dystroglycan complex also occurs on perivascular glial end-feet (Hallmann et al. 2005, Milner et al. 2008, Tian et al. 1996), and is essential in the gliovascular connections.

The dystrophin is an actin-binding protein. The full-length dystrophin is 427 kD. There are promoters which start the transcription of shorter dystrophins, which have less amino acids at their N-terminal. These isoforms are distinguished by their molecule mass. In the central nervous system the predominant form is the Dp71 (Austin et al. 1995, Bar et al. 1990, Greenberg et al. 1996, Lederfein et al. 1992). Its splicing variants are Dp71d and Dp71f. Their mapping in glial cells has not been performed yet.

Like epithelial cells, glia are also covered by basal lamina where they are adjacent to connective tissue, i.e. to the pia mater. Where it is not between a cell layer and connective tissue, but between two cell layer, the basal lamina has a modification. There is no reticular lamina which completes it into a basement membrane, the two basal laminae of the cell layers fuse together with their outer surfaces, and a common, ‘composite’ basal lamina is formed. It occurs e.g. in the brain, between the perivascular glia and the endothelium.

The gradually narrowing connective tissue space (space of Virchow-Robin) therefore separates the vessels from the pia mater, and the glial (parenchymal) basal lamina. Where the penetrating vessels have reduced into endothelial tubes the endothelial and glial

(parenchymal) basal laminae fuse, the perivascular space ceases. The fusion does not occur (i.e. the perivascular space persists) at he immature vessels, and in the circumventricular organs, and around lesions.

Lesions in the central nervous system provoke a glial reaction. The glial reaction can be monitored by the immunohistochemical reactions of GFAP (Bignami and Dahl 1976, Mathewson and Berry 1984). The lesions result in the loosening of gliovascular connections, the 'detachment' of the glial endfeet, the leakiness of the blood-brain barrier. Later vascular regeneration occurs and a new membrane limitans (secundaria) is formed, which is thicker, coarser and consists of more layers of glial processes than the primary one did (Carbonell and Boya 1988). These phenomena involve the rearrangement of the connections between glia and connective tissue.

3. Aims of the study

The scientific background emphasized the importance of the glia-connective tissue connections in the formation of the border layers of the central nervous tissue, in the vascularization, and post-lesional reaction. The aim of the study is therefore to find immunohistochemical reactions which are characteristic of the state and functional alterations of the glia-connective tissue connections, especially the gliovascular connections.

Investigations covered:

- In intact, adult animals the cerebral distribution of laminin, β-dystroglycan and Dp71f dystrophin isoform
- During development the immunohistochemical signs of the formation of the gliovascular connections *via* the immunostainings of laminin, β-dystroglycan, fibronectin, nestin and GFAP.
- Gliovascular alterations following lesions *via* the immunostainings of laminin, β-dystroglycan and GFAP.
- Trials to find similar signs by the immunohistochemical reaction of the most frequent integrin subunits αV and β1.

4.Methods

The animals were albino adult and developing (E12-E20, and P0-P20) rats. (E0 – the day of spermapozitivity of vaginal smears after mating, P0 – the day of birth.). Lesions were performed in ketamine-xylasine narcosis by sterile disposable needles, following drilling the skull. The postoperative periods are given at the results of the corresponding experiments. Following intracardial perfusion with buffered 4% paraformaldehyde, and post-fixation, series of coronal sections were cut by Vibratome. Floating sections were processed for fluorescent immunohistochemistry. Investigations targeted the parietal cortex, unless otherwise specified. The photomicrographs were taken by an Olympus BX 51 microscope equipped with a DP50 digital camera, unless otherwise specified.

5. Results:

5.1. Intact, adult animals:

Laminin immunopositivity was not found in their cerebral vessels, either polyclonal anti-laminin-1 or monoclonal anti-β1, -γ1, -α2 and -β2 chain antibodies were applied. There are, however, exceptions: those parts of vessels which were just entering the brain, as well as the meningeal surface itself, and the meningeal vessels, and those circumventricular organs in which there is no blood-brain barrier: subfornical organ, area postrema, median eminence, organon vasculosum laminae terminalis. It is to be noted that neurons proved to be immunopositive in the majority of cases.

Integrin immunoreactivity was not found, either anti-αV- or anti-β1-integrin antibodies were applied. Immunopositivity was not detected in the developing animals either.

Following immunohistochemical reactions against β-dystroglycan the vascular network was labeled thorough the brain, including the circumventricular organs. Meningeal surface but no meningeal vessels were labeled. Neither astrocytes nor their processes were visualized.

To detect dystrophin, the antibodies labeled 5f3 and Dys2 were used. The former one recognizes the splicing variant Dp71f, the latter one recognizes the Dp71d, and every other isoforms, including

the full-length Dp427, by their common C-terminal. On the distribution of the Dp71f no mapping study has been performed yet. Dys2-antibody labeled neurons as 5f3 did it, but no astrocytes. Immunostaining with 5f3 labeled the glia limitans along the meningeal surface, the Bergmann glia, and perivascular glia, including that of the circumventricular organs. Ependyma proved to be immunoreactive along the ventricular system, including the central canal of the spinal cord. Subependymal astrocytes were also visualized. According to the subventricular zone a honeycomb-like pattern was visible, probably due to the labeled cell contours.

Astrocytes independent from the meningeal surface, the ventricular system and the vessels were labeled only few areas: pallidum, habenula, substantia nigra and interpeduncular nucleus

5.2 Developing animals:

At the beginning of the period examined, at intrauterine day 12 (E12), the cerebrovascular network could be visualized well by immunostaining against laminin or fibronectin. The first weak β-dystroglycan immunoreactivity was found on the meningeal surface and in the vessels of the ganglionic hill at E15, but at E16 it appeared throughout the brain. When double labeling of nestin and laminin was applied and investigated by confocal microscope, glial endfeet were recognized at E18, but not yet at E16. By E20 the endfeet had covered the vessels almost completely.

During the first postnatal days at first fibronectin (P0-P5) then laminin (P5-P12) immunopositivity of vessels disappeared, starting in the middle of the cortex and progrediating to the superficial and deep cortical zones. Dystroglycan preserved its immunoreactivity during the whole developmental period as well as in the adult animals.

Concerning the glia, at P7 still radial glia predominate, immunopositive to nestin, but GFAP immunopositive astrocytes were also appearing along the meningeal surface and the vessels. By P12 the radial glia disappeared. Despite of the parallel intervals, no local coincidence was found between the changes in the glial system and the disappearance of either laminin or fibronectin.

5.3. Glial reactions to lesions

Following lesion, by day 2 the β-dystroglycan-immunopositivity disappeared from the neighbouring vessels. GFAP-immunopositive astrocytes demarcated the lesioned area. During the acme of the glial reaction the territories of the β-dystroglycan-immunonegative vessels and that of the reactive astrcytes coincided. Later on, in parallel with the formation of the new glia limitans to cover the lesion, β-dystroglycan-immunopositive vessels were also found in the territory of the glial reaction. By the end of the 3rd week all the vessels displayed immunoreactivity to dystroglycan. No reactive astrocytes proved to be dystroglycan-positive.

Laminin immunopositivity was detected in the vessels around the lesion in three days. Double-labeling studies revealed that immunopositivity to laminin could usually be found in vessels lacking β-dystroglycan, and persisted approximately in the same period. In the lesioned area the vessels displayed laminin immunopositivity only that part where they had not passed yet the new glia limitans, e.g. where gliovascular connections had not formed yet. Having passed the glia limitans and penetrating into the reactive glia, the laminin immunopositivity gradually decreases and replaced by the GFAP immunopositivity of perivascular glia.

Dystrophin immunoreactivity was checked only at postoperative day 7. Near the lesion immunostaining against Dp71f visualized reactive astrocytes, whereas Dys2 antibody was not effective.

When integrin was investigated, near the lesion reactive astrocytes proved to be immunopositive to the αV subunit,but not to the β1 subunit, between the 4 and 14 postoperative days.

6. Discussion

6.1 The explanation of the appearance and disappearance of the laminin immunoreactivity

Since the laminin is an ubiquitous component of vessels, the problem seems to be in its detectability rather than in its absence. Ther are different opinions published:

a) The laminin (at least its determining epitopes) becomes unaccessible for the antibodies (Jucker et al. 1992).

The vessels in either the cavity of the lesion or the intact meningeal surface, however, had been more exposed to the post-fixation than the vessels embedded deeply in the brain tissue, but they are labeled intensely. The laminin immunopositivity of the maturing vessels or those of the circumventricular organs [Krum and Rosenstein 1989, Krum et al 1991], see also our results, also challenges the decisive effect of fixation.

b) In other opinions the laminin immunoreactivity depends on the characteristics of the vessels (Hagg et al. 1989, Krum and Rosenstein 1991, Zhou 1990). There are two possibilities:

At first, the vascular laminin is of that type, which cannot be detected with the reagents used by us. The polyclonal antiserum against laminin 1, which was applied by us, could have detected any laminin containing either $\alpha 1$, or $\beta 1$, or $\gamma 1$ chain,. Neither this, nor monoclonal antibodies against $\alpha 2$, $\beta 1$, $\beta 2$, and $\gamma 1$ chains did not label vessels of the intact brain tissue. There is only one, laminin 5, which has neither $\alpha 1$, nor $\beta 1$, nor $\gamma 1$ chain. In cerebral vessels, however, it was found only by Wagner et al. (1997). It forms, however, basal lamina only in the presence of laminin 6 (Aumailley and Gayraud 1998, Champliaud et al. 1996, Ekblom et al. 1998), which contains $\beta 1$ and $\gamma 1$ chains.

The other possibility is that the lack of laminin immunopositivity in the vessels of the mature and intact brain may be attributed to the unaccessibility of the epitopes for antibodies which cannot enter between the vascular and glial (parenchymal) basal laminae which are fused to each other during development. According to several observations, however, laminin immunopositivity can be detected when the two laminae are not

fused completely: in immature vessels, in the entering segments of vessels at the brain surface (Virchow-Robin spaces), in circumventricular organs, and following lesions (Krum et al. 1991, Shigematsu et al. 1989ab, Zhou 1990)

The laminin immunopositivity was intense in the brain vessels in newborn rats (Krum et al. 1991, Zhou 1990), but gradually disappeared by postnatal day 11 (Krum et al. 1991), i.e. about the cessation of the vascular growth and the formation of astroglio-vascular connections (Bär and Wolff 1972, Caley and Maxwell 1970, Marin-Padilla 1985). Laminin immunopositivity persisted only in the circumventricular organs, where the two basal laminae remained separate (Krum et al. 1991). Our observations are in accordance with this.

Following lesions the gliovascular connections decouple (Jaeger and Blight 1997) Therefore the temporary post-lesion immunopositivity of cerebral vessels to laminin (Krum et al. 1991, Shigematsu et al. 1989ab, Sosale et al. 1988) can be explained by the temporary post-lesion separation of glial and vascular basal laminae. The reactive astrocytes demarcate the destroyed tissue with a new glia limitans (Carbonell and Boya 1988). They also express laminin and form a basal lamina (Bernstein et al. 1985, Jucker et al. 1996, Toti et al. 1998). These structures were clearly observed in our material, as well as the fusion of basal laminae (glial and vascular), in parallel with the disappearance of vascular immunopositivity to laminin. Therefore the disappearance of laminin immunoreactivity may indicate the regeneration of the gliovascular connections, or the maturation of new vessels

In conclusion, the lack of laminin immunopositivity in the vessels of the mature and intact brain may be attributed to the unaccessibility of the epitopes for antibodies in consequence of the formation of gliovascular connections and fused, common vascular/glial basal laminae. Therefore the disappearance of laminin immunopositivity may indicate these processes (a vessel „maturation") during the vascularization of the brain.

The phenomenon that β-dystroglycan immunopositivity is missing in the territory of laminin immunopositive vessels, also suggests the detaching of the gliovascular connection (see later).

Matrix metalloproteinases can cleave β-dystroglycan (see later), and activity promotes the immunohistochemical detectability of cerebrovascular laminin (Mauro et al. 1984).

The laminin immunopositivity of neurons has been mentioned by several authors (Hagg et al. 1989, Jucker et al. 1992 1996, Powell and Kleinmann 1997, Yamamoto et al. 1988, Zhou 1990), although its explanation is still not clear.

6.2. Loss of β-dystroglycan immunostaining is a phenomenon of gliovascular decoupling?

Markedly reduced or absent β-dystroglycan immunostaining was observed recently by Agrawal et al. (2006), in experimental autoimmune encephalomyelitis, at the sites of leukocyte infiltration, and Milner et al. (2008) following focal ischemia. The phenomenon was attributed to the cleavage of β-dystroglycan by matrix metalloproteinases, which could result in a decrease of β-dystroglycan immunoreactivity, as *in vitro* Milner et al. (2008) proved it. Metalloproteinase-2 and -9 remove that fragment of β-dystroglycan, which connects α-dystroglycan, and through it, laminin (Yamada et al. 2001; Zhong et al. 2006) which could result in the decoupling of the gliovascular connections.The gliovascular connections are necessary for the endothelial cells to form blood-brain barrier. The integrity of the dystroglycan-dystrophin complex and the dystroglycan-laminin connection are required for the gliovascular coupling, the stabilization of the cerebral vascular structure, and the sustaining of the blood-brain barrier.

Our results, which were obtained in a different type of lesion than the observations of Agrawal et al. (2006) and Milner et al. (2008) support the postulation that loss of vascular β-dystroglycan immunopositivity is a general phenomenon following cerebral lesions. Therefore, it may be regarded as an indirect marker of the decoupling of the gliovascular connections in different brain damages.

6.3. Mutual enhancement between glial reaction and gliovascular decoupling?

The glial reaction was intense and prolonged in the area, in which vessels lost immunopositivity to β-dystroglycan. Here a mutual enhancement may take place. Under the influence of substances diffusing from the vessels following lesion, microglia (Del Zoppo et al. 2007, Lee et al. 2007), later the reactive astrocytes express matrix

metalloproteinases (Gotschall and Deb 1996; Swanson et al. 2004) which may cleave, among others, β-dystroglycan. In return, the cleavage of β-dystroglycan may enhance both the 'leakage' from the cerebral vessels and the glial reaction. According to Moore et al. (2002), in the absence of β-dystroglycan (in null-mutant mice), the GFAP expression increases. Reactive astrocytes therefore can sustain and prolong the lack of β-dystroglycan and *vice versa*.

These effects may underlie the phenomenon that there is a period, when the territory of the glial reaction coincided with the area where vessels lost their immunoreactivity to β-dystroglycan. This period is comparable to the immunohistochemical and biochemical data of Mathewson and Berry (1984) and Hozumi et al. (1990ab), who localized the plateau of the glial reaction to the second post-lesion week. Hsu et al. (2006) found increased activity of matrix metalloproteinase 2 during the second week in the astrocytes bordering the lesion.

6.4 Immunohistochemical reaction to β-dystroglycan helps to define stages in the post-lesion process

1) Lack of β-dystroglycan immunopositivity precedes the increase of GFAP immunopositivity.
2) In an early phase the GFAP immunopositivity increases even in areas distant to the site of the lesion, where vessels preserve immunopositvity to β-dystroglycan.
3) The territory of glial reaction coincides with the area, in which vessels lost immunopositivity to β-dystroglycan.
4) Immunopositvity to β-dystroglycan is detected again even vessels adjacent the lesion, within the reactive astrocytes.

According to Jaeger and Blight (1997), sealing of the blood brain barrier leakage was not found until day 17 following lesion. This finding is congruent with our observation on the return of the β-dystroglycan immunopositivity following day 18.

6.5 Distribution of Dp71 in the brain of adult rat

The Dp71f dystrophin splicing variant was found in those astrocytes which always express GFAP immunopositivity (Hajós and Kálmán 1989, Kálmán and Hajós 1989): glia limitans, perivascular glia, cirkumventricular organs, subependymal astrocytes, hippocampus, substantia nigra, habenula, interpeduncular nucleus, pallidum, and reactive glia. The astrocytes of the glia limitans (including Bergmann glia) and the perivascular glia (including the circumventricular organs) are basal lamina-producing as well as the reactive glia.

The localization of 5f3 in the dorsolateral corner of the lateral ventricle corresponds to the residual subventricular zone, in which extracellular matrix remains similar to that of embryonic brain, contains laminin as well (Gates et al. 1995, Gressens et al. 1992, see also Mercier et al. 2002: 'fractons'). Here cell production takes still place even in adult animals. The 'honeycomb' pattern visible here can be attributed to the labeling of the cell contours (ependyma was also labeled in this form). The question arises whether the neural stem cells reported several times in the last decade (see e.g. Doetsch 2003, Doetsch et al. 1999, Seri 2001) are Dp71f-containing.

The expression of Dp71f in the reactive glia is never so extensive than that of GFAP (Mathewson and Berry 1984, Moumdijan et al. 1991).

6.6 Investigation of developing animals

Most interestingly laminin has already appeared along the cerebral vessels at embryonic day 12, i.e. earlier than its receptor β-dystroglycan. On the other hand, appearance of β-dystroglycan precedes that of glial end-feet. It seems to contradict to the authors, who localize the β-dystroglycan on the glial endfeet, but not on the endothel of the vessels (Culligan et al. 2001, Hallmann et al. 2005, Sixt et al. 2001, Tian et al. 1996).

The gradual disappearance of fibronectin and later laminin may refer to the gradual narrowing and disappearing of the perivascular space due to the fusion of the vascular and glial (parenchymal) basal laminae until the postnatal day 12 (Krum et al. 1991) during the 'maturation' of the vessels.

The disappearance of the laminin immunopositivity (postnatal days 7-12) occurs in the same time interval with the

replacement of the nestin-containing radial glia by GFAP-expressing astrocytes There is no local coincidence, however, although it would be check easily, because the disappearance of laminin starts in the middle of the cortex and progresses to the superficial and deeper zones.

The immunohistochemical patterns help to distinguish the following stages:
Before E16: fibronectin, laminin
E16-P3: fibronectin, laminin, β-dystroglycan
After E18 – appearance and increase of nestin-positive glial endfeet
P3 – P5 laminin, β-dystroglycan, decreasing fibronectin,
P5 – P7 laminin, β-dystroglycan
P7-P12 β-dystroglycan, decreasing laminin,
P7-P12 nestin/GFAP, radial glia/astrocyta 'switch'
After P12 – like in adult animals.
The data refer to the neocortex, in other areas the processes may follow different schedules.

6.7. Integrins

The subunits αV and β1 which were investigated have importance in the development of vessels, as well as the maintenance of the gliovascular connections (Del Zoppo and Milner 2006; Del Zoppo et al. 2006, Francis and mtsai 2002, Milner and Campbell 2002ab). Despite of it none of the two subunits proved to be detectable in the vessels of developing or intact adult animals. Since integrins are usually detectable by light microscopic immunohistochemistry, technical problems are to be supposed.

The increased immunoreactivity of αVβ3 integrin was found in focal ischemie (Okada et al. 1996), but in endothelial cells. In astrocytes αVβ5 and αVβ8 were detected in immature cell, and in cell cultures (Milner et al. 2001). Appearance of αV subunit in reactive glia seems to be a new finding. It is to be noted that the peak of the αV immunoreactivity was in the same time interval in which the territorial 'coincidence' of the β-dystroglycan-immunonegativity and GFAP-positivity has not formed yet (see 6.3). The increase integrin immunoreactivity may be attributed to a compensation of the eliminated dystroglycan (see 6.2) or the increased access of the immunoreagents to the integrins during the gliovascular decoupling like in the case of laminin (ld. 6.1).

7. New experimental results and conclusions

7.1 List of new experimental results

1) First mapping on the distribution of the Dp71f dystrophin *in situ.* Its localization showed correlations to the GFAP-expression, and to the basal lamina production by astrocytes, including reactive astrocytes. Ependyma and subventricular zone were also labeled.
2) Following lesions, correlations were found between
   a) the appearance of the laminin immunopositivity and the disappearance of the β-dystroglycan immunopositivity;
   b) the disappearance of the β-dystroglycan immunopositivity and the GFAP expression of reactive glia;
   c) the disappearance of laminin immunopositivity and the formation of new gliovascular connections and basal lamina, in the vessels
3) During development the β-dystroglycan immunopositivity appears between the appearance of the laminin immunopositivity and the formation of the early gliovascular connections.
4) The disappearance of the laminin immunopositivity occurs in parallel with the replacement of nestin-containing radial glia with GFAP-containing astrocytes, but there is no local coincidence.
5) The immunoreactivity of the αV integrin-subunit increases in the reactive glia.

7.2 Conclusions

Beyond the new experimental data obtained, let me emphasize the general consequence of our investigations, i.e. applying different histochemical markers the vascularization and the vascular reactions following lesions or other pathological alterations can be monitored and its stages can be distinguished. It may have a great importance in the pathology, especially because the inhibition of vascularization is a possibility in the therapy of tumors, including brain tumors (see e.g. Puduvalli and Sawaya 2000, Wei et al. 2001).

8. Publications of the author

Publications in peer-reviewed papers and concerning the theses:

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Szabó A, Kálmán M Post traumatic lesion absence of β-dystroglycan-immunopositivity in brain vessels coincides with the glial reaction and the immunoreactivity of vascular laminin. *Curr Neurovasc Res* (2008) 5: 206-213 **IF: 2,826**

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