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ARTICLE

Early phenomena following cryogenic lesions of rat brain – a preliminary study

László Tóth[1], Dávid Szöllősi[1], Katalin Kis-Petik[2], Erzsébet Oszwald[1], Mihály Kálmán[1]*

[1]Department of Anatomy, Histology and Embryology, Semmelweis University, Budapest, Hungary,
[2]Department of Biophysics and Radiation Biology, Semmelweis University, Budapest, Hungary

ABSTRACT The cerebrovascular laminin becomes detectable following lesions, whereas the lamina basalis-receptor β-dystroglycan disappears. These alterations may be indirect markers of a glio-vascular detachment which may result in the impairment of blood-brain-barrier. The present study estimates the correlations between the *post-lesion* exudation and the aforementioned phenomena. Lesions were performed in ketamine-xylazine anaesthesia with a copper rod cooled with dry ice. Immediately, or in 5 or 10 min brains were fixed in buffered 4% paraformaldehyde. Immunohistochemical reactions were performed in floating sections. Exudation was estimated with immunohistochemical detection of plasma-fibronectin and immunoglobulins. Glio-vascular connections were investigated with immunohistochemistry (GFAP, S100, glutamine synthetase), and electron microscopy. Laminin immunoreactivity appeared already at immediate fixation. Exudate was found only around the vessels. β-dystroglycan was still detectable. At five-ten minutes the territory of exudate became confluent and dystroglycan disappeared. Some but not all vessels were free of astrocytes. Electron microscopy demonstrated wide perivascular 'spaces'. '*In vivo*' monitoring was attempted with a multiphoton microscope in the Department of Biophysics of Semmelweis University. Astrocytes were labeled supravitally with sulforhodamine 101 so glio-vascular connections were visible. However, neither in the intact brain nor in 30-min *post-lesion* period astrocyte motility was observed.

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KEY WORDS

blood-brain barrier
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post-lesion exudation

Introduction

The blood-brain barrier (BBB) becomes 'leaky' following lesions. The astrocytes, the glio-vascular connections have a basic importance in the formation and maintenance of the blood-brain barrier (Janzer and Raff 1987; Abbott 2002; Abbott et al. 2010). According to Jaeger and Blight (1997), the astrocytic end-feet 'decouple' (detach) from the vessels and a substantial expansion of perivascular spaces becomes evident. It may have role in the impairment of BBB.

Former studies revealed characteristic immunohistochemical alterations of cerebral vessels following various lesions (*e.g.*, stab wounds, cryogenic lesion, excitotoxic lesion, arterial occlusions). Cerebrovascular laminin which usually not detectable in the formaldehyde fixed intact brain tissue except some circumventricular organs becomes detectable in the area of lesion (Sosale et al. 1988; Shigematsu et al. 1989; Krum et al. 1991; Szabó and Kálmán 2004), whereas the immunoreactivity of the lamina basalis-receptor β-dystroglycan which delineates the vessels of intact brain (Uchino et al. 1995; Zaccaria et al. 2001) disappeares from the lesioned area (Szabó and Kálmán 2008; Kálmán et al. 2011; Wappler et al. 2011). These alterations are supposed to be indirect markers of glio-vascular detachment. The phenomena proved to be reversible.

Dystroglycan consists of two subunits: the α-dystroglycan binds laminin and other basal lamina components; the β-dystroglycan is a transmembrane protein that anchors α-dystroglycan to the cell membrane. The β-dystroglycan immunoreactivity delineates the brain vessels but the β-dystroglycan itself is localized in the perivascular glial end-feet (Tian et al. 1996). The β-dystroglycan and other proteins (dystrophin, syntrophin, etc.) form a complex, which is responsible for the proper distribution of ion channels and receptors including the water-pore channel protein aquaporin-4. (For recent reviews, see *e.g.*, Wolburg et al. 2009; Waite et al. 2012). The dystroglycan-laminin connection is necessary for the glio-vascular coupling which is in turn a condition of the formation and maintenance of blood-brain barrier by the endothelial cells (Janzer and Raff 1987; Abbott

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*Corresponding author. E-mail: misimiska@gmail.com

2002; Abbott et al. 2010). Deletion of β-dystroglycan in mice results in discontinuity of the cerebrovascular basal laminae (Moore et al. 2002).

The aims of the present study were to investigate in rats following brain lesion: 1) the correlation between the exudation, the breakdown of the blood-brain barrier and the alteration of the vascular β-dystroglycan and laminin immunoreactivity; 2) whether the change of the vascular β-dystroglycan and laminin immunoreactivity is really a marker of a glio-vascular decoupling; 3) the sequence of these phenomena.

The area of *post-lesion* exudation can be estimated with various tracers. Intrinsic tracers can be immunoglobulins of the animal or the plasma fibronectin (Sundström et al. 1985; Nag et al. 2002) which leave the vessels following the breakdown of the blood-brain barrier, and can be detected by immunohistochemical reaction. The territories labeled by the abovementioned tracers are to be compared in double-labeling experiments to the territory where the cerebral vessels are immunoreactive to laminin but not to β-dystroglycan.

Perivascular glia can be detected by the immunohistochemical staining of GFAP (glial fibrillary acidic protein), the characteristic intermediate filament protein of mature astrocytes (Bignami et al. 1980). However, according to several studies, not all the astrocytes are detectable by this way. It is necessary, therefore, to examine other astroglial markers, *i.e.*, glutamine synthetase, and S100 protein (Ludwin et al. 1976; Martinez-Hernandez et al. 1977). Glio-vascular connections can be studied by confocal microscopy on specimens double-labelled for glial markers *plus* laminin, β-dystroglycan or endothelial marker (*e.g.*, RECA; Duijvestijn et al. 1992).

Electron microscopy and electron microscopic immunohistochemistry were also applied to investigate the correlation between the absence of β-dystroglycan and/or presence of laminin immunoreactivity and presence/absence of glio-vascular connections.

The lesions were cryogenic lesions. A previous study already described the alterations of laminin and β-dystroglycan immunopositivities following longer survival periods (Kálmán et al. 2011). In the present study the *post-lesion* time intervals were: immediate *post-lesion* fixation, or 5, 10, 30 min. There is, however, a time limit, within the immediate *post-lesion* events are impossible to follow confidently with classic, *post-mortem* methods. This early events are to be *in vivo* monitored. The multiphoton microscope allows examination of thick specimens, including living brain. The *in vivo* investigation, however, needs *in vivo* staining. A possibility is a supravital fluorescent labeling. The astrocytes can be labeled with sulforhodamine 101 (Nimmerjahn et al. 2004) whereas the exudation can be detected applying *e.g.,* fluoresceine isothiocyanate (FITC)-dextran. Before the lesion, the observation of the intact brain is also interesting. It can be visualized, whether the glio-vascular connections are stable or dynamic (attaching/detaching), whether the decoupling of the cell processes precedes or follows the breakdown of the blood-brain barrier.

Materials and Methods

Animals

Adult rats (Wistar) of either sex weighing 250 to 300 g were to be used, supplied with food and water *ad libitum,* and kept in artificial 12/12 h light-and-dark periods. All experimental procedures were performed in accordance with the guidelines of European Community Council Directive (86/609/EEC). For multiphoton experiments mice were used kept in similar circumstances since the holder of the instrument was not large enough for rats.

Cryogenic lesions

Cryogenic lesions were performed in deep ketamine-xylazine anaesthesia (20 and 80 mg/kg body weight, resp., intramuscularly). The skin was opened and a bone piece was removed by drilling, so a 'window' was formed on the skull. The lesion was performed on the dorsoparietal cortex by a copper rod adjusted to a stereotaxic instrument and cooled in carbondioxyde 'snow', contacted for 20 sec the brain surface covered only with leptomeninx. Preliminary experiments suggested that the optimal time is 20 sec. In this case the lesions were quite large and similarly sized. Then the bone piece was reposed and the skin sutured. Survival periods were 5, 10, 30 min or in some cases the brains were fixed immediately *post-lesion*.

Fixation and sectioning

Anaesthesia was similar as before. The animals were perfused through the aorta with 100 ml 0.9% sodium chloride followed by 300 ml 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). After perfusion brains were removed and post-fixed in the same fixative for 1-2 days at 4 °C. Then serial coronal sections of 50 μm were cut with Vibratome through the lesioned area, and washed in phosphate buffered-saline 0.1 M, pH 7.4 (PBS, Sigma) overnight. To check whether perfusion has an influence on the distribution of exudate, some experiments were repeated with immersive fixation but it did not affect the immunohistochemical reactions.

Immunohistochemistry

Floating sections were pretreated with normal goat serum diluted to 20% in PBS for 90 min to block the non-specific binding of antibodies. This and the following steps were fol-

lowed by an extensive wash in PBS (30 min, at room temperature). Primary antibodies were diluted to as described in Table 1, in PBS containing 0.5% Triton X-100 and 0.01% sodium azide. Sections were incubated for 40 h at 4 ºC. Fluorescent secondary antibodies (Life Technologies, Eugene, OR, USA; fluorogens: Alexa 488 and 555) were used at room temperature for 3 h. The sections were finally washed in PBS at room temperature for 1 h, mounted onto glasses, cover-slipped in a mixture of glycerol and bi-distilled water (1:1), and sealed with lacquer. Control sections were done by substituting the primary antibody with normal serum. No structure-bound fluorescence was observed in these specimens.

Area of *post-lesion* exudation was estimated with immunohistochemical detection of plasma-fibronectin or immunoglobulins exudated. These labelings were combined with immunohistochemical reactions against laminin or β-dystroglycan.

In double-labeling experiments the corresponding primary antibodies were applied together, otherwise the protocol was similar as before.

Fluorescent microscopy and digital imaging

Slides were photographed by a DP50 digital camera mounted on an Olympus BX-51 microscope (both from Olympus Optical Co. Ltd, Tokyo, Japan), or, in the case of double labelings, by a Radiance-2100 (BioRad, Hercules, CA) confocal laser scanning microscope. Digital images were processed using Photoshop 9.2 software (Adobe Systems, Mountain View, CA) with minimal adjustments for brightness and contrast.

Preembedding electron microscopic immunohistochemistry

In this case 0.5% glutaraldehyde is added to the perfusive solution for a better fixation. The immunohistochemical reaction was performed on vibratome sections according to the avidin-biotinylated peroxidase (ABC) method against β-dystroglycan and laminin. In this case endogenous peroxidase was inactivated with 3% H_2O_2 in PBS, for 5 min, at room temperature, followed by an extensive washing in PBS at room temperature for 30 min. Incubations with normal goat serum and primary antibodies (see in Table 1) occurred as above, except for that Triton X-100 detergent was reduced to 0.1% to decrease the tissue destruction. Procedure continued by applying biotinylated antibodies, followed by the avidin-biotinylated peroxidase complex (both from Vector Laboratories, Burlingam, CA, USA). Both incubations lasted for 90 min at room temperature, and were followed by PBS at room temperature for 30 min. To visualize the immunohistochemical reaction product 0.05% 3,3'-diaminobenzidine-tetrahydrochloride (DAB) and 0.01% H_2O_2 in Tris-HCl buffer (0.05 M, pH 7.4, at room temperature) were used. The peroxidase-reaction was stopped at visual color control replacing the solution by changes of PBS.

Electron microscopic investigation

Following the immunoreactions tissue areas were selected under light microscope, and were cut from the vibratome sections. They were immersed for 30 min into 1% osmium tetroxide solution in phosphate buffer (0.1 M, pH 7.4), then washed in phosphate buffer and dehydrated through a graded series up to absolute ethanol. Following immersion in propylene oxyde (10 min), the tissue pieces were embedded flat into epoxy resin (Durcupan, Fluka). Semithin sections were cut with a Reichert Ultracut S ultramicrotome, the proper wereas were selected and the samples were adequately trimmed, then ultrathin sections were prepared with the same ultramicrotome. Finally, ultrathin sections were mounted on grids. The photomicrographs were taken by a JEOL 100B elecron microscope equipped with a Sys Morada digital camera.

Multiphoton microscopy

The multiphoton microscope is based on the phenomenon that the energy of two photons may randomly summed to achieve the absorption thus the required excitation wavelength is approximately twice of the wavelength required in the case of a one-photon absorption of the dye. That means, red-infrared range can excite molecules in UV-blue-green range. Deep tissues are accessible due to the less light scattering of the longer wavelength light applied. This method makes possible the microscopic investigation of the living, *in situ* brain in the depth of hundreds of micrometers. The instrument (Femtonics 2D-Inverted Multi-Photon Laser Scanning Microscope, INMIND 278850) was provided by the Institute of Biophysics and Radiation Biology of Semmelweis University.

In 2 h before the experiment supravital dyes (sulphorhodamine 101 and FITC-dextran, 70 kDa) were administered through the caudal vein. The skull and envelopes have been removed, and the intact brain was also studied. The lesion was performed as described above. Immediately after lesion the animals were positioned into the instrument.

Results

Area of exudation corresponded to the area of altered vascular laminin and β-dystroglycan immunoreactivity

The lesioned tissue was well recognizable in transparent illumination as a lens-like light area with darker border zone (Fig. 1a). Following a 30-min postoperative period this territory was completely penetrated by exudate (Fig. 1b). Anti-rat

Table 1. The primary antibodies applied in the study.

Against	Type	Supplier	Code	Dilution	Final concentration (μg/mL)
β-Dystroglycan	Mouse*	Novocastra, Newcastle upon Tyne, UK	ncl-b-dg	1:100	0.19
Fibronectin	Rabbit**	Sigma, San Louis, MO, USA	F 3648	1:200	***
GFAP	Mouse*	Novocastra, Newcastle, upon Tyne, UK	ga5	1:100	100
Glutamine synthetase	Mouse*	Transduction Labs., Erembodegem, Belgium	610518	1:100	2.5
Rat immuno-globulin	Goat**	Thermo Scientific, Rockford, IL, USA	31680	1:200	***
Laminin 1	Rabbit**	Sigma, San Louis, MO, USA	l 9393	1:100	5
RECA-1	Mouse*	ABCAM, Cambridge, CO, USA	ab9774	1:1000	100
S100	Rabbit**	Sigma, San Louis, MO, USA	s-2644	1:200	81

*monoclonal, **polyclonal, ***no data (the original concentration is not given by the supplier)

immunoglobulin and anti-fibronectin marked the same area. In the intact brain tissue some vessels are also marked with anti-rat immglobulin and anti-fibronectin immunostaining.

Vascular laminin immunoreactivity was detectable throughout in the area of lesion and the vascular β-dystroglycan immunoreactivity has disappeared (Fig. 1c and d). When laminin and β-dystroglycan immunostainings were combined vascular laminin immunopositivity was usually confined to the vessels not labeled for β-dystroglycan (not shown, see Kálmán et al. 2011). Sections that were double stained against laminin and rat immunoglobulin revealed that laminin marked the vessels in the territory penetrated by immunoglobulin (compare Fig. 1b and c). Similarly, double immunostaining for β-dystroglycan and fibronectin revealed that lack of β-dystroglycan immunopositivity corresponded to the area of exudation.

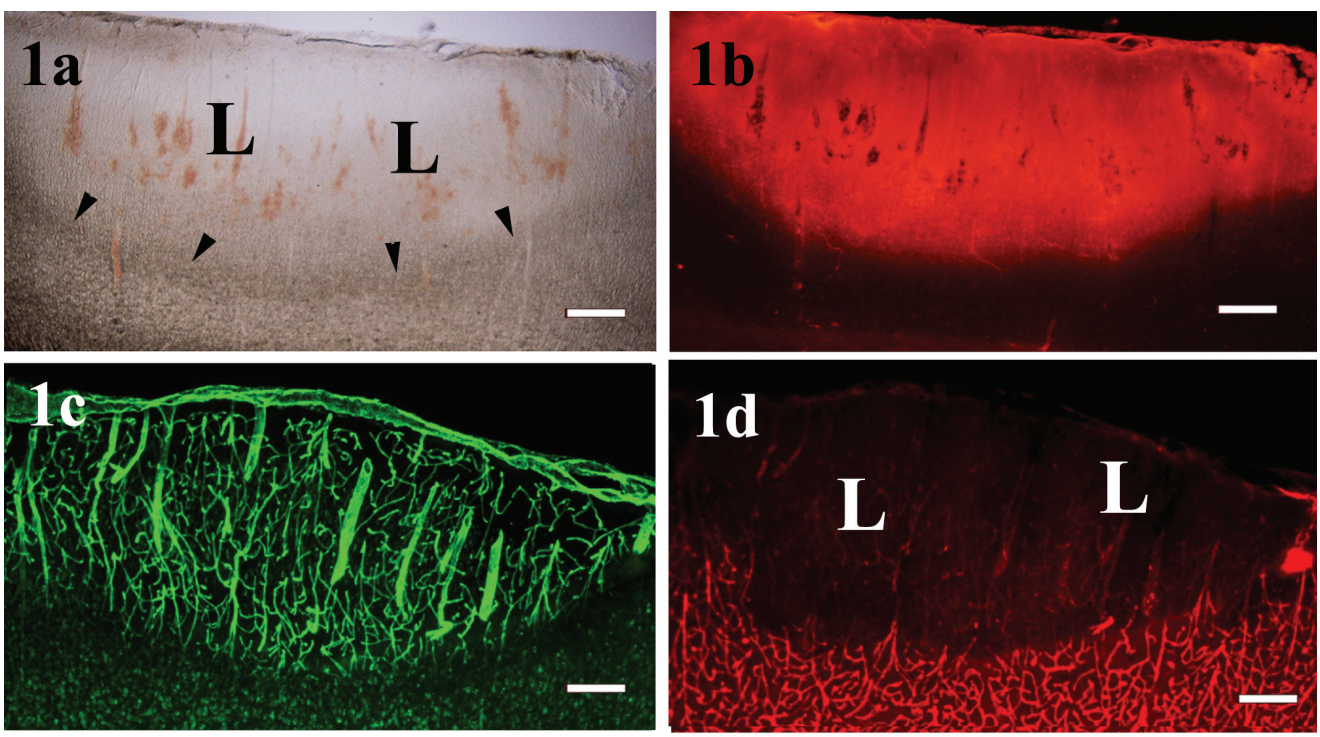

Figure 1. Cryogenic lesions in 30 min after operation. a) Transparent illumination. The area of lesion (L) is lighter, surrounded by a darker border zone (arrowsheads). b) Immunostaining for rat immunoglobulin demonstrates exudation in the whole area of lesion. c) Laminin immunostaining, a number of vessels (probably all of them) are immunopositive to laminin in the territory of lesion but not in the intact tissue. d) β-dystroglycan immunoreactivity is missing in the territory of lesion (L). Apart from this territory the vasculature is intensely labeled. Scale bars: 200 μm.

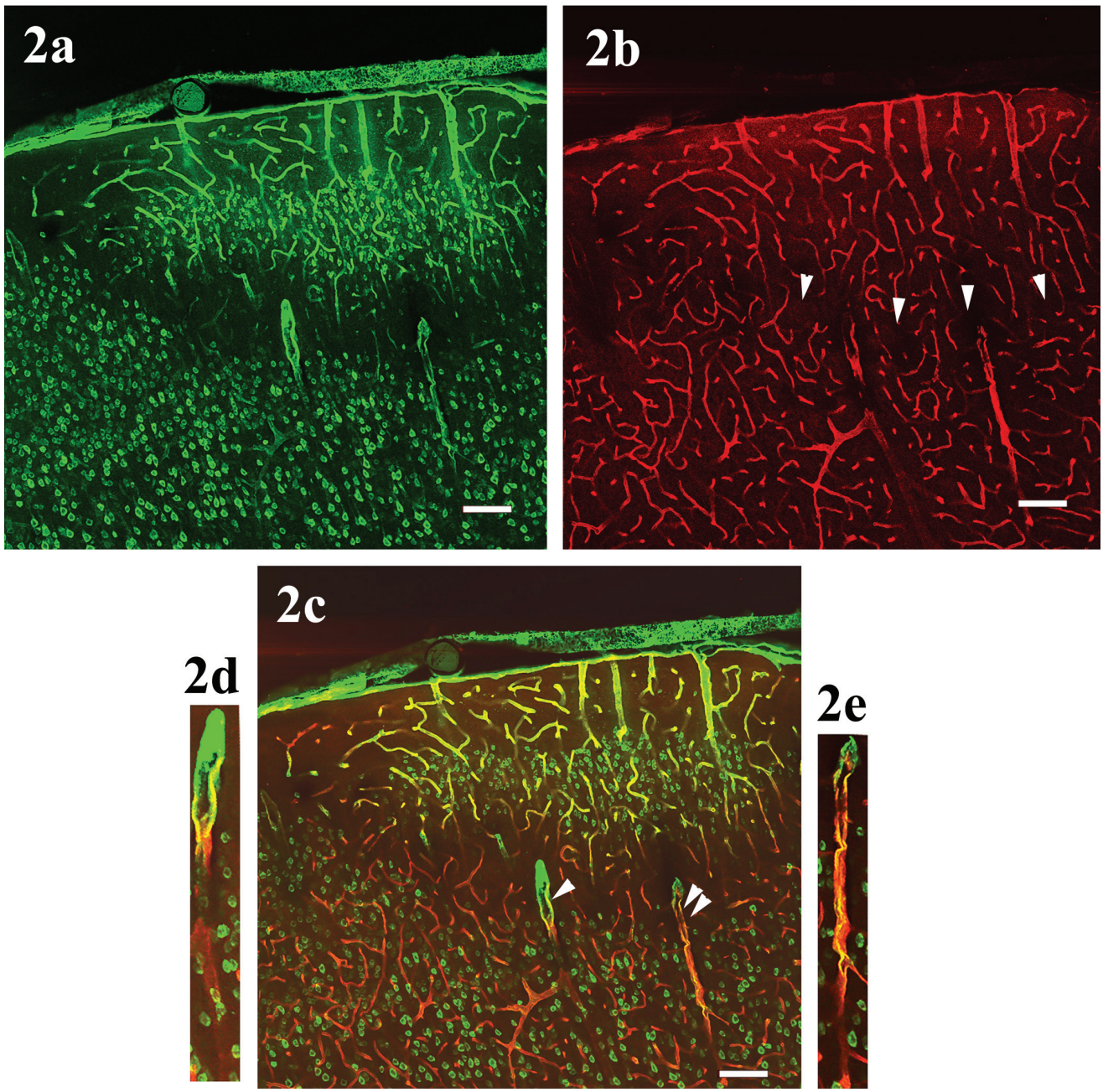

Figure 2. Double labeling for laminin and β-dystroglycan following immediate post-lesion fixation. a) Vascular laminin immunopositivity has appeared. The small green spots (arrowheads) are laminin-immunopositive neurons, it also occurs in the intact brain (see *e.g.*, Powell and Kleinmann 1997). b) The vessels are still immunopositive for β-dystroglycan. c) The merge picture demonstrates that the two immunopositivities are co-localised. d and e) High-power photomicrographs show that change of immunopositivity is gradual. Scale bars: 100 μm; for panels d and e: 50 μm.

Effects of immediate and short-timed fixations: laminin changes abrupt, exudation gradually, later dystroglycan

In the brains fixed immediately following the lesion the laminin immunopositivity already was found in the vessels througout the area of lesion. However, the β-dystroglycan immunopositivity had not disappeared. Even in five minutes several vessels were still immunopositive and even at ten minutes some immunopositive vessels were found. When

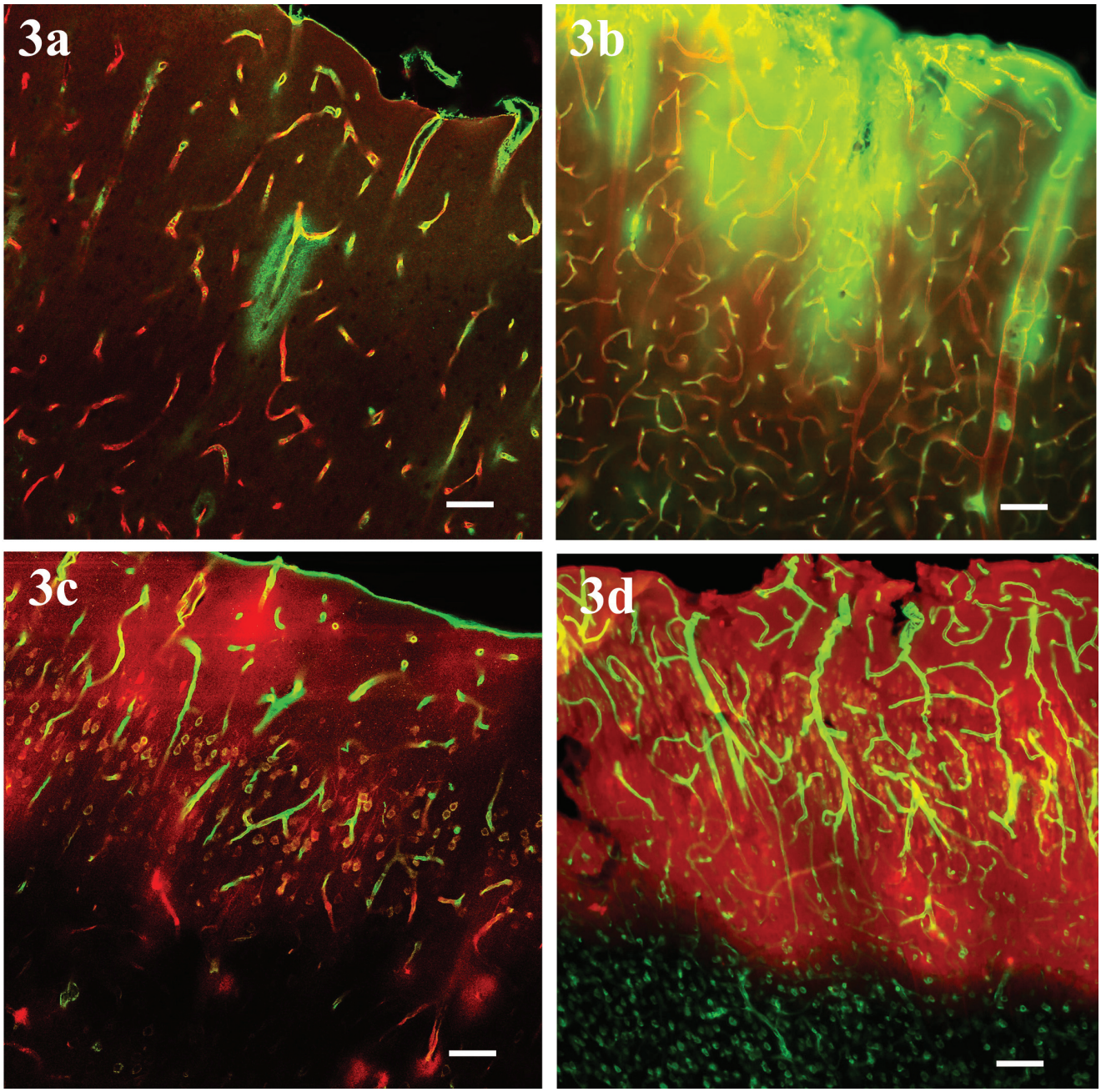

Figure 3. Double labeling for vessels and exudate. a) Immediate fixation. Vascular β-dystroglycan immunostaining (red) still exists but some vessels are surrounded with exudated fibronectin (green). b) In ten minutes the exudate-penetrated areas are almost confluent with each other. c) Immediate fixation, labeling of laminin (green) and rat immunoglobulin (red). The exudate is still confined to strips adjacent the vessels, even not each of them. d) In ten minutes the exudate has penetrated almost the whole area of lesion. Scale bars: 70 μm.

double labeling was applied for laminin and β-dystroglycan immunopositivity (Fig. 2), laminin-immunopositive vessels also proved to be immunopositive for β-dystroglycan. Confocal microscopy demonstrated that the former one appeared at first only discontinuously (Fig. 2c-e).

In these brains the exudate was still confined to stripes adjacent to vessels (Fig. 3a and c), even not each of them. In brains fixed by ten minutes the exudate penetrated continuously almost the whole area of lesion (Fig. 3b and d). Despite the exudation, β-dystroglycan immunopositivity was still detectable in the vessels surrounded with exudate (Fig. 3a and b).

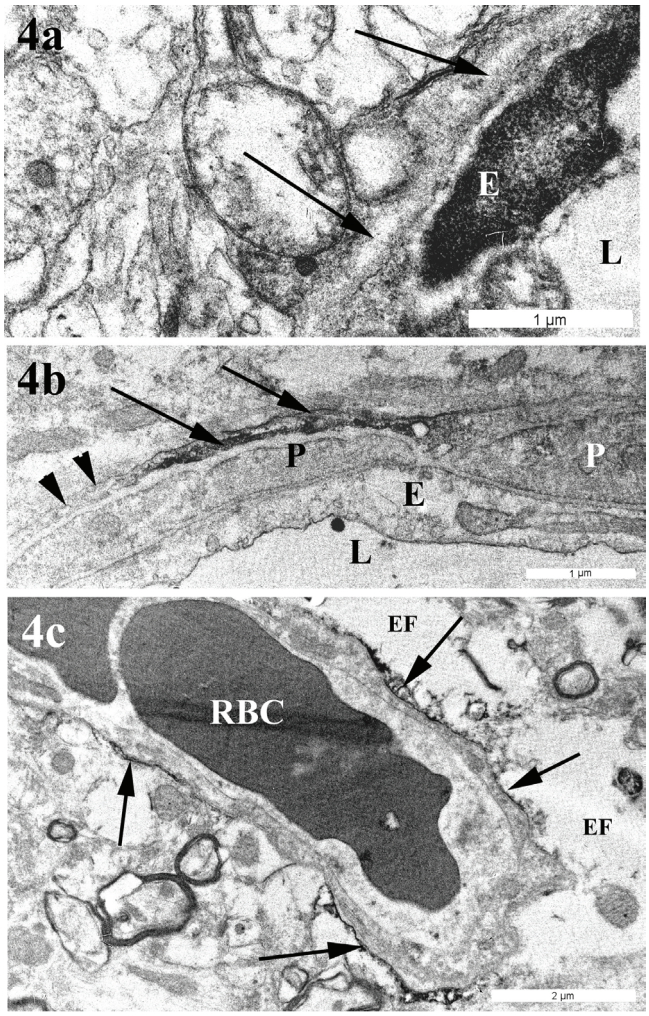

Figure 4. Electron microscopic investigation following immediate post-lesion fixation. a) Gap (arrows) between the glial and endothelial basal laminae in the lesioned area. b) Laminin immunopositivity (arrows) between the separate glial and endothelial basal laminae. Arrowheads point to tight cell contact without immunohistochemical reaction product. c) β-dystroglycan immunopositivity (arrows) around a capillary. Swollen astrocyte endfeet (EF) may imitate 'perivascular gaps'. E – endothelial cell, L – capillary lumen, P – pericyte, RBC – red blood cell.

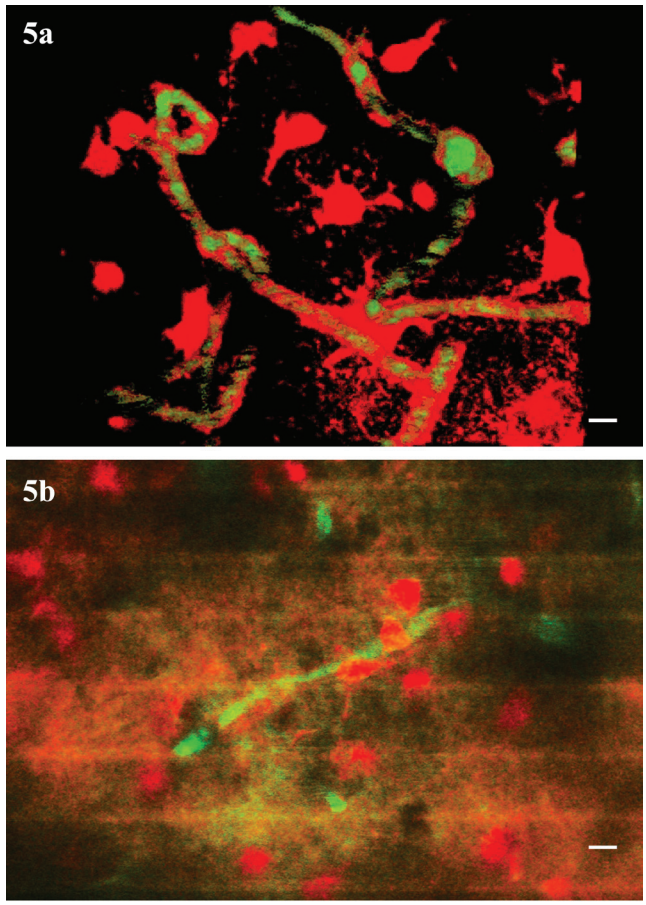

Figure 5. Multiphoton microscopic investigation. a) Intact brain, note the glio-vascular connections. b) Immediately following cryogenic lesion. Exudate almost covers the vessel and the astrocytes. Glia-covered vascular surface is smaller but connections still exist. Astrocytes are labeled with sulphorhodamine 101, vessels with FITC-dextran (Mw 70 kD). Scale bars: 10 µm.

When glial markers were applied, connections were detected on the vessels visualized with RECA or labeled for laminin or β-dystroglycan, although the visualized glial processes did not completely surrounded the vessels (not shown). Astrocytes had not got yet the classical shape of the reactive glia which appears only in a few days *post-lesion* (see, *e.g.,* Kálmán et al. 2011).

Electron microscopic observations

Two different alterations were found around the vessels in the area of the lesion: the glial and endothelial basal laminae were going to separate and the perivascular glial endfeet were swallen so they seemed to be 'perivascular gaps' (Fig. 4a-c). The electron microscopic observations supported the data on the localisations of laminin and β-dystroglycan. Laminin immunoreactivity appeared between the separate glial and endothelial basal laminae (Fig. 4b) whereas β-dystroglycan immunoreactivity was found within the endfeet (Fig. 4c). Such wide, true perivascular spaces, however, which were shown by Jaeger and Blight (1997) we never seen.

Multiphoton microscopy

In the intact brain the FITC-isothiocyanate delineated the vessels, and sulforhodamine 101 visualized astrocyte processes so glio-vascular connections were visible (Fig. 5a).

In the intact brain the glio-vascular connections proved to be stable, no spontaneous detaching/attaching of the processes was observed. Following lesion the bloodstream stopped, hemostasis formed, the vessels became 'leaky' and the tracers penetrated the surrounding area. The glio-vascular connections were not clearly detectable. At the margin of the area of lesion bloodstream and glio-vascular connections were still observed. No motion of astroglial processes were found during 30-min *post-lesion* periods, which overlap the delay resulted by the fixation in the *post-mortem* investigations was observed.

Discussion

Correlations between the phenomena investigated

The results presented here are in accordance with previous data on the immunoreactivities observed in the intact brain, as well as on the post-lesion alterations of laminin and β-dystroglycan (Kálmán et al. 2011). New result is that the territory, where laminin gained immunopositivity and β-dystroglycan lost it corresponded to the area of exudation marked by either fibronectin or rat immunoglobulin immunostaining. Cryogenic lesion caused an immediate leakage of plasma proteins. These alterations become near complete by 10 min. The laminin immunoreactivity appeared before the leakage of the blood-brain barrier which, however, preceded the disappearance of β-dystroglycan immunoreactivity. These phenomena, both β-dystroglycan immunonegativity and laminin immunopositivity, can be regarded as indirect signs of glio-vascular decoupling.

The mechanisms supposed

To explain these phenomena we accept the opinions of Krum et al. (1991) and Milner et al. (2008). In the brain there are two basal laminae around the vessels, an astroglial one and an endothelial one which fuse into a common glio-vascular basal lamina, except in the circumventricular organs and in the Virchow-Robin spaces where laminin immunoreactivity remains detectable. It is supposed therefore that the fusion of the basal laminae has a "hiding" effect on laminin epitopes (Krum et al. 1991) making them unavailable for immunoreagents. The phenomenon that the vascular and glial laminins are detected following lesions may be attributed to a *post-lesion* separation of glial and vascular basal laminae (Krum et al. 1991; Sixt et al. 2001; Hallmann et al. 2005): the laminin epitopes become temporarily 'uncovered'.

Post-lesion disappearance of the β-dystroglycan immunoreactivity is attributed to the cleavage of β-dystroglycan by matrix metalloproteinases (MMP 2 and 9), as Milner et al. (2008) suggested it, demonstrating a decrease of β-dystroglycan level with Western-blot studies. MMP (collagenase) activity also promotes the immunohistochemical detectability of cerebrovascular laminin (Mauro et al. 1984).

Electron microscopic and multiphoton microscopic investigations, however, do not support an abrupt withdrawal of the perivascular glial end-feet in such a manner as figures of Jaeger and Blight (1997) demonstrated it following longer *post-lesion* periods.

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