Neuroprotective effect of Fn14 deficiency is associated with induction of the granulocyte-colony stimulating factor (G-CSF) pathway in experimental stroke and enhanced by a pathogenic human antiphospholipid antibody

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Abstract

Using a transgenic mouse model of ischemic stroke we checked for a possible interaction of antiphospholipid antibodies (aPL) which often cause thromboses as well as central nervous system (CNS) involvement under non-thrombotic conditions and the TWEAK/Fn14 pathway known to be adversely involved in inflammatory and ischemic brain disease. After 7 days, infarct volumes were reduced in Fn14 deficient mice and were further decreased by aPL treatment. This was associated with strongest increase of the endogenous neuroprotective G-CSF/G-CSF receptor system. This unexpected beneficial action of aPL is an example for a non-thrombogenic action and the double-edged nature of aPL.

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1. Introduction

The antiphospholipid syndrome (APS) is an autoimmune disorder characterized by systemic clinical manifestations—including arterial and/or venous thromboses and pregnancy morbidity—as well as by the presence of laboratory markers, such as antiphospholipid antibodies (aPL) or lupus anticoagulant (LA). The syndrome occurs as an isolated autoimmune phenomenon in patients without any underlying systemic autoimmune disease (primary APS) or in combination with other autoimmune diseases, like systemic lupus erythematosus (SLE) (secondary APS) (Krause et al., 2007; Miyakis et al., 2006; Shoenfeld et al., 2008). aPL belong to a heterogeneous group of autoantibodies and it is well known that aPL induce hypercoagulability and proinflammatory responses via direct and/or indirect upregulation of procoagulant and proinflammatory factors in the vascular system. It has been demonstrated that aPL induce activation by direct binding of platelets (Khamashta et al., 1988) and endothelial cells (ECs) (Vega-Ostertag et al., 2007), upregulation of monocyte chemoattractant protein 1 (MCP-1) and Interleukin 8 (IL-8) on ECs, upregulation of tissue factor on monocytes (Clemens et al., 2009), and vitamin binding to phospholipids or phospholipid-binding proteins like beta2-glycoprotein I (beta2-GPI) (Cabezas et al., 1995; Simantov et al., 1995), which may well cause occlusion of blood vessels as for instance in the brain (Rouby and Hoffman, 1997). The presence of LA and elevated aPL levels, e.g. beta2-GPI-dependent antibodies against cardiolipin (aCL) and phosphatidylserine (aPS), is described as a risk factor for a first episode of ischemic stroke (Brey et al., 2001, Saidi et al., 2009). At present, of all the many different neurological symptoms described in APS patients, only cerebral ischemia is accepted for diagnosing APS (Brey et al., 2003). There is substantial evidence that after stroke, apart from procoagulant and proinflammatory antibody-mediated vascular effects, aPL may directly...
interact with neural tissue or microthrombosis-mediated opening of the blood-brain barrier. This pathogenic role of aPL may lead to immediate tissue or cell injury followed by several neurological manifestations (e.g., cognitive dysfunction and dementia). Recent studies have revealed that aPL are able to permeabilize and depolarize brain synaptoneuroses and, thus, lead to cognitive impairment (Chapman et al., 1999, Shoenfeld et al., 2003). Furthermore, there is direct evidence of a pathogenic role of aPL in brain dysfunction in experimental APS (APS) animal models (Katzav et al., in press; Menachem et al., 2009; Tanne et al., 2008). These results corroborate the hypothesis that aPL may play a direct role in the pathogenesis of neurological manifestations of APS.

Worldwide, stroke ranks third among the most common causes of death and is a leading cause of morbidity (Rosamond et al., 2007). In APS patients, it is one of the top three common clinical manifestations and causes of morbidity and mortality (Cervera et al., 2009, Shah et al., 1998). Brain injury due to cerebral ischemia is triggered by a cascade of events, including excitotoxicity, peri-infarct depolarization, inflammation, and apoptotic cell death (Dirmagl et al., 1999). Whether inflammation primarily mediates neuroprotective or neurotoxic events is currently under discussion (Kadhim et al., 2008, Kriz and Lalancette-Hébert, 2009). In this context, the cytokine TWEAK (tumor necrosis factor-like weak inducer of apoptosis), a member of the tumor necrosis factor (TNF) superfamily described for the first time in 1997 (Chicheportiche et al., 1997), and its receptor Fn14, a member of the TNF receptor superfamily (Wiley and Winkles, 2003), are known to be upregulated during stroke in mice but also in humans (Inta et al., 2008; Yepes et al., 2005; Potrovita et al., 2004). Their role in neurotoxicity is presently an area of considerable interest. In vitro TWEAK has been shown to stimulate various cellular responses including proliferation, survival, apoptosis, migration, and differentiation (Polek et al., 2003; Wiley and Winkles, 2003). In neuroinflammation, TWEAK/Fn14-mediated signaling, in vitro and in vivo, has proved to play a major role (Chicheportiche et al., 1997, 2002; Desplat-Jégo et al., 2002; Harada et al., 2002; Kim et al., 2004; Saas et al., 2000; Xu et al., 2004).

In experimental stroke in mice, blocking of TWEAK or deficiency of its receptor Fn14 is thought to have a neuroprotective effect since it invariably leads to a significant decrease of cerebral infarct volumes (Polarvarapu et al., 2005; Potrovita et al., 2004; Yepes et al., 2005; Zhang et al., 2007). Recent work indicates that the TWEAK/Fn14 pathway contributes not only to the pathogenesis of ischemic damage but is also critically involved in the pathophysiology of systemic inflammatory/autoimmune disorders. Blocking this pathway has been shown to significantly reduce the severity of experimental autoimmune myelitis (EAE). In systemic lupus erythematosus, which can be accompanied by secondary APS, TWEAK/Fn14-mediated effects seem to be important for end-organ pathology (for review see Zheng and Burkly, 2008, Serafini et al., 2008). Our experiments were therefore designed first to check for an adverse effect of aPL on postschismic outcome. Secondly, we checked whether interrupting the TWEAK/Fn axis could reverse the hypothesized effect. Using Fn14 receptor knock-out mice (Fn14−/−), we investigated the role of Fn14 in long-term stroke outcome in a model of intracerebroventricularly injected (ICV) administered pathogenic human aPL (HLSB) (von Landenberg et al., 1999) compared to that of a monoclonal IgG control antibody. We further checked for a potentially different regulation of the granulocyte-colony stimulating factor (G-CSF) system which, in most of the studies, has been shown to provide robust neuroprotective effects after experimental ischemic stroke (Minnerup et al., 2008).

2. Materials and methods

2.1. Animal experiments

All animal procedures were carried out according to the guidelines of the German animal protection law. Fn14−/− mice backcrossed for more than five generations on a C57BL/6 background were provided by Biogen Idec (Jakubowski et al., 2005). Wildtype C57BL/6 mice (WT) served as controls. In our present study, altogether 17 adult male Fn14−/− mice and 19 adult male WT mice were used. Mice of both groups were randomly subjected to intracerebroventricular (ICV) injection of either the human monoclonal antiphospholipid IgG2 antibody HLSB (von Landenberg et al., 1999) or a human monoclonal IgG2 control antibody without reactivity to phospholipids/phospholipid-binding proteins (both endotoxin-free). Therefore, mice were anesthetized by intraperitoneal injection of 2.5% tribromoethanol (15 µl per g body weight), HLSB or IgG (1.5 µg in 3 µl phosphate-buffered saline (PBS)) were slowly injected into the left cerebral ventricle (2 mm posterior to the bregma and 2 mm lateral to the midline at the depth of 3 mm) using 10-µl Hamilton syringes. Ten minutes after ICV injection, ischemia was initiated by permanent distal middle cerebral artery occlusion (dMCAO) under the microscope, as previously described (Potrovita et al., 2004). Briefly, a 2 cm-long skin incision was made between the ear and the orbit on the left side. The temporal muscle was removed using electrical coagulation (Modell ICC 50, Erbe, Tuebingen, Germany) and a Burr hole was drilled into the skull. The middle cerebral artery was exposed and occluded by bipolar coagulation (Modell ICC 50, Erbe, Tuebingen, Germany). During the surgery, a body temperature of 37 °C was maintained using a heating pad. Eyes were protected with panthenol ointment (Bepanthen, Roche, Mannheim, Germany). After surgery, the mice were placed under infrared light until full recovery and, then, individually housed in institutional standard cages for 7 days. On day 7, the mice were reanesthetized by intraperitoneal injection of 2.5% tribromoethanol (20 µl per g body weight) and transcendally perfused, at first with Ringer’s solution and then with 4% paraformaldehyde (PFA). Brains were carefully removed and postfixed in 2% PFA for 24 h and, afterwards, stored in 0.5% PFA until sectioning.

Non-ischmic WT mice and non-ischmic Fn14−/− mice (n=3/group) were used for additional assessment of the cerebral vasculature and of baseline hippocampal neuronal cell densities. The mice were asphyxied with CO2 and transcendally perfused with 0.9% NaCl followed by 5 ml of a 10% suspension of black ink in 0.9% NaCl according to Barone et al. (1993). Brains were carefully removed, fixed in 4% PFA overnight at 4 °C and stored in 0.5% PFA until tissue processing.

2.2. Brain tissue processing

Brains of non-ischmic mice were cut at the level of the dorsal hippocampus and one coronal section of 3 mm thickness was subsequently paraffin-embedded. Brains from ischemic mice were first cut into 8 coronal sections of 1 mm thickness using a commercially available rodent brain matrix (Rodent Brain Matrix, Adult Mouse, 30 g, coronal Item no. RMB - 2000 C; ASI Instruments, USA) and were subsequently paraffin-embedded. Four micrometer-thick coronal sections were serially cut and mounted on triethoxysilylpropylamine (TESPA)-coated slides.

2.3. Infarct volume analysis

For determination of brain infarcts, slides were immunohistochemically stained with a MAP2 antibody as described below (Popp et al., 2009). Using the MCID image analysis system (Imaging Research Inc, St. Catharines, Ontario, Canada), the ischemic lesions could be marked on the monitor and infarct areas were automatically determined. At first, total infarct volumes were calculated via multiplying sectional infarct areas by interval thickness, and subsequently expressed as percentage of the ipsilateral hemisphere ± standard deviation (SD).

2.4. Neuropathological evaluation

For assessment of the cerebral vasculature the Circle of Willis was identified in non-ischmic WT and Fn14−/− (n=3/group) animals.
Middle cerebral artery (MCA) trunk and branch as well as the presence/absence of the posterior communicating arteries (PComAs) or other anastomosing vessels were examined as recently described (Barone et al., 1993; Kleinschnitz et al., 2010). The development of left and right PComAs was scored individually as follows: absent: 0; present but hypoplastic or other anastomosing vessels: 1; present and well formed: 2. A single PComA development score was calculated for each animal by averaging both, left and right scores.

For detailed analysis of the hippocampus, sections were Nissl-stained according to standard protocols. Neuronal cell densities within the hippocampal CA1 pyramidal layer were quantitatively assessed at the level of the dorsal hippocampus. Sections were scanned at a magnification of ×280 using a Leica Microscope (Leica, Germany), digitized, and transferred to a computer screen. Viable neurons on two sets of three adjacent regions per area (strictly localized within the pyramidal cell layer of CA1) per hemisphere were recorded, averaged and expressed as mean number of neuronal cells/mm² (‘neuronal density’) ± SD as recently described (Frauenknecht et al., 2009).

In addition, for analysis of white matter changes, sections at the levels of the striatum and the dorsal hippocampus were Klüver-Barrera stained according to standard protocols.

2.5. Immunohistochemistry

Immunohistochemistry was performed using antibodies against MAP2 (monoclonal mouse, HM-2, Sigma Aldrich, St. Louis, MO, USA), Iba-1 (polyclonal, rabbit, Wako, Osaka, Japan), GFAP (polyclonal, rabbit, DAKO, Glostrup, Denmark), C-GSF (polyclonal, rabbit, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and G-CSF receptor (polyclonal, rabbit, Santa Cruz Biotechnology, Santa Cruz, CA, USA), respectively. After dewaxing, sections were incubated with a target retrieval solution (pH 9.0) (DAKO, Glostrup, Denmark) at 95 °C. Then, endogenous peroxidase was blocked by 3%H₂O₂ solution (Merck, Darmstadt, Germany) and sections were treated with FICIN (Zymed, South San Francisco, USA) at 37 °C for digestion of formalin-fixed paraffin-embedded tissues. Non-specific biotin binding was blocked using a commercially available kit (Avidin/biotin blocking kit, Vector, Burlingame, CA, USA). Subsequently, sections were incubated with primary antibodies for 60 min at 21 °C in a humidified chamber using the following dilutions: 1:200 (Iba-1), 1:100 (GFAP), and 1:100 (G-CSF). Immunoreactivity was visualized by the avidin–biotin complex method. Sections were developed in diaminobenzidine (Sigma, St. Louis, USA) with 0.03% hydrogen peroxide and counterstained with Mayer’s Hematoxylin. MAP2 and G-CSF receptor staining was performed using an immunostainer (Dako Autostainer Plus, DAKO, Glostrup, Denmark). Endogenous peroxidase was blocked by peroxidase–block solution (DAKO, Glostrup, Denmark) and sections were treated with primary antisera (MAP2: 1:1000 and G-CSF receptor, 1:150, respectively) at 21 °C for 60 min. Immunoreactivity was visualized by universal enzime–dye polymer method (Nichirei Biosciences, Tokyo, Japan). Finally sections were developed in diaminobenzidine (Lab Vision Cooperation, Fermoant, CA, USA). Omission of the primary antisera in a subset of control slides resulted in no immunostaining at all.

For semiquantitative analysis of hippocampal dendritic integrity, MAP2-stained slides were used (Mateic and Lin, 1994). Within the hippocampal CA1 subfield in both hemispheres, the dendritic layers (stratum oriens and stratum radiatum) were defined as region of interest (ROI). For semiquantitative analysis of microglia (Iba-1) as well as of astrocytes (GFAP), sections at the level of the largest infarct area square dimension were used and six ROIs were defined within the ipsilateral hemisphere (1. hemisphere without infarct; 2. peri-infarct area; 3. remaining hemisphere; 4. dorsal cortical peri-infarct area; 5. subcortical peri-infarct area; and 6. ventral cortical peri-infarct area) and then compared to the corresponding equal areas within the unaffected contralateral side (Fig. 1A). For semiquantitative analysis of G-CSF–R immunoreactivity, four ROIs at the level of the largest infarct area square dimension were defined, including three cortical regions and the region of the striatum in the ipsilateral hemisphere (Fig. 2A). The cellular and regional distribution of G-CSF immunoreactivity was qualitatively assessed at the level of the largest infarct area square dimension.

Images were captured on a Leica Microscope (Leica, Germany) at a magnification of ×17.5 (MAP2), ×1.75 (G-CSF-R) and ×28 (G-CSF) under equal light conditions, digitized, and transferred to a computer screen. Total Iba-1– as well as GFAP-stained brain sections were scanned under equal light conditions with the digital CoolSNAP camera (Roper Scientific, Photometrics CoolSNAP™ MC, Ottobrunn/Munich, Germany). Within the hemispheres, the above-mentioned ROIs were marked on the monitor and optical densities (OD) of MAP2-, Iba-1-, GFAP–, and G-CSF–R-stained slices were determined using the MCID image analysis system (Image Research Inc, St. Catharines, Ontario, Canada). For analysis of MAP2 and G-CSF-R immunoreactivity the OD of the corpus callosum was used as reference value for background staining (ODUSP) and subtracted from total OD in the ROI (ODROT), resulting in specific OD (ODSP). OD values were expressed as mean ± SD.

For analysis of Iba-1 and GFAP immunoreactivity, OD of the negative control, carried along for each animal, was used as reference value for background staining (ODUSP) and subtracted from total OD in the respective ROI (ODROT), as described above. The ratio of OD of each ipsilateral vs. each contralateral ROI was calculated as ‘ROI ipsilateral/ROI contralateral’ and expressed as mean ratio ± SD. G-CSF-positive cells ipsi- and contralateral to the side of the infarct were evaluated. For analysis only viable healthy-looking cells with a vesicular nucleus and positively stained cytoplasm were marked as positive. The G-CSF protein expression was graded by gross visual inspection. Staining intensity of neurons and neuropil was rated as none, mild, moderate, or severe.

2.6. Statistical analysis

Data were analyzed using non-parametric Mann–Whitney test. Post hoc Holm–Sidak two-way ANOVA method was used for all multiple comparisons between the different groups to show interactions between treatment and genetic background. When interaction between factors was significant (‘p < 0.05’), t test comparisons were performed to determine significance between the different groups. Statistical analysis was performed using SigmaStat software (SPSS Inc., Chicago, IL). Data are expressed as mean ± standard deviation (SD).

3. Results
3.1. Animal experiments

In WT mice, one out of eight of the IgG-treated animals and, in Fn14+/− mice, one out of seven IgG-treated and three out of ten HLSB-treated mice died shortly after surgery. Due to the fact that brain slices of 1 mm thickness were difficult to handle during brain tissue processing, brains of one WT-IgG mouse and of two Fn14+/−/−HLSB mice were fragmented. The fragmented brains had to be singled out and excluded from further analysis. In the end, 17 WT mice (WT-IgG: n = 7; WT-HLSB: n = 10) and 11 Fn14+/−/− mice (Fn14+/−/−-IgG: n = 6; Fn14+/−/−-HLSB: n = 5) were included in further analysis.

3.2. Assessment of the cerebral vasculature in non-ischemic mice

Major cerebral arteries were examined in non-ischemic WT and Fn14+/−/− mice. The black ink perfusion technique showed that MCA trunk and branch appeared to be anatomically identical in both genotypes. Differences in PComA development were found among both strains. Patent PComAs as well as hypoplasia or absence of one
PComA could be detected in both mouse strains. The mean PComA scores of the WT mice were nearly identical (0.67±0.58) compared to that of the Fn14−/− mice (0.83±0.29; p=0.678).

### 3.3. Infarct size

Seven days after ICV injection of HL5B or IgG control antibody and subsequent dMCAO, Fn14−/− mice showed a decreased infarct volume in comparison to WT mice (Fig. 3). The largest infarct volumes (% of the ipsilateral hemisphere) were detectable in WT-IgG (n=7), followed by WT-HL5B (n=10) with a 16% decrease in infarct volumes, which did not become statistically significant (15.2±2.8 vs 12.8±4.3; p=0.187). Genetic deficiency of the Fn14 receptor resulted in a significant 52% reduction of infarct volumes in HL5B-injected animals (n=5) compared to WT-IgG animals (n=7) (7.3±4.1 vs. 15.2±2.8; *p=0.003) and a significant 43% reduction of infarct volumes compared to WT-HL5B (n=10) animals (7.3±4.1 vs. 12.8±4.3; *p=0.012). Consistent with previous studies, the infarct volumes in Fn14−/−-IgG (n=6) animals were smaller than those in WT-IgG animals (11.2±4.2 vs 15.2±2.8), with a 26% decrease in infarct volumes of Fn14−/−-IgG animals, although this difference did not achieve statistical significance (p = 0.060). Differences between Fn14−/−-IgG and Fn14−/−-HL5B animals, with a 34% decrease in infarct volumes of HL-5B animals, were statistically insignificant (p = 0.093).

### 3.5. Activation of microglia (Iba-1 immunohistochemistry)

In general, in all experimental groups, Iba-1-stained cells were more frequently detectable within the ischemic hemisphere. Consequently, semiquantitative analysis of Iba-1 protein expression as a sensitive marker for ischemic damage in the dendritic layers of the vulnerable CA1 hippocampal subfield revealed significantly lower cell densities in non-ischemic Fn14−/− mice compared to non-ischemic WT mice (*p = 0.042; data not shown). These significantly lower cell densities were also detectable in the ipsilateral (*p = 0.017) as well as in the contralateral (*p = 0.036) CA1 hippocampal subfield of ischemic Fn14−/− mice compared to ischemic WT mice. Multiple comparisons revealed no significant influence of antibody treatment on neuronal cell densities (p > 0.05; Table 1).

In Kluever-Barrera-stained slices, qualitative analysis of white matter changes outside of the infarct, at the level of the striatum and the dorsal hippocampus, did not reveal any differences between the four groups (not shown).
did not show any significant differences, neither in the various ipsilateral ROIs nor when comparing ROIs in the contralateral hemisphere \( (p > 0.05; \text{data not shown}). \) However, calculation of the ratio between the OD in the ipsilateral and corresponding contralateral ROI exhibited generally higher values in Fn14 deficient mice. The differences between Fn14 \(-/-\) mice and WT mice were statistically significant in ROI 1 \((p = 0.018)\) and ROI 3 \((p = 0.014)\). Multiple comparisons revealed no statistically influence of treatment on these higher microglia activation in Fn14 \(-/-\) mice \((p > 0.05)\) \(\text{(Fig. 1b).}\)

### 3.6. Activation of astrocytes (GFAP immunohistochemistry)

Similar to Iba-1 immunohistochemistry, GFAP-stained cells were more frequently detectable within the ischemic hemisphere in all

### Table 1

Neuronal cell densities per mm² in the vulnerable CA1 subfield.

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>n</th>
<th>Ipsilateral</th>
<th>Contralateral</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT-IgG</td>
<td>7</td>
<td>1375 ± 75</td>
<td>1377 ± 72</td>
</tr>
<tr>
<td>WT-HL5B</td>
<td>10</td>
<td>1363 ± 63</td>
<td>1390 ± 62</td>
</tr>
<tr>
<td>Fn14/-IgG</td>
<td>6</td>
<td>1324 ± 28</td>
<td>1337 ± 36</td>
</tr>
<tr>
<td>Fn14/-HL5B</td>
<td>5</td>
<td>1319 ± 37</td>
<td>1325 ± 62</td>
</tr>
</tbody>
</table>

Note that there are significantly lower cell densities in the CA1 hippocampal subfield in Fn14 deficient mice ipsilateral \((p = 0.017)\) and contralateral \((p = 0.036)\) compared to WT mice but multiple comparisons revealed no significant influence of treatment on these differences \((p > 0.05)\). Neuronal densities are expressed as mean ± SD.
experimental groups. GFAP-stained sections of Fn14-deficient mice treated with HL5B showed significantly lower intensity values in the ischemic hemisphere in ROIs 1, 3 and 5 (not shown) while comparing the other groups and analyzing the contralateral hemisphere did not reveal any differences. Calculating the ratio between ROIs of the ipsilateral and corresponding ROIs of the contralateral hemisphere, as done for Iba-1, did not result in any differences between the four experimental groups. (p > 0.05, Fig. 1c).

3.7. G-CSF-R protein expression

In general, G-CSF-R immunohistochemistry in WT mice revealed positively stained neurons only within a thin rim around the necrotic tissue while the neuropil remained largely unstained (Fig. 2b). Only one animal out of seven (14%) in the WT-IgG group and three animals out of 10 (33%) in the WT-HL5B group showed a larger, positively stained area surrounding the infarct and a positively labeled neuropil in the ipsilateral hemisphere. In Fn14−/− mice, numerous G-CSF-R-positive cortical and striatal neurons as well as visibly stronger staining of the neuropil in the hemisphere containing the infarct were seen in 66% (4/6) of IgG-treated and in all (5/5) HL5B-treated mice. In general, G-CSF-R immunohistochemistry revealed Fn14 receptor knock-out-dependent increase in IR within the ipsilateral hemisphere compared to WT mice. Interestingly, especially HL5B treatment led to the strongest and more widespread G-CSF-R IR compared to the other three experimental groups (Fig. 2b). The contralateral non-ischemic hemisphere remained unstained in all experimental groups.

Semiquantitative analysis of G-CSF-R IR demonstrated overall higher values in the ipsilateral dorsal cortex (ROI 1), cortical peri-infarct regions (ROI 2) and ROI 3 (ROI 1 + ROI 2) and in the striatum (ROI 4) in Fn14−/−, which were even significantly higher after treatment with HL5B in ROI 1 (WT-IgG vs. Fn14−/−-IgG: p = 0.854 and vs. Fn14−/−-HL5B: *p = 0.032, WT-HL5B vs. Fn14−/−-HL5B: *p = 0.024). Remarkably, not only Fn14−/−-HL5B but also WT-HL5B mice revealed significantly higher G-CSF-R IR in the peri-infarct cortex (ROI 2) compared to the WT-IgG group (WT-IgG vs. WT-HL5B: *p = 0.031; vs. Fn14−/−-IgG: *p = 0.004; vs. Fn14−/−-HL5B: *p = 0.008). The striatum (ROI 4) showed significantly higher G-CSF-R staining in Fn14 receptor-deficient mice treated with IgG (*p = 0.007) and higher IR after HL5B antibody treatment in WT (p = 0.055) and Fn14−/− mice (p = 0.052).

3.8. G-CSF protein expression

In HL5B-treated Fn14−/− mice a markedly enhanced neuronal G-CSF staining could be detected in the infarct area, within the peri-infarct tissue and the contralateral hemisphere (not shown). In these Fn14−/−-HL5B animals (Fig. 4d), over 50% of viable neurons in both

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**Fig. 4.** Representative G-CSF stains of the ipsilateral cortical peri-infarct area of all four experimental groups. After administration of IgG in WT mice, (a) less than 10% of neurons were slightly stained (arrowhead) in the cortical peri-infarct area and no staining of the neuropil. HL5B ICV injection (b) revealed more than 10% of moderately stained neurons (arrowhead), and staining of neuropil and cytoplasm was stronger than after IgG treatment. In IgG-treated Fn14 knock-out mice (c) neuropil and positive cells (arrowhead) revealed nearly the same intensity in neurons (slightly decreased) and neuropil compared to WT-HL5B mice. Treatment of Fn14−/− mice with HL5B (d) revealed the strongest staining of neuropil as well as of neuronal cytoplasm, and more than 50% of the neurons in the cortical peri-infarct area stained positive for the G-CSF protein (bar corresponds to 20 µm).
hemispheres exhibited a more intense G-CSF protein expression in the cytoplasm while neurons in the other groups were only slightly (WT-IgG, Fig. 4a) or moderately (WT-HL5B, Fig. 4b; Fn14−/−IgG, Fig. 4c) labeled. Furthermore, staining of the background/neuropil was more prominent compared to the other three experimental groups (Fig. 4a–d). The contralateral hemispheres of Fn14−/−/HL5B animals showed strong IR for G-CSF compared to slight (WT-IgG; WT-HL5B) or moderate (Fn14−/−IgG) IR (not shown).

4. Discussion

While in the past the relevance of aPL was reduced to their thrombogenic potential there is growing evidence for a more complex role of these autoantibodies in a variety of diseases (Horstman et al., 2009). Our present study was designed to check for the impact of the HL5B aPL as compared to a control IgG antibody after experimental ischemic stroke in order to elucidate pathways potentially leading to neurological deficits in patients with APS. One major finding of our present experiments was that genetic deficiency of Fn14 in ischemic stroke was associated with reduced infarct volumes and increased protein expression of G-CSF and its receptor which was surprisingly enhanced by HL5B aPL. These results support the notion that the effects of aPL may be double-edged depending on the respective context including variations in the genetic background.

It is known that the cerebral vasculature varies considerably between different mouse strains and even the degree of PComA plasticity varies among individual mice of a single strain. Recent studies have shown that the variability of intracranial vasculature at the circle of Willis can influence the degree of ischemia in experimental stroke models in mice (Barone et al., 1993; Connolly et al., 1996; Kitagawa et al., 1998) whereas another study showed that the genetic background seems not to be a major confounding factor of infarct size in a MCAO model (Pham et al., 2010). In the direct ligation model of the MCA, Majid and coworkers could show that the presence and patency of PComAs, although variable among different strains, had no influence on the extent of ischemic injury (Majid et al., 2000). In our present study MCA trunk and branch appeared to be anatomically identical among both genotypes and within the different strains whereas differences in PComA plasticity between mouse strains and even among individual mice of each strain could be seen. Scoring of the PComA plasticity showed no significant differences between WT and Fn14 deficient mice. These results support that intrinsic factors (e.g. Fn14 receptor deficiency), other than vascular variability, are considered to contribute to the different ischemic vulnerability among strains.

Due to its thrombogenic properties HLS5 aPL has been primarily suggested to deteriorate outcome after stroke—so the question raised by our study is how is HLS5 able to enhance the neuroprotective effect in Fn14 transgenic mice. The TWEAK/Fn14 cytokine-receptor axis is known to be involved in the regulation of numerous cellular events, including proliferation, differentiation, migration, and cell death (for review see Burdly et al., 2007 and Winkles, 2008). Recent studies revealed that TWEAK and Fn14 receptor expression are increased after focal cerebral ischemia not only in mice but also in humans (Inta et al., 2008; Potrovita et al., 2004; Yepes et al., 2005; Zhang et al., 2007). In this context, inhibition of the TWEAK/Fn14 pathway has consistently been reported to show neuroprotective effects. Using the same model of permanent distal MCAO in mice, as we did in our present study, Potrovita and colleagues could show that intraperitoneal injection of a neutralizing anti-TWEAK antibody significantly reduced infarct volumes after 48 h (Potrovita et al., 2004). Treatment by intracerebroventricular injection of a Fn14-Fc decoy receptor in mouse models of focal cerebral ischemia resulted in marked preservation of the blood-brain barrier (Zhang et al., 2007) with consecutive infarct volume reduction (Yepes et al., 2005). Finally, mice deficient for Fn14 subjected to MCAO developed smaller infarcts after 48 h, compared to wildtype animals (Zhang et al., 2007). In our present study, infarcts in Fn14 deficient mice treated with control IgG antibody were also smaller compared to those in wildtype mice, 7 days after onset of ischemia, although this difference did not reach significance level. Surprisingly, stronger protection of Fn14 deficiency was observed in the current model with the ICV injection of HL5B aPL just prior to ischemia, with significant reduction in infarct volumes as compared to WT mice treated either with control IgG or HLS5 antibody.

Notably, infarct reduction in Fn14-deficient mice was associated with increased postsischemic protein expression of G-CSF and G-CSF receptor which was further enhanced by application of the HL5B aPL. The neuroprotective effect of the hematopoietic growth factor G-CSF after focal cerebral ischemia has now been demonstrated by various groups in a large number of experimental studies (Minnerup et al., 2008). Apart from a reduction of infarct volumes, application of G-CSF has been shown to stimulate neural progenitor cells and improve functional outcome after ischemic stroke in various animal models of ischemic stroke (Schneider et al., 2005). Primarily known as hematopoietic growth factor and commonly used to treat neutropenia, recent work could verify an important role of this factor and its receptor also in the healthy brain (Diederich et al., 2009). Therefore, a causal link can be assumed between increased protein expression of G-CSF and its receptor and the corresponding decrease in infarct volumes.

The link between induction of the G-CSF system and the HLS5 aPL is easy to explain by its known procoagulant and proinflammatory activity. In vitro data indicate that HLS5 induces several cytokines, such as tumor necrosis factor alpha (TNF-alpha), interleukin-18 (IL18), tissue factor (TF), or adhesion molecules (e.g. inter cellular adhesion molecule 1 (ICAM-1)) in different cell types of the vascular system (Clemens et al., 2009; Döring et al., 2010; Hurst et al., 2009). Unpublished in vitro experiments from our group have shown a significant upregulation of G-CSF mRNA as well as a significant release of G-CSF protein from endothelial cells stimulated with HLS5, in contrast to stimulation with monoclonal IgG. Our present study indicates that HLS5 is also able to induce G-CSF and G-CSF receptor protein expression in vivo in neuronal cells thereby providing resistance against ischemic damage. Concerning the effects of autoantibodies in the brain, only one study could supply evidence of a direct cross-reaction of lupus anti-DNA antibodies with NR2 glutamate receptors thereby triggering neuronal death (DeGiorgio et al., 2001).

We also checked whether the neuroprotective effect of HLS5 in Fn14-deficient mice could be explained by a specific modulation of the glial and/or microglial response to the ischemic injury but did not find evidence to support this possibility.

In conclusion, the increased G-CSF and G-CSF receptor protein expression in Fn14-deficient mice, 7 days after focal cerebral ischemia, is suggestive of an additional neuroprotective mechanism, apart from turning off the nuclear factor-κB pathway. Induction of the G-CSF system in Fn-14 deficient mice was further enhanced by the HLS5, in contrast to stimulation with monoclonal IgG. Our present study indicates that HLS5 is also able to induce G-CSF and G-CSF receptor protein expression in vivo in neuronal cells thereby providing resistance against ischemic damage. Concerning the effects of autoantibodies in the brain, only one study could supply evidence of a direct cross-reaction of lupus anti-DNA antibodies with NR2 glutamate receptors thereby triggering neuronal death (DeGiorgio et al., 2001).

Conflict of interest

C. Sommer is the inventor on a patent application concerning treatment of neurological diseases with G-CSF. L. Burkly and M. Schwaninger are the inventors on a patent application concerning treatment of neurological diseases with TWEAK inhibitors.
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