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SUBACUTE TREATMENT WITH VASCULAR ENDOTHELIAL GROWTH FACTOR AFTER TRAUMATIC BRAIN INJURY INCREASES ANGIOGENESIS AND GLIOGENESIS

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Abstract—Vascular endothelial growth factor (VEGF) is neuroprotective and induces neurogenesis and angiogenesis when given early after traumatic brain injury (TBI). However, the effects of VEGF administration in the subacute phase after TBI remain unknown. Mice were subjected to TBI and treated with vehicle or VEGF beginning 7 days later for an additional 7 days. The animals were injected with BrdU to label proliferating cells and examined with a motor-sensory scale at pre-determined time points. Mice were killed 90 days post injury and immunohistochemistry was used to study cell fates. Our results demonstrate that lesion volumes did not differ between the groups confirming the lack of neuroprotective effects in this paradigm. VEGF treatment led to significant increments in cell proliferation (1.9 fold increase vs. vehicle, $P<0.0001$) and angiogenesis in the lesioned cortex (1.7 fold increase vs. vehicle, $P=0.0001$) but most of the proliferating cells differentiated into glia and no mature newly-generated neurons were detected. In conclusion, VEGF induces gliogenesis and angiogenesis when given 7 days post TBI. However, treated mice had only insignificant motor improvements in this paradigm, suggesting that the bulk of the beneficial effects observed when VEGF is given early after TBI results from the neuroprotective effects.

Key words: angiogenesis, growth factor, neurogenesis, neuroprotection, traumatic brain injury, vascular endothelial.

Traumatic brain injury (TBI) is a major cause of morbidity and mortality (Langlois et al., 2004). Neuroprotective strategies have been largely disappointing despite a plethora of mechanisms and drugs explored (Leker and Shohami, 2002; Flierl et al., 2009).

Repair mechanisms based on the proliferation of endogenous cells have been explored after brain injury (Dash et al., 2001; Chirumamilla et al., 2002). However, spontaneous neurogenesis is not sufficient to induce meaningful recovery (Shetty et al., 2004). Therefore, augmenting neurogenesis is a reasonable approach to improve functional outcome following TBI. Vascular endothelial growth factor (VEGF) is expressed in the CNS following injury (Sköld et al., 2005; Wang et al., 2005; Lafuente et al., 2006; Dore-Duffy et al., 2007) and induces angiogenesis (Nag et al., 1997; Nieto et al., 2001; Kim et al., 2004). VEGF may also have beneficial effects on the survival of newborn neuronal precursors (Widenfalk et al., 2003) and has been implicated in neurogenesis (Wang et al., 2007; Xiao et al., 2007) and neurite outgrowth (Jin et al., 2006). Furthermore, VEGF has important implications in increasing the size of the subventricular zone (SVZ) (Gotts and Chesselet, 2005) and inhibition of VEGF expression after injury may actually exacerbate outcome (Sköld et al., 2006). In a previous study we have shown that exogenous VEGF induces neuroprotection, angiogenesis, and neurogenesis when given immediately after TBI (Thau-Zuchman et al., 2010). In the current set of experiments we wished to extend our observations by differentiating between these effects. To this end administration of VEGF was begun at 7 days post TBI, thus, eliminating the neuroprotective effects and isolating the neurogenic and angiogenic mechanisms.

EXPERIMENTAL PROCEDURES

Animals and traumatic brain injury model

The study was conducted according to Institutional Animal Care and Use Committee guidelines in compliance with NIH guidelines. Adult Sabra male mice weighing 40 g were used for these experiments and treated with VEGF or vehicle. Food and water were provided *ad libitum*. TBI was induced in mice using the closed head injury model (CHI) (Chen et al., 1996; Flierl et al., 2009). This is a highly reproducible model of TBI that results in isolated fronto-parietal cortical injury and is associated with a very low frequency of mortality (<5%). Briefly, after induction of isoflurane anesthesia, a midline longitudinal incision was performed, the skin was retracted and the skull exposed. The left anterior frontal area was identified and a tipped Teflon cone was placed 1 mm lateral to the midline. The head was fixed and a 95 g weight was dropped on the cone (Chen et al., 1996; Flierl et al., 2009). After 7 days post TBI, mice were anesthetized again with isoflurane and were implanted with an Alzet mini-osmotic pump secreting vehicle (Saline) or VEGF for 7 days at a rate of 0.5 μl/h for a total dose of 0.84 μg (Vehicle $n=10$ and VEGF $n=9$) into the right lateral ventricle. The coordinates for intraventricular injections were 0.3 mm caudal to bregma, 0.75 mm lateral to the midline, and 2.5 mm below dura (Franklin and Paxinos, 1997). We specifically chose this location, as the skull overlying the left lateral ventricle may deform following the injury and this may affect the accurate placement of the cannula. Furthermore, since the two lateral ventricles are interconnected, placement of the cannula into the intact ventricle as-

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Abbreviations: CHI, closed head injury model; DCX, doublecortin; NSS, neurological severity score; SVZ, subventricular zone; TBI, traumatic brain injury; VEGF, Vascular endothelial growth factor.

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sures exposure of the cells in the ipsi-lateral SVZ to the exogenous VEGF. After recovery from anesthesia, the mice were returned to their home cages with postoperative care and free access to food and water. We specifically choose the time point of 7 days post TBI for initiation of VEGF therapy because we wanted to ensure the lack of neuroprotective effects and to be still within the time frame of active neurogenesis.

BrdU injections

From day 7 post injury, and during 10 days, all animals received IP injection of the tracer BrdU (50 mg/kg twice a day) to label dividing cells.

Evaluations

Functional outcome. One hour after TBI, the functional status of the mice was evaluated according to a set of 10 neurobehavioral tasks (neurological severity score, NSS), which examine reflexes, alertness, coordination, motor abilities and balancing (Beni-Adani et al., 2001). The pathological scores correlate very well with clinical disability scores and with the degree of brain edema (Tsenter et al., 2008). Failure to perform a task scores one point and a success scores 0. Hence, normal animals score 0, reflecting healthy mice, whereas a score of 10 reflects maximal neurological impairment. The first NSS, obtained at 1 h after TBI, reflects the initial severity of injury, is predictive of both mortality and morbidity and correlates well with the extent of damage seen on MRI (Tsenter et al., 2008). In order to achieve a homogenous group we aimed (by adjusting the height of the fall) to create a mild to moderate injury and therefore, we selected only animals with an NSS 6–7 at 1 h, for this test (NSS 1 h). Only one mouse with an NSS of 9 was excluded from the study. The extent of recovery is calculated as the difference between the NSS at 1 h and at any subsequent time point (ΔNSS). Immediately after initial evaluation of NSS the mice were randomly assigned to treatment groups (Vehicle $n=10$ and VEGF $n=9$), and NSS was re-evaluated on days 1, 3, 5, 7, and 14 and in 1 week intervals up to 90 days after TBI. The analyses were performed by an investigator who was blinded to treatment.

Memory test—object recognition test (ORT). The object recognition test was performed as previously described (Tsenter et al., 2008) 3, 14, 21, and 30 days after CHI. This is a sensitive and reproducible test for measuring cognitive abnormalities in this model as was shown in several studies using neuroprotective drugs (Yatsiv et al., 2005; Biegon, 2004; Yaka et al., 2007). Mice were placed for 1 h habituation period in an open glass aquarium-like transparent box, each at a time, in a sound-isolated room. On the following day they were re-introduced in the box for 5 min with two identical clean plaster objects, placed in two different corners of the box. Four hours later, one of the objects was replaced with a new one of the same size and texture, and the mice were re-introduced for additional 5 min in the same cage. Time spent by the mouse in object exploration was recorded manually, and the cumulative time spent at each of the objects was recorded. Exploration of an object was defined as directing the nose to the object at a distance of 2 cm and/or touching it with the nose. The percentage of the total exploration time that the animal spent investigating the new object out of total exploration time was the measure of recognition memory.

Injury size. At 90 days after the surgery the animals were deeply anesthetized and perfusion fixed with 4% paraformaldehyde. Brains were frozen-sectioned at 10 μm. Brain slices 200 μm apart between bregma +1.42 and bregma −0.8 were stained with Giemsa stain modified solution (Fluka, Sigma-Aldrich Corporate, St. Louis, MO, USA; 1:1) and digitally photographed. The volume of injured tissue was measured with image J.40 g software (National Institutes of Health, USA). Damaged tissue volume was calculated by dividing the volume of the injured hemisphere by that of the non-lesioned hemisphere (Fig. 2) (Swanson et al., 1990; Lin et al., 1993). The results are expressed as a percentage of hemispheric tissue:

$$\frac{\text{Area of contralateral hemisphere} - \text{Area of ipsilateral hemisphere}}{\text{Area of contralateral hemisphere}} \times 100 = \text{lesion volume}\ (\%)$$

Immunohistochemistry. Overall, at day 90 post injury, brains were perfusion fixed and frozen sections were prepared at 10 μm. Evenly spaced slices ($n=12$) were counted for each brain between bregma +1.42 and bregma −0.8 and in each slice 10 evenly spaced high power magnification fields (×400) in the entire area surrounding the infarct were counted. Because by 90 days post CHI, the lesion core had already liquefied, the area forming the outer boundary of the brain represents the border zone that survived. This area included mainly cortex and subcortical tissue but not striatum. Brain slices were double or triple stained for immunohistochemical evaluation using fate specific antibodies that included rat anti BrdU (marker for cell proliferation; Accurate, New York, NY, USA; 1:200), goat anti doublecortin (DCX, marker for migrating neuroblasts; Santa-Cruz Biotechnology, Santa Cruz, CA, USA; 1:200), rabbit anti GFAP (marker for astrocytes; Dako, Glostrup, Denmark; 1:200), mouse anti NeuN (marker for mature neurons 1:100), rabbit anti Gal-C (marker for oligodendrocytes; 1:200) were all from Chemicom, Temecula, CA, USA, and mouse anti CD31 (marker for endothelial cells; Abcam Inc., Cambridge, MA, USA; 1:100). Alexa 488 and Alexa 555 conjugates were used as secondary antibodies (Molecular Probes, Leiden, The Netherlands; 1:200), and DAPI (Sigma, Israel) was used to visualize nuclei.

Cell counting. Immuno-positive cells were counted using an epifluorescent Olympus microscope in pre-specified regions of interest (ROI) including the SVZ, corpus callosum, and the area surrounding the lesioned cortex. Specifically, we choose the SVZ because it is the most important generator of newborn cells to the cortex (Leker et al., 2007). The corpus callosum was chosen as the major migration tract for newborn cells from the SVZ to the injured cortex and the peri-lesion cortex was chosen as the target for newborn cell migration and differentiation (Leker et al., 2007). Cells were counted in a semi-quantitative manner meaning counting the actual numbers of cell per high power field (×400). Specifically, we studied equidistant slices, 100 μm apart, from bregma+1.42 to bregma −0.8. In each slice, cells were counted in 10 equidistant fields per region of interest (30 fields per slide and 360 fields per brain at ×400 magnification). Confocal Z sections on a Zeiss LSM system (10 z-slices per cell) were used to determine co-localization.

Statistical analysis

Analysis was performed with the Sigma-Stat software package (SYSTAT, Richmond, CA, USA). Data are presented as mean±SEM as indicated in the legends. Values were compared using one-way analysis of variance (ANOVA) followed by post test Student–Newman–Keuls method. P-values ≤0.05 were considered significant for all comparisons.

RESULTS

Administration of VEGF 7 days post TBI does not have a significant effect on functional outcome and lesion volume

Neurological deficits were measured at pre-determined time points after TBI with the NSS. No differences in NSS