March 25, 2011

Ms. Mary Ray
New Jersey Commission on Brain Injury Research
P.O. Box 360
5th Floor, room 502
Trenton, New Jersey 08625

Re: Final Report - NJCBIR Grant #09-3206-BIR-E
Role of Bone Marrow cells in Repair of the Blood Brain Barrier after Injury
John Glod, MD, PhD, PI

Dear Ms. Ray,

Attached please find an original and five copies of my final report for the above referenced grant.

I would like to thank the New Jersey Commission on Brain Injury Research for providing funding to support this important research.

Should you have any questions, please do not hesitate to contact me at 732-235-8864 or glodjo@umdnj.edu

Thank you again.

Sincerely,

John Glod, MD, PhD
Assistant Professor of Pediatrics
UMDNJ-Robert Wood Johnson Medical Schol

Attachments: original and five copies
1. Program Director’s Name, Address, Telephone Number:

   John Glod, MD, PHD
   Cancer Institute of New Jersey
   Division of Pediatric Hematology/Oncology
   195 Little Albany Street
   New Brunswick, NJ 08903
   732-235-8864

2. Name of Institution:

   UMDNJ-Robert Wood Johnson Medical School

3. Grant Title:

   Role of Bone Marrow cells in Repair of the Blood Brain Barrier after Injury

4. Grant Number:

   09-3206-BIR-E-2

5. Grant Period Covered by the Report:

   6/1/2008 – 5/31/2010; No cost extension approved to 12/31/2010

6. Date of Submission of Report:

   3/25/2011
I. Original aims of the project:

The repair of damaged vasculature is an important component of the pathophysiology of traumatic brain injury. Recent evidence suggests that bone marrow cells such as macrophages and mesenchymal stromal cells (MSCs) are critical for this process. Mesenchymal stromal cells localize to areas of injury in the brain as well as tumors within the central nervous system. Evidence from other laboratories show that the bone marrow is a source of pericytes and our preliminary data demonstrate that glial factors induce the expression of pericyte-like features by MSCs, suggesting that they are the precursors to pericytes. **We hypothesize that:**

1) **MSCs are a source of pericytes during vascular repair in the brain**
2) **Macrophages present at a site of brain injury are required for effective recruitment of MSCs and their incorporation into repairing vasculature.**

In order to test this hypothesis we have pursued the following specific aims:

**Specific Aim #1:** Determine whether MSCs adopt a pericyte phenotype in response to the microenvironment at a site of brain injury.

**Specific Aim #2:** Define the role of macrophages in recruitment of MSCs to the site of brain injury.

2. Project Successes

We have submitted a manuscript for publication. The manuscript is currently in revision.

We have demonstrated that macrophages are important in the reformation of the blood brain barrier following injury. Our work shows that blood vessels are less abundant following injury in the context of macrophage depletion. Additionally, experiments suggest that very large diameter vessels may be less impacted in the setting of macrophage depletion. We have shown that human MSCs show in vitro migration in response to factors produced by macrophages in both a cell line model (U937) and primary culture human macrophages, identified soluble factors that are involved in macrophage-induced MSC migration, and identified the JNK signaling pathway as an important component in macrophage / MSC interactions. We have also begun to identify changes in MSC expression of cytokines and cytokine receptors in response to soluble factors produced by macrophages.
3. Project Challenges

We have not identified significant changes in pericyte phenotype related to macrophage depletion. We have also not demonstrated conclusively that mesenchymal stromal cells develop into pericytes following injury.

4. Implications for future research and/or clinical treatment

The requirement for macrophages for optimal repair of the BBB following injury has important clinical implications. Treatments that increase macrophage mobilization such as administration of hematopoietic growth factors may have the potential of improving recover following traumatic brain injury. This work also suggests that factors that impair macrophage production such as infection may lead to increased tissue repair following brain injury.

5. Plans to continue the research, including applications submitted to other sources for ongoing support.

We are continuing with work looking at the interaction between macrophages and mesenchymal stromal cells at the site of injury. We further plan to better characterize changes in the time course and architecture of vascular remodeling after traumatic brain injury and better defining the role of macrophages in this process.

We plan to use the work funded by the NJCBIR as the basis for an NIH grant application.

6. Explain how you have leveraged NJCBIR funding to obtain additional federal or other support for brain injury research and list the appropriate funding organizations.

NJCBIR funding has been used to generate data that will form the basis of planned applications for federal NIH funding.

7. List and including a copy of all publications emerging from this research including those used in preparation.


Final Financial Report was submitted on 1/13/11 by the UMDNJ Grants Office – see attached.
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# PAYMENT VOUCHER

**Vendor Invoice**

**State of New Jersey**

**PO#**

**PV Date**

**Contract No.**

**Agency Ref.**

**Buyer**

**Terms**

**Total Amount**

## Payee Name and Address

**UMDNJ**

**Grants and Contracts**

**Liberty Plaza**

**PO Box 2685**

**New Brunswick, NJ 08903 2685**

## Payee Declarations

I certify that the within payment voucher is correct in all its particulars, that the described goods or services have been furnished or rendered and that no bonus has been given or received on account of said document.

**Signature**

Anthony Mayo

Manager - Grants & Contracts

01/31/11

**Payee Title**

**Billing Date**

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**Item No.**

**Description of Item**


FOR THE PERIOD ENDED 12/31/10

**Total** $15,047.00

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**Certification by Receiving Agency:** I certify that the above articles have been received or services rendered as stated herein.

**Signature**

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**Certification by Approval Officer:** I certify that this Payment Voucher is correct and just, and payment is approved.

**Authorized Signature**

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PV 0/893 MAY 05
John Glod, MD, PhD  
Grant # 09-3206-BIR-E-2  
Role of Bone Marrow cells in Repair of the Blood Brain Barrier after Injury

**PUBLICATIONS AND PRESENTATIONS**

All papers, presentations, chapter, and abstracts should mention that the research was supported by a grant from the New Jersey Commission on Brain Injury Research. Copies must be sent to the NJCBIR office. Copies must be sent to the NJCBIR office, even if you inadvertently forgot to cite NJCBIR support.

See attached.
Macrophages Play a Key Role in early Blood Brain Barrier Reformation after Hypothermic Brain Injury

Rajeth Koneru³, David Kobiler³, Shoshona Lehrer³,⁵, Jiadong Li³, Nico van Rooijen⁴, Debabrata Banerjee¹,², and John Glod²,³

Departments of ¹Medicine, ²Pharmacology and ³Pediatrics, The Cancer Institute of New Jersey. Robert Wood Johnson Medical School, University of Medicine and Dentistry of New Jersey; ⁴Department of Molecular Cell Biology, Vrije Universiteit Medical Center, Amsterdam, The Netherlands. ⁵Current address IIBR, Ness Ziona, Israel.

Key words: blood brain barrier (BBB); macrophage

Running title: Macrophage depletion delays BBB reformation after injury

Correspondence: John Glod, M.D., Ph.D., glodjo@umdnj.edu; The Cancer Institute of New Jersey, RWJMS/UMDNJ, 195 Little Albany street, New Brunswick, New Jersey 08903, USA. Telephone: 732-235-9854; Fax: 732-235-8234
Abstract

The repair of damaged vasculature is an important component of the pathophysiology of traumatic brain injury. In addition to re-establishing perfusion to areas supplied by damaged vessels, the integrity of the blood brain barrier (BBB) must be reformed at sites of injury. Recent evidence suggests that bone marrow-derived cells such as macrophages are critical for this process. Cells of the monocyte/macrophage lineage may play a role in formation of the BBB through modulation of the phenotype of microvascular endothelial cells or potentially through a structural role in the vasculature. We have used liposomal clodronate to deplete monocytes and tissue macrophages. This method led to a marked reduction in the accumulation of F4/80-expressing cells at sites of hypothermic brain injury in a murine model. The reduction in macrophages at the injury site was accompanied by a decrease in neovascularization following the injury. We also evaluated the reformation of the blood brain barrier after injury. In control animals the permeability of the BBB at the injury to FITC-labeled albumin returned to normal levels by seven days post-injury. In macrophage-depleted mice leakage of albumin was still observed at seven days post-injury. These results suggest that macrophages play an important role in both post-traumatic revascularization as well as early repair of the BBB after injury.

Introduction

A critical component of tissue repair following traumatic brain injury (TBI) is the reformation of the brain vasculature. Evidence suggests that reformation of the vascular network following traumatic injury to the CNS is important for functional recovery. Inhibition of angiogenesis after TBI in a rodent model through vascular endothelial growth factor (VEGF) blockade led to increases in the area of hemorrhage and increases in serum markers of neuronal injury as well as a decrease in vascular density (Skold et al., 2006). Conversely, increased angiogenesis following experimental CNS injury after treatment with atorvastatin or VEGF led to
improved functional recovery in a TBI model (Lu et al., 2004) and decreased secondary tissue degeneration in a spinal cord contusion model (Widenfalk et al., 2003). Another important component to vascular repair in the CNS is restoration of blood brain barrier (BBB) integrity. The BBB is a complex system contributed to by endothelial cells, astroglia, perivascular macrophages, and pericytes that regulates the movement of substances from the circulation to the brain parenchyma and vice versa (Abbott, 2000; Kniesel and Wolburg, 2000; Lee et al., 2009; Rubin and Staddon, 1999). Impaired BBB function is seen after brain injury and is related to neuronal injury (Dinapoli et al., 2007; Ping et al., 2005). In addition to TBI, impaired BBB function has been implicated in the pathophysiology of diseases including Alzheimer disease, multiple sclerosis and CNS infection (Adams et al., 2002; Annunziata, 2003; Floris et al., 2004; Jellinger, 2002; Ujiie et al., 2003). Thus, outcome after brain injury may be impacted by both revascularization as well as reformation of the blood brain barrier.

Cells of the monocyte / macrophage lineage play an important role in blood vessel growth. An increase in tissue macrophages accompanies angiogenesis after stroke (Manoonkitiwongsa et al., 2001) and VEGF mediated angiogenesis in the brain (Croll et al., 2004) and depletion of peripheral blood monocytes inhibits vascularization of the choroid of the eye (Espinosa-Heidmann et al., 2003; Sakurai et al., 2003) and collateral artery growth in a limb ischemia model (Heil et al., 2002). Macrophages may perform multiple functions during angiogenesis including production of pro-angiogenic factors such as VEGF and placenta-derived growth factor (PDGF) and disruption of the extracellular matrix during vessel growth (Fujiyama et al., 2003; Moldovan et al., 2000). Populations of monocytes and macrophages may also play a structural role in new blood vessels. Myeloid cells have been reported to assume endothelial characteristics (Fernandez Pujol et al., 2000; Fernandez Pujol et al., 2001; Glod et al., 2006; Harraz et al., 2001; Havemann et al., 2003; Nakul-Aquaronne et al., 2003; Rehman et al., 2003; Schmeisser et al., 2001) and perivascular macrophages contribute to both the blood-retinal and blood-nerve barriers (Gray et al., 2007; Mendes-Jorge et al., 2009).
We sought to determine the importance of macrophages in repair of brain vasculature following traumatic brain injury. Treatment of mice with liposomal clodronate led to decreased peripheral blood monocytes and a marked reduction in the accumulation of F4/80 expressing cells at sites of hypothermic brain injury. Macrophage depletion led to decreased vascularization following injury and also a delay in the reconstitution of the blood brain barrier as measured by FITC-albumin leakage. These results suggest that macrophages play an important role in both vascular growth and reconstitution of the BBB following traumatic brain injury.

Methods

Animal Studies/Injury Model
Three to four week old female Swiss-Webster mice were purchased from Taconic Farms and utilized in the brain injury model. The experimental group was treated with liposomal clodronate, a monocyte depleting agent (van Rooijen et al., 1996). Clodronate (a gift from Roche Diagnostics 120 GmbH, Mannheim, Germany) was encapsulated into liposomes as described previously (Van Rooijen and Sanders, 1994). Mice were injected via tail vein with 100μl of either liposomal clodronate or PBS liposomes 3-4 days prior to hypothermic injury, on the day of injury, and every 3-4 days thereafter until the time of sacrifice. Hypothermic brain injury was performed as previously described (Nag, 1996). Animals were deeply anesthetized and placed in a David Kopf stereotaxic apparatus. The skin surface was cleansed with 100% ethanol. A small incision was made in the skin of the skull. The coordinates of the bregma were noted and the injury was located 1mm anterior and 1.5 mm lateral of bregma. A cold stainless steel 2 mm diameter cylindrical rod was set in liquid nitrogen for a few minutes. After removal from liquid nitrogen the rod was immediately placed on the injury site for 60 seconds. The site was then cleansed with betadine and the skin was closed with a surgical staple. All animal procedures were approved by the Animal Care and Use Committee of RWJMS.
Vessel Quantitation

On the day of sacrifice mice were deeply anesthetized and perfused with phosphate buffered saline (PBS) through the left ventricle. Brains were removed, placed in a dry-ice cooled solution of isopentane for 10 seconds, and then placed in a cryomold on dry-ice in the proper orientation for coronal tissue sectioning. The mold was filled with Tissue-Tek O.C.T. compound (Sakura Finetek). After the O.C.T. solidified, the tissue was placed in a Leica cryostat for 1 hour at -20 degrees. Ten micron sections were cut and adhered to microscope slides and placed in methanol at 4 degrees for 10 minutes then washed in cold PBS. Tissue sections were then stained for expression of vonWillebrand factor to label blood vessels.

All tissue sections were imaged using a Nikon Eclipse C1 Confocal Microscope. Images were obtained using a 60X oil lens. Vessel quantitation was performed using Adobe Photoshop CS3 software. Each injury site was divided into fields of 3000 pixels X 1800 pixels. Vasculature was quantitated in sections at the injury site as well as sections of uninjured cortex. The number of pixels that were labeled for von Willebrand factor (VWF) was determined for each field using a magic wand tool. The von Willebrand staining per field was averaged across a minimum of seven fields for each animal.

Immunoflourscence

The following primary antibodies were used in these experiments: anti-human von Willebrand Factor (Dako), anti-mouse F4/80 (Abcam), and rabbit anti-GFAP (Sigma). Labeled goat anti-rabbit and anti-rat secondary antibodies were purchased from Molecular Probes. Slides were immersed in a dish containing blocking buffer (consisted of 10% goat serum (Dako) + 0.01% Triton X-100 (Sigma) in PBS) for 1 hour in a humidified chamber at 37 degrees. The tissue sections were covered with primary antibody diluted in blocking buffer. After incubation for 1
hour at 37°C in humidified chamber, excess liquid was blotted from slides and then rinsed three times in PBS for five minutes per wash. Secondary antibody labeling was performed in a similar manner. Slides were mounted in Vectashield with DAPI (Vector Labs). All tissue sections were imaged using a Nikon Eclipse C1 Confocal Microscope. Images were obtained using a 60X oil lens.

**BBB leakage**

Swiss-Webster mice (female) approx 3-4 weeks old were purchased from Taconic Farms and utilized in Blood Brain Barrier leakage study. Mice were injected through the tail vein with either liposomal clodronate or liposomes containing PBS 3-4 days prior to cold shock injury, on the day of injury, and every 3-4 days thereafter until the time of sacrifice. Hypothermic brain injury was performed as described above. On the day of sacrifice mice were injected i.p. with an anesthetic (Ketaset(100mg/ml) /Acepromazine(1:2)). Thirty minutes prior to the time of sacrifice mice were injected via tail vein with 100μl of Sodium Flourescein labeled albumin (2.5% in PBS). Five minutes prior to the time of sacrifice mice were injected via tail vein with 100μl of Rhodamine Dextran (2.5% w/v in PBS). At the time of sacrifice, mice were decapitated and the brain hemispheres were removed, weighed and stored in separate tubes on dry ice. The tissue was homogenized and centrifuged. The supernatant was collected and the concentration of Fluorescien albumin and rhodamine dextran in each hemisphere were determined using a fluorimeter. The concentrations of Rhodamine dextran and FITC-albumin in the uninjured hemisphere were used to calculate the amount of FITC-albumin present in the vasculature. The intravascular FITC-albumin was then subtracted from the total FITC-albumin in the injured hemisphere to calculate the amount of leakage.

\[
\text{Amount of leakage} = \text{Alb}_{(\text{inj})} - \left( \frac{\text{Alb}_{(\text{con})} * \text{Rhod}_{(\text{inj})}}{\text{Rhod}_{(\text{con})}} \right)
\]
Results

Treatment with liposomal clodronate results in decreased accumulation of F4/80 expressing cells after brain injury.

Swiss-Webster mice were treated with liposomal clodronate every four days beginning four days prior to injury. Animals were sacrificed seven days post-injury. Hematoxylin and eosin staining of tissue sections through the injury site demonstrated an overall decrease in cellularity in animals treated with liposomal clodronate (Figure 1a). More specifically, immunohistochemical staining for the macrophage marker F4/80 showed a decrease in the accumulation of macrophages at the injury site in animals that were treated with liposomal clodronate (Figure 1b). Tissue sections through injury sites were also stained for glial fibrillary acidic protein. Both control animals as well as mice treated with liposomal clodronate had a brisk gliosis present at day 7 after injury (Figure 1c).

Macrophage depletion decreases the angiogenic response following cold injury.

Angiogenesis following injury was quantitated. Tissue sections from control animals and animals treated with liposomal clodronate were stained for the endothelial protein von Willebrand factor. In preliminary experiments vessel density following hypothermic injury increased until day seven following injury and then plateaued (Figure 2a). Other investigators have reported a similar time-course of vascularization following CNS injury (Nag, 2002). Revascularization following injury was then compared between animals treated with clodronate and controls. A decrease in vessel density between control animals and macrophage-depleted animals was evident at day 7 after injury and persisted throughout the length of the experiment (Figure 2b and 2c). Animals treated with liposomal clodronate had an approximately 45% decrease in von Willebrand factor staining at day 7 after injury (5392 +/- 3113 pixels per field compared to 9890 +/- 4161 pixels per field; p=0.0147) (Figure 2b). There was no significant difference in the vascular
density between liposomal clodronate treated and control mice in an area of normal brain adjacent to the injury site (2267 +/- 1718 pixels per field compared to 3058 +/- 1924 pixels; p=0.0967) (B). At day fourteen mice treated with liposomal clodronate had an area of staining of 7866 +/- 872 pixels per field compared to 11918 +/- 1052 pixels per field for control mice (p = 0.003). Again there was no significant difference in the vascular density in areas of normal brain adjacent to the injury site (6621 +/- 289 pixels per field compared to 5191 +/- 1448 pixels per field; p=0.15) (n= 5 for mice treated with liposomal clodronate and n=3 for control mice) (C).

Macrophage depletion causes a delay in blood brain barrier repair after injury

In order to assess the role of macrophages in BBB repair, the leakage of FITC-labeled albumin was measured after injury. In control mice albumin leakage was seen at 3 days after injury, but by 7 days after injury the degree of albumin extravasation at the injury site was indistinguishable from the contra lateral hemisphere (Figure 3). This rate of reformation of the BBB after injury is similar to results seen by others (Nag, 2002). In mice that were treated with liposomal clodronate albumin leakage continued through day 7 after injury. At day three following injury the leakage of FITC-albumin was the same in both the liposomal clodronate treated and control animals (301 +/- 126 compared to 259 +/- 153; p=0.7). A non-significant trend toward increased FITC-albumin leak was seen in the liposomal clodronate treated animals at day 5. At seven days after injury there was continued leakage of FITC-albumin in the clodronate treated animals and minimal leakage in the control animals (314 +/- 51 compared to 22 +/- 70; p=0.005). These data indicate that the presence of macrophages is important for reformation of the BBB following injury.

Discussion

Angiogenesis and reformation of the blood brain barrier play a critical role in the re-establishment of homeostasis in the central nervous system following traumatic injury. While the
role of macrophages in blood vessel formation has been described for a number of organ systems, the impact of macrophages on the reformation of the specialized vasculature of the brain is not completely understood. A relationship between macrophage accumulation and cerebrovascular angiogenesis following injury has been described by several groups and accumulation of macrophages coincides with changes in microvessel density following focal cerebral ischemia as well as cold injury in animal models (Manoonkitiwongsa et al., 2001; Nourhaghighi et al., 2003). Macrophages are likely to have a variety of important functions during this process including the “Clean-up” of necrotic debris (Manoonkitiwongsa et al., 2001) and regulation of blood vessel growth and barrier integrity through the elaboration of factors such as angiopoietin-1 and angiopoietin-2 (Nourhaghighi et al., 2003). Here we provide direct evidence that macrophages are required for vascular proliferation following injury by demonstrating that macrophage depletion impairs the formation of new blood vessels. Additionally, we demonstrate directly that decreased levels of macrophage infiltration impairs another critical component of vascular repair in the central nervous system, the reformation of the blood brain barrier.

Our data show that depletion of macrophage accumulation at an injury site perturbs the increase in vascular density that is seen following brain injury. Macrophages are likely to promote angiogenesis through a number of mechanisms. Macrophages secrete important pro-angiogenic cytokines such as IL-8 (Carmi et al., 2009) and IL-1 (Hong et al., 2009) and enhance degradation and reformation of the extracellular matrix through the production of matrix metalloproteinases (Giraudo et al., 2004; Johnson et al., 2004). A more precise understanding of the exact role of macrophages in these critical aspects of brain angiogenesis is complicated and awaits further study.

Our data also provide evidence for an important role for macrophages in reformation of the blood brain barrier. Brain macrophages have been implicated previously in maintenance of BBB integrity. Using in vitro co-culture systems it has been reported that peripheral blood
macrophages enhance the tightness of a BBB model (Glod et al., 2006; Zenker et al., 2003) and perivascular microglial cells within the CNS have been shown to participate in the CNS vascular through the uptake of macromolecules(Mato et al., 1996). Others have reported that perivascular macrophages migrate to areas of vascular leak and participate in barrier formation in the blood retinal barrier (Mendes-Jorge et al., 2009) and the area postrema (Willis et al., 2007). Additionally, macrophage infiltration into the CNS in an experimental autoimmune encephalomyelitis model was associated with areas with a more intact BBB (Ladewig et al., 2009) suggesting that macrophages may be associated with local BBB repair.

Interestingly, the degree of angiogenesis, macrophage infiltration, and the degree of BBB dysfunction have all been related to neuronal damage or functional recovery after injury. However, the relationship between these processes and recovery are complicated. While angiogenic agents such as vascular endothelial growth factor have been reported to have neuroprotective effects (Carmeliet and Storkebaum, 2002; Ferrara and Gerber, 2001; Sun et al., 2003), recent information suggests that increased angiogenesis facilitated by the administration of VEGF may be accompanied by increased macrophage infiltration as well as neuronal damage(Manoonkitiwongsa et al., 2006). A subset of macrophages have been shown to play an important anti-inflammatory role following spinal cord injury(Shechter et al., 2009). It is possible that subtle changes in the inflammatory response have a significant impact on the extent and timing of revascularization and reinstitution of the blood brain barrier following traumatic injury.

In summary, macrophages play an extensive role in vascular repair in the brain after injury. Their impact is not confined to elaboration of pro-angiogenic factors but also includes roles in the regulation of the vascular architecture and BBB integrity. A better understanding of the interactions between macrophages and the repairing CNS vasculature could identify therapeutic targets for improving recovery after brain injury.
Acknowledgment

This work was supported by the New Jersey Commission on Brain Injury; grant No.08-3206-BIR-E-1 to J.G.

Author Disclosure Statement

No competing financial interests exist.

Figure Legends

**Figure 1:** Treatment with liposomal clodronate leads to decreased accumulation of macrophages and impaired resolution of necrosis following hypothermic brain injury. Mice were treated with liposomal clodronate of phosphate buffered saline before and after hypothermic brain injury. Seven days after injury the animals were sacrificed and tissue sections were taken through the injury sites. H & E staining shows a decrease in the level of cellularity at the injury site of animals treated with clodronate as well as an accumulation of necrotic debris compared to controls (A). Staining for the macrophage marker F4/80 demonstrates a marked decrease in the accumulation of macrophages at the injury site in animals that were treated with liposomal clodronate compared to control animals (B). Additionally, immunohistochemical staining for glial fibrillary acidic protein shows brisk gliosis after injury in both the treated and untreated animals(C).
**Figure 2:** Depletion of peripheral blood monocytes leads to delayed neovascularization after injury. Mice were subjected to hypothermic brain injury and the time course of neovascularization following injury was determined through the quantitation of von Willibrand expressing vessels at the injury site. The number of vessels per field was counted for a minimum of seven fields at each time point. (A) The majority of neovascularization occurs by day seven after injury. There was no increase in von Willibrand Factor staining at the injury site at 3 weeks after injury compared to seven days after injury. The impact of macrophage depletion with liposomal clodronate on revascularization following injury was then determined. Tissue sections through the injury site at day seven and 14 were stained for von Willibrand Factor. The total pixels per field exhibiting staining for vWF at each injury site was quantitated. At day seven mice treated with liposomal clodronate had a decreased area of staining compared to control mice treated with liposomal PBS (5392 +/- 3113 pixels compared to 9890 +/- 4161 pixels; p = 0.0147) (B). There was no significant difference in the vascular density between liposomal clodronate treated and control mice in an area of normal brain adjacent to the injury site (2267 +/- 1718 pixels per field compared to 3058 +/- 1924 pixels; p = 0.0967) (n=3 for liposomal clodronate treated mice and n=3 for control mice) (B). The difference in vascular density at the injury site between liposomal clodronate treated mice and control mice was less pronounced 14 days after injury. At day fourteen mice treated with liposomal clodronate had an area of staining of 7866 +/- 872 pixels per field compared to 11918 +/- 1052 pixels per field for control mice; p = 0.003). Again there was no significant difference in the vascular density in areas of normal brain adjacent to
Figure 3: Reformation of the blood brain barrier is delayed in mice treated with liposomal clodronate. At various time points following hypothermic brain injury animals were injected with FITC-labeled albumin and the leakage of albumin in the injured hemisphere was calculated. Animals were injected intravenously with FITC-labeled albumin. One hour later animals were injected intravenously with rhodamine-labeled dextran (average MW approximately 70,000) and immediately sacrificed. The brains were bisected and homogenized and the amount of FITC-albumin present in the circulation was calculated using a ratio of rhodamine to fluorescene in the non-injured hemisphere. The amount of FITC-albumin that had leaked into the parenchyma of the injured hemisphere was then calculated in arbitrary fluorescence units. (A) In normal controls a peak of albumin leak was observed on day two following injury with the integrity of the BBB returning to baseline levels by day seven. (B) At day three following injury the leakage of FITC-albumin was the same in both the liposomal clodronate treated and control animals (301 +/- 126 compared to 259 +/- 153; p=0.7). A non-significant trend toward increased FITC-albumin leak was seen in the liposomal clodronate treated animals at day 5. At seven days after injury there was continued leakage of FITC-albumin in the clodronate treated animals and minimal leakage in the control animals (314 +/- 51 compared to 22 +/- 70; p=0.005). (n=4 mice in each group)
References


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