Published in final edited form as:

Brain Res. 2014 July 29; 1574: 70–76. doi:10.1016/j.brainres.2014.06.003.

Brain CD47 Expression in a Swine Model of Intracerebral Hemorrhage

Xiang Zhou, MD, Qing Xie, MD, Ph.D., Guohua Xi, MD, Richard F. Keep, Ph.D, and Ya Hua, MD

Department of Neurosurgery, University of Michigan, Ann Arbor, MI, USA

Abstract

CD47 contributes to neuronal death, inflammation and angiogenesis after brain ischemia. The role of CD47 in intracerebral hemorrhage (ICH) has not been investigated and the current study examined brain CD47 expression in a pig ICH model. Pigs received a blood injection or needle insertion into the right frontal lobe and were euthanized at different times to examine CD47 expression. Pigs were also treated with an iron chelator, deferoxamine, (50 mg/kg, i.m.) or vehicle and killed at day-3 to examine the effects on CD47. ICH resulted in upregulation of brain CD47 in both white and grey matter by both immunohistochemistry and Western blot. A time-course showed ICH-induced CD47 upregulation from 4 hours to day-14, with a peak at day-3. CD47 positive cells were neurons, microglia/macrophage and oligodendrocytes. Brain CD47 levels were lower in the ipsilateral white and grey matter in pigs which had deferoxamine treatment. In conclusion, CD47 expression was increased in the perihematomal white and grey matter after ICH. Deferoxamine and iron may modulate CD47 expression.

Keywords

cerebral hemorrhage; CD47; deferoxamine; pigs

1. Introduction

Cluster of Differentiation 47 (CD 47), also called integrin associated protein, is a transmembrane protein belonging to the immunoglobulin superfamily. Recent studies found that CD47 plays a critical role in focal cerebral ischemia, being involved in neuronal death, inflammation and angiogenesis (Jin et al., 2009; Xing et al., 2009). Overexpression of CD47 in neurons induced apoptosis (Koshimizu et al., 2002). Activation of CD47 signaling

© 2014 Elsevier B.V. All rights reserved.

Corresponding to: Ya Hua, MD, R5018 BSRB, University of Michigan, 109 Zina Pitcher Place, Ann Arbor, Michigan 48109-2200, Phone: 734-764-1207, yahua@umich.edu.

Disclosures:

None.

The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

induced caspase-dependent and caspase-independent cell death mechanisms in neurons (Xing et al., 2009).

The impact of intracerebral hemorrhage (ICH) on CD47 has not been examined. However, in kidney there is evidence that iron overload (induced by Fe-nitrilotriacetic acid) causes CD47 upregulation (Nishiyama et al., 1997), and ICH causes perihematomal iron accumulation (Gu et al., 2009; Wu et al., 2003). In addition, known ligands for CD47 are thrombospondin (TSP)-1 and -2 (Xing et al., 2009) and signal regulatory protein (SIRP) alpha (Rogers et al., 2012). Brain TSP-1 and -2 levels are increased after ICH (Zhou et al., 2010) and SIRP α is expressed on phagocytes (Han et al., 2012). Phagocytes, microglia and invading macrophages, are important in clearing the cerebral hematoma.

The present study examined brain CD 47 expression in a pig model of ICH. It also examined whether the CD47 response is modulated by treatment with deferoxamine, an iron chelator currently in clinical trial for ICH.

2. Results

At 3 days after sham operation, CD47 immunoreactivity was very low in both ipsi- and contralateral white and gray matter. In contrast, after ICH, some CD 47 positive cells were found in ipsilateral white and grey matter and contralateral grey matter by 4 hours and CD47 immunoreactivity increased in both ipsi- and contralateral white and grey matter, and was stronger in the ipsilateral hemisphere, from day-1 to day-7 (Fig. 1).

As assessed by Western blot, CD47 protein levels in both ipsilateral white (Fig. 2A) and gray matter (Fig. 2B) were upregulated after ICH. In white matter, CD47 levels increased quickly at 4 hours, were maintained at high levels at day-1 and day-3, decreased at day-7 and reached basal levels at day-14 after ICH (Fig. 2A). In gray matter, CD47 protein levels were increased by 4 hours and peaked at day-3. Levels then decreased gradually, but were still higher than basal levels at day-14 (Fig. 2B). Compared to sham-operated animals and contralateral hemisphere, ICH induced significantly higher CD47 protein levels in the ipsilateral white matter (CD47/beta-actin: 0.62 ± 0.30 vs. 0.26 ± 0.03 in sham and 0.30 ± 0.05 in the contralateral, $p < 0.05$, Fig. 3A) and grey matter (0.94 ± 0.27 vs. 0.31 ± 0.16 in sham and 0.48 ± 0.21 in the contralateral, $p < 0.05$, Fig. 3B) at 3 days after ICH.

Triple immunofluorescence staining showed that CD47 positive cells were primarily neurons, microglia/macrophages and oligodendrocytes, but not astrocytes. Thus, CD47 co-localized with NeuN (a neuronal marker, Fig. 4 A), MBP (an oligodendrocyte marker, Fig. 4 B) and Iba1 (a microglia/macrophage marker, Fig. 4 D), but not GFAP (an astrocyte marker, Fig. 4 C).

Systemic treatment with deferoxamine (started at 2 hours after ICH and every 12 hours for 3 days), an iron chelator, reduced CD47 levels in the ipsilateral white matter (CD47/beta-actin: 0.35 ± 0.08 vs. 0.73 ± 0.09 in vehicle-treated pigs, $p < 0.01$, Fig. 5A) and grey matter (0.64 ± 0.16 vs. 1.19 ± 0.20 in vehicle-treated pigs, $p < 0.01$, Fig. 5B) at day-3 after ICH.

3. Discussion

The present study found that: 1) brain CD47 levels were increased after ICH; 2) CD47 was expressed on neurons, microglia/macrophages, and oligodendrocytes, but not on astrocytes; and 3) deferoxamine reduced ICH-induced CD47 upregulation.

The current study demonstrates that brain CD47 levels are increased significantly in both white and grey matter following ICH. CD47 has been found in neurons and plays a critical role in the central nervous system (Numakawa et al., 2004; Reinhold et al., 1995). For example, CD47 is important to synaptic function in developing cortical neurons (Numakawa et al., 2004). Studies have shown that viral overexpression of CD47 in neurons induces apoptosis (Koshimizu et al., 2002) and ligand-mediated activation of CD47 is neurotoxic (Xing et al., 2009). Genetic deletion of CD47 reduces infarct volume and brain edema after stroke by reducing neutrophil extravasation and MMP-9 expression (Jin et al., 2009) and induces resistance to experimental autoimmune encephalomyelitis (Han et al., 2012). Given those results and the data on ICH-induced CD47 presented in this study, future experiments should determine the role of CD47 in brain injury after ICH.

Iron accumulation occurs in the brain after ICH and results in brain injury (Bodmer et al., 2012; Keep et al., 2012; Wu et al., 2003; Xi et al., 2006). We have previously reported that systemic treatment with deferoxamine reduces brain edema, neuronal death and neurological deficits in experimental studies (Gu et al., 2009; Nakamura et al., 2004; Okauchi et al., 2009; Song et al., 2007). In this study, we found that deferoxamine treatment attenuated brain CD47 expression after ICH, which suggests a role for iron in ICH-induced upregulation of brain CD47. One study showed over expression of CD47 in rat kidney treated with ferric nitrilotriacetate (Fe-NTA), but not with NTA (Nishiyama et al., 1997), and Fe-NTA induced iron overload (Morales et al., 2012).

Several ligands bind to CD47, including TSP-1 and -2 and SIRP α (Rogers et al., 2012). Ligand binding to CD47 can activate multiple cell survival and cell death pathways (Rogers et al., 2012). It can also regulate phagocytosis, with CD47 interacting with SIRP α on macrophages providing a 'don't eat me signal' (Matozaki et al., 2009). In addition, recent evidence indicates that CD47 and leukocyte interactions play an important role in regulating leukocyte entry into brain at the blood-brain barrier (Martinelli et al., 2013). Although development of the CD47 KO mouse (Jin et al., 2009) can provide important information on the role of CD47 in these processes and in neurological diseases, understanding the importance of CD47 interactions in translational models, such as the pig ICH model, requires the development and validation of reagents to block those interactions.

In conclusion, CD47 expression was increased in perihematomal white and grey matter after ICH. The role of parenchymal cell CD47 in ICH-induced brain injury remains to be determined but deferoxamine may be a method of modulating CD47 expression.

4. Materials and Methods

Animal preparation and intracerebral infusion

Animal use protocols were approved by the University of Michigan Committee on the Use and Care of Animals. A total of 52 male pigs (8 to 10 kg; Michigan State University, East Lansing) were used in this study. Pigs were sedated with ketamine (25 mg/kg, IM) and anesthetized with isoflurane. After a surgical plane of anesthesia was reached, animals were orotracheally intubated. The right femoral artery was catheterized for monitoring of blood pressure, blood gases, and glucose concentrations. Body temperature was maintained at $37.5 \pm 0.5^{\circ}\text{C}$.

A cranial burr hole (1.5 mm) was then drilled 11 mm to the right of the sagittal and 11 mm anterior to the coronal suture. An 18-mm-long 20-gauge sterile plastic catheter was placed stereotactically into the center of the right frontal cerebral white matter and cemented in place. One mL of autologous arterial blood was infused over 15 minutes and another 1.5 mL of blood was injected over 15 minutes after a 5 minutes break with an infusion pump (Gu et al., 2009). Sham pigs underwent the same procedure without blood infusion.

Experimental Groups

There were two sets of experiments in this study. First, pigs had 2.5 ml blood injected into the right front lobe. Brains were perfused by 10% formalin for histological examination at 4 hours or days 1, 3 and 7 (n=4 each), or were frozen *in situ* for immunoblotting at 4 hours or days 1, 3, 7 and 14 (n=4 each). Control pigs had insertion of a needle and were euthanized at day-3. Brains were also perfused by 10% formalin for histology (n=4) and had *in situ* frozen for immunoblotting (n=4). In the second set, pigs received deferoxamine (50 mg/kg, i.m.) or vehicle (saline) treatment at 2 hours after ICH and then every 12 hours for 3 days. Pigs were euthanized at day-3 and brains used for immunoblotting (n=4 per group).

Immunohistochemistry and immunofluorescence staining

Immunohistochemical staining was performed using the avidin-biotin complex method. Paraffin-embedded brains were cut into 10- μm -thick sections. The sections were deparaffinized in xylene and rehydrated in a graded series of alcohol dilutions. Antigen retrieval was performed by the microwave method using citrate buffer (10mM, pH 6.0). All sections were then treated with 0.3% hydrogen peroxide to neutralize endogenous peroxidases. Sections were then permeabilized in 0.3% Triton X-100 (v/v) for 15 min and washed three times in PBS. Non-specific binding was blocked by 30 min treatment in 10% normal horse serum/PBS. The sections were incubated with primary antibody at 4°C overnight. The primary antibody was monoclonal mouse anti-human CD47 (1:100, MCA911, AbD serotec). After three-time washes in PBS, sections were incubated with biotinylated horse anti-mouse IgG (1:200; Vector Laboratories) for 2 hours at room temperature, followed by 2 hours incubation in avidin-biotinylated peroxidase complex (1:150; Vectorstain Elite Kit) at room temperature. Diaminobenzidine (DAB; Invitrogen) was used as chromagen, with reactions sustained for 2–5 min at room temperature. For negative controls, the primary antibody was replaced with normal horse serum and no specific positive staining was detected. Stained sections were examined on an Olympus

BX51 microscope. Images used for analysis were captured at the same contrast setting and exposure times.

For triple labeling, paraffin-embedded sections were treated with the same protocol as described above. Following overnight incubation at 4°C with primary antibodies, sections were rinsed with PBS and incubated at room temperature for 2 hours with secondary antibodies. The primary antibodies were monoclonal mouse anti human CD47 (1:100, MCA911, AbD serotec), polyclonal rabbit anti-FOX3/NeuN (1:500, ab104225, Abcam), monoclonal rat anti-myelin basic protein (MBP; 1:50, MCA409S, AbD serotec), polyclonal goat anti-glial fibrillary acidic protein (GFAP; 1:400, sc-6170, Santa Cruz Biotechnology), polyclonal goat anti-Iba1 (1:100, ab5076, Abcam). Secondary antibodies were Alexa Fluor 488 donkey anti-mouse IgG (1:200, Life Technologies), Alexa Fluor 594 donkey anti-rabbit IgG (1:200, Life Technologies), Alexa Fluor 594 donkey anti-rat IgG (1:200, Life Technologies), and Alexa Fluor 594 donkey anti-goat IgG (1:200, Life Technologies). Hoechst 33258 was used for nuclear labeling.

Western Blot Analysis

Western blot analysis was performed as previously described (He et al., 2012; Wu et al., 2003). Ipsi- and contralateral white and gray matter tissues were sampled. The primary and secondary antibodies were monoclonal mouse anti human CD47 (1:1000, MCA911, AbD Serotec) and HRP-conjugated goat anti-mouse IgG (1:2000, Bio-Rad).

Statistical Analysis

Data from different animal groups were expressed as mean $\pm$ SD and analyzed by ANOVA and Student's t-test. Differences were considered significant at $p < 0.05$.

Acknowledgments

Sources of Funding:

This study was supported by grants NS-073595, NS-079157 and NS-084049 from the National Institutes of Health (NIH).

Abbreviations

ICH	intracerebral hemorrhage
CD47	cluster of differentiation 47
MBP	myelin basic protein

References

- Bodmer D, et al. The Molecular Mechanisms that Promote Edema After Intracerebral Hemorrhage. *Translational Stroke Research*. 2012; 3:S52–S61.
- Gu Y, et al. Deferoxamine reduces intracerebral hematoma-induced iron accumulation and neuronal death in piglets. *Stroke*. 2009; 40:2241–3. [PubMed: 19372448]
- Han MH, et al. Janus-like opposing roles of CD47 in autoimmune brain inflammation in humans and mice. *The Journal of experimental medicine*. 2012; 209:1325–34. [PubMed: 22734047]

- He YD, et al. Ischemic Preconditioning Attenuates Brain Edema After Experimental Intracerebral Hemorrhage. *Translational Stroke Research*. 2012; 3:S180–S187.
- Jin G, et al. CD47 gene knockout protects against transient focal cerebral ischemia in mice. *Experimental neurology*. 2009; 217:165–70. [PubMed: 19233173]
- Keep RF, Hua Y, Xi G. Intracerebral haemorrhage: mechanisms of injury and therapeutic targets. *Lancet neurology*. 2012; 11:720–731. [PubMed: 22698888]
- Koshimizu H, et al. Expression of CD47/integrin-associated protein induces death of cultured cerebral cortical neurons. *Journal of neurochemistry*. 2002; 82:249–57. [PubMed: 12124426]
- Martinelli R, et al. Novel role of CD47 in rat microvascular endothelium: signaling and regulation of T-cell transendothelial migration. *Arterioscler Thromb Vasc Biol*. 2013; 33:2566–76. [PubMed: 23990210]
- Matozaki T, et al. Functions and molecular mechanisms of the CD47-SIRPalpha signalling pathway. *Trends in cell biology*. 2009; 19:72–80. [PubMed: 19144521]
- Morales NP, et al. Hepatic reduction of carbamoyl-PROXYL in ferric nitrilotriacetate induced iron overloaded mice: an in vivo ESR study. *Biological & pharmaceutical bulletin*. 2012; 35:1035–40. [PubMed: 22791149]
- Nakamura T, et al. Deferoxamine-induced attenuation of brain edema and neurological deficits in a rat model of intracerebral hemorrhage. *Journal of Neurosurgery*. 2004; 100:672–8. [PubMed: 15070122]
- Nishiyama Y, et al. Overexpression of integrin-associated protein (CD47) in rat kidney treated with a renal carcinogen, ferric nitrilotriacetate. *Japanese journal of cancer research : Gann*. 1997; 88:120–8. [PubMed: 9119739]
- Numakawa T, et al. Neuronal roles of the integrin-associated protein (IAP/CD47) in developing cortical neurons. *The Journal of biological chemistry*. 2004; 279:43245–53. [PubMed: 15297459]
- Okauchi M, et al. Effects of deferoxamine on intracerebral hemorrhage-induced brain injury in aged rats. *Stroke*. 2009; 40:1858–63. [PubMed: 19286595]
- Reinhold MI, et al. In vivo expression of alternatively spliced forms of integrin-associated protein (CD47). *Journal of cell science*. 1995; 108(Pt 11):3419–25. [PubMed: 8586654]
- Rogers NM, et al. Activated CD47 regulates multiple vascular and stress responses: implications for acute kidney injury and its management. *Am J Physiol Renal Physiol*. 2012; 303:F1117–25. [PubMed: 22874763]
- Song S, et al. A new hippocampal model for examining intracerebral hemorrhage-related neuronal death: effects of deferoxamine on hemoglobin-induced neuronal death. *Stroke*. 2007; 38:2861–3. [PubMed: 17761912]
- Wu J, et al. Iron and iron-handling proteins in the brain after intracerebral hemorrhage. *Stroke*. 2003; 34:2964–9. [PubMed: 14615611]
- Xi G, Keep RF, Hoff JT. Mechanisms of brain injury after intracerebral haemorrhage. *Lancet Neurol*. 2006; 5:53–63. [PubMed: 16361023]
- Xing C, et al. Role of oxidative stress and caspase 3 in CD47-mediated neuronal cell death. *Journal of neurochemistry*. 2009; 108:430–6. [PubMed: 19012741]
- Zhou HJ, et al. Alteration of thrombospondin-1 and -2 in rat brains following experimental intracerebral hemorrhage. *Laboratory investigation. J Neurosurg*. 2010; 113:820–5. [PubMed: 20136391]

Highlights

- Effects of intracerebral hemorrhage (ICH) on CD47 examined in a swine model
- Brain CD47 levels were increased in both grey and white matter after ICH
- CD47 was expressed on neurons, microglia/macrophages, and oligodendrocytes, but not on astrocytes
- Deferoxamine, an iron chelator, reduced ICH-induced CD47 upregulation

Figure 1. CD47 immunoreactivity in contra- and ipsilateral white and gray matter at different times after ICH and at day-3 after sham operation. Scale bar=50 μ m, * indicates the hematoma.

Figure 2.

The time-course of the CD47 protein levels in ipsilateral white (A) and gray (B) matter after a needle insertion or ICH.

Figure 3. CD47 levels in the ipsi- or contralateral white matter (A) and grey matter (B) at day-3 in pigs with a sham operation or ICH. Values are mean $\pm$ SD, n=4, *p<0.05.

Figure 4. Triple immunofluorescence staining showing co-localization of CD47 positive cells with NeuN (A), MBP (B) GFAP(C) and Iba-1 (D) positive cells in the perihematoma area at 3 days after blood injection. Scale bar= 20 μ m at (A–C) and 50 μ m at (D), * indicates the hematoma.

Figure 5.

CD47 levels in the contralateral or ipsilateral white (A) and grey (B) matters at 3 days after 2.5ml blood injected into the right frontal lobe of the pig with the treatment of deferoxamine or vehicle. Values are mean $\pm$ SD, n=4, #p<0.01.