

DESCRIPTION: State the application's broad, long-term objectives and specific aims, making reference to the health relatedness of the project. Describe concisely the research design and methods for achieving these goals. Avoid summaries of past accomplishments and the use of the first person. This abstract is meant to serve as a succinct and accurate description of the proposed work when separated from the application. If the application is funded, this description, as is, will become public information. Therefore, do not include proprietary/confidential information. **DO NOT EXCEED THE SPACE PROVIDED.**

Spontaneous intracerebral hemorrhage (ICH) is a common and often fatal stroke subtype. If the patient survives the ictus, the resulting hematoma within the brain parenchyma triggers a series of events leading to secondary insults and severe neurological deficits. Brain edema plays an important role in the secondary brain injury following ICH. It is the long-term goal of our laboratory to identify the mechanisms involved in brain edema formation following ICH. Our previous studies indicate that edema formation following ICH may involve several phases. An early phase involving the clotting cascade and thrombin production. Although our data indicate that thrombin inhibition is a therapeutic target, concerns over rebleeding may limit the utility of this approach. It is, therefore, important to understand the downstream mechanisms activated by thrombin. Our preliminary studies have found: 1) thrombin activity increases immediately after ICH; 2) complement cascade is activated in the brain following ICH and thrombin injection; 3) thrombin upregulates protease-activated receptors (PARs); 4) thrombin increases brain TNF- $\alpha$  levels; 5) thrombin potentiates iron-mediated damage. These results lead us to test the following specific aims:

1) To determine whether thrombin formation following ICH activates the complement cascade in the brain resulting in blood-brain barrier disruption and edema formation. These experiments will employ complement inhibitors as well as C3 and C5 deficient mice.

2) To determine whether thrombin causes brain edema through activation of PARs. These experiments will employ PAR-1, 3 and -4 agonists and antagonists as well as PAR-1 knockout mice.

3) To determine whether thrombin exacerbates brain edema induced by iron.

The purpose of our project is to investigate the mechanisms involved in thrombin-induced brain edema. If our hypotheses are correct, these experiments may lead to novel methods of treating ICH.

PERFORMANCE SITE(S) (*organization, city, state*)

University of Michigan  
Ann Arbor, Michigan

KEY PERSONNEL. See instructions. *Use continuation pages as needed* to provide the required information in the format shown below. Start with Principal Investigator. List all other key personnel in alphabetical order, last name first.

Name	Organization	Role on Project
HOFF, JULIAN T. M.D.	UNIVERSITY OF MICHIGAN	PI
HUA, YA, M.D	UNIVERSITY OF MICHIGAN	CO-INVESTIGATOR
KEEP, RICHARD F., PH.D.	UNIVERSITY OF MICHIGAN	CO-INVESTIGATOR
XI, GUOHUA, M.D.	UNIVERSITY OF MICHIGAN	CO-INVESTIGATOR

**Disclosure Permission Statement.** Applicable to SBIR/STTR Only. See instructions.  Yes  No

**RESEARCH GRANT**

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*Appendices NOT PERMITTED for Phase I SBIR/STTR unless specifically solicited.*

Number of publications and manuscripts accepted for publication (*not to exceed 10*) \_\_\_\_\_ 10 \_\_\_\_\_  
 Other items (list): \_\_\_\_\_



Check if  
Appendix is  
Included

<b>BUDGET JUSTIFICATION PAGE MODULAR RESEARCH GRANT APPLICATION</b>				
<b>Initial Budget Period</b>	<b>Second Year of Support</b>	<b>Third Year of Support</b>	<b>Fourth Year of Support</b>	<b>Fifth Year of Support</b>
<b>\$ 225,000</b>	<b>\$ 225,000</b>	<b>\$ 225,000</b>	<b>\$ 225,000</b>	<b>\$ 225,000</b>
<b>Total Direct Costs Requested for Entire Project Period</b>				<b>\$ 1,125,000</b>

**Personnel**

Julian T. Hoff, M.D., Principal Investigator, (15% effort) will have overall responsibility for the project. He will design the experiments, set the priorities and interpret results.

Ya Hua, M.D., Co-Investigator, (60% effort) will have day-to-day responsibility for the project. She will perform Western blots and immunohistochemistry, carry out behavioral tests, and participate in the experimental design and manuscript writing.

Richard F. Keep, Ph.D., Co-Investigator, (15% effort) will aid in the design and performance of the blood-brain barrier integrity experiment.

Guohua Xi, M.D. Co-Investigator, (20% effort) will perform animal surgery and measure brain edema.

Takehiro Nakamura, M.D., Ph.D. Research Fellow (100% effort) will be involved in the day-to-day work. The Research Fellow will perform or assist in animal surgery, edema measurement, behavioral test, RT-PCR, in situ hybridization, and immunohistochemistry.

**Consortium**

N/A

Fixed Fee (SBIR/STTR Only)

**BIOGRAPHICAL SKETCH**

Provide the following information for the key personnel in the order listed for Form Page 2.  
Follow the sample format on preceding page for each person. **DO NOT EXCEED FOUR PAGES.**

NAME		POSITION TITLE	
Julian T. Hoff, MD		Professor and Chairman	
EDUCATION/TRAINING ( <i>Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.</i> )			
INSTITUTION AND LOCATION	DEGREE ( <i>if applicable</i> )	YEAR(s)	FIELD OF STUDY
Stanford University, Palo Alto California	A.B.	1954-1958	Biology, Chemistry
Cornell University Medical College, New York	M.D.	1958-1962	Medicine
New York Hospital, New York	Intern	1962-1963	Straight Surgery
New York Hospital, New York	Resident	1963-1964	General Surgery
New York Hospital, New York	Resident	1966-1970	Neurosurgery

**A. Positions and Honors**Professional Positions:

- 1968-70 Fellow, Department of Surgery, Memorial-Sloan Kettering Medical Center, New York
- 1969-70 Instructor, Department of Surgery, Cornell University Medical College, New York
- 1970-74 Assistant Professor, Neurological Surgery, University of California, San Francisco
- 1974-78 Associate Professor, Neurological Surgery, University of California, San Francisco
- 1975-80 Vice-Chairman, Department of Neurological Surgery, University of California, San Francisco
- 1977-78 Acting Chairman, Department of Neurological Surgery, University of California, San Francisco
- 1978-81 Professor, Department of Neurological Surgery, University of California, San Francisco
- 1981-01 Professor and Head, Section of Neurosurgery, Department of Surgery, University of Michigan, Ann Arbor
- 1992-01 Richard C. Schneider Professor, Head, Section of Neurosurgery, Department of Surgery, University of Michigan, Ann Arbor
- 2001- Richard C. Schneider Professor, Chair, Department of Neurosurgery, University of Michigan, Ann Arbor

Awards and Other Professional Activities:

- 1977-80 Editorial Board Stroke
- 1978-83 Editorial Board Neurosurgery
- 1987- Editorial Board Acta Neurochirurgica
- 1990-95 Editorial Board Journal of Neuroimaging
- 1990-present Editorial Board Journal of Neurosurgery
- 1997-99 Co-Chairman Journal of Neurosurgery
- 1993-2000 Editorial Board Canadian Journal of Neurological Sciences
- 1993-2000 Editorial Board Journal of Trauma
- 1994- present Editorial Board Journal of American College of Surgeons
- 1985-92 Jacob Javitz Neuroscience Award, NINDS
- 1992-99 Jacob Javitz Neuroscience Award, NINDS
- 1996-97 President, American Academy of Neurological Surgeons
- 1993-94 President, American Association of Neurological Surgeons
- 1991-92 Chairman, American Board of Neurological Surgeons
- 1981-85 Program Project Review Committee A, NINDS, NIH
- 1992-97 Board of Scientific Councilors, NINDS, NIH
- 1998-02 National Advisory Council, NINDS, NIH
- 1999- Institute of Medicine, National Academy of Science

**B: Selected Publications** (selected of 260+):

1. Menzies SA, Betz AL, Hoff JT: Contribution of ions and albumin to the formation and resolution of ischemic brain edema. *J Neurosurg* 78:257-266, 1993
2. Tucci DL, Telian SA, Kileny PR, Hoff JT, Kemink JL: Stability of hearing preservation following acoustic tumor surgery. *Am J Otolaryngol* 15:1-5, 1994
3. Hoff JT: Towards better balance. The 1994 AANS Presidential Address. *J Neurosurg* 81:651-655, 1994
4. Yang G-Y, Betz AL, Hoff JT: Experimental intracerebral hemorrhage. Relationship between brain edema, blood flow, and blood-brain barrier permeability in rats. *J Neurosurg* 81:93-102, 1994
5. Lee K, Betz AL, Keep R, Hoff J: Intracerebral infusion of thrombin as a cause of brain edema. *J Neurosurg* 83:1045-1050, 1995
6. McLelland ML, Woster P, Sweasey T, Hoff JT: Continuous midazolam/atracurium infusions for the management of increased pressure. *J Neurosci Nursing* 27:96-101, 1995
7. Lee KR, Kawai N, Kim S, Sagher O, Hoff JT: Mechanisms of edema formation after intracerebral hemorrhage: Effects of thrombin in cerebral blood flow, blood-brain-barrier permeability, and cell survival in a rat model. *J Neurosurg* 86:272-278, 1997
8. Lee KR, Drury I, Vitarbo E, Hoff JT: Seizures induced by intracerebral injection of thrombin: A model of intracerebral hemorrhage. *J Neurosurg* 87:73-78, 1997
9. Colon G, Hoff JT, Ross DA: Sequential outer-table craniotomy in hyperostosing meningioma. Technical Note. *J Neurosurg* 88:346-348, 1998
10. Rodas RA, Fenstermaker RA, Hopkins LN, McKeever PE, Blaivas M, Papadopoulos SM, Hoff JT, Greenberg HS: Intraluminal thrombosis in brain tumor vessels correlates with postoperative deep vein thrombosis. *J Neurosurg* 89:200-205, 1998
11. Figueroa BE, Keep RF, Betz AL, Hoff JT: Plasminogen activators potentiate thrombin-induced brain injury. *Stroke* 29:1202-1208, 1998
12. Xi G, Wagner KR, Keep RF, Hua Y, deCourten-Myers GM, Broderick JP, Brott TG, Hoff JT: The role of blood clot formation on early edema development following experimental intracerebral hemorrhage. *Stroke* 29: 2580-2586, 1998
13. Xi G, Keep RF, Hoff JT: Erythrocytes and delayed brain edema formation following intracerebral hemorrhage in rats. *J. Neurosurg.* 89: 991-996, 1998
14. Boland M, Hoff J: Neurosurgery, Chapter 104, in Greenfield L, et al. (eds): *Surgery*, 2d Ed. Philadelphia: Lippincott, 1997, pp 755-763
15. Xi G, Keep RF, Hua Y, Xiang J, Hoff JT. Attenuation of thrombin-induced brain edema by cerebral thrombin preconditioning. *Stroke* 1999;30 (6):1247-1255
16. Hua Y, Xi G, Keep RF, Hoff JT. Complement activation in the brain after experimental intracerebral hemorrhage. *J Neurosurg* 2000;92:1016-1022
17. Xi G, Hua Y, Keep RF, Younger JG, Hoff JT. Systemic complement depletion diminishes perihematoma brain edema. *Stroke* 2001;32(1):162-167
18. Xi G, Hua Y, Bhasin RR, Ennis SR, Keep RF, Hoff JT. Mechanisms of edema formation following intracerebral hemorrhage: Effects of extravasated red blood cells on blood flow and blood-barrier integrity. *Stroke* 2001;32:2932-2938
19. Huang FP, Xi G, Keep RF, Hua Y, Nemoianu A, Hoff JT. Brain Edema After Experimental Intracerebral Hemorrhage: Role of Hemoglobin Degradation Products. *J Neurosurg* 2002;96:287-293
20. Hua Y, Xi G, Keep RF, Wu J-M, Jiang Y-J, Hoff JT. Plasminogen Activator Inhibitor-1 Induction after Experimental Intracerebral Hemorrhage. *J Cereb Blood Flood Metab* 2002;22:55-61
21. Xi G, Keep RF, Hoff JT. Pathophysiology of Edema. *Neurosurg Clinics of North America.* 2002;13:371-383.
22. Jiang Y-J, Wu J-M, Keep RF, Hua Y, Hoff JT, Xi G. Hypoxia Inducible Factor-1 $\alpha$  Accumulation in the Brain after Experimental Intracerebral Hemorrhage. *J Cereb Blood Flood Metab* 2002;22:689-696
23. Hua Y, Schallert T, Keep RF, Wu J-M, Hoff JT, Xi G. Behavioral tests after intracerebral hemorrhage in the rat. *Stroke* 2002;33:2478-2484.
24. Nakamura T, Keep RF, Hua Y, Schallert T, Hoff JT, Xi G. Deferoxamine attenuates brain edema and neurological deficits in a rat model of intracerebral hemorrhage. *Neurosurgical Focus.* Oct. 15, 2003
25. Hua Y, Keep KF, Hoff JT, Xi G. Thrombin preconditioning attenuates brain edema induced by erythrocyte and iron. *J Cereb Blood Flow Metab*, 2003;23:1448-1454.
26. Wu J-M, Hua Y, Keep RF, Nakamura T, Hoff JT, Xi G. Iron and iron handling proteins in the brain after intracerebral hemorrhage. *Stroke*, 2003;34:2964-2969.

**C: Research Projects Ongoing or Completed During Last 3 Years****Ongoing:**

'Mechanisms of brain edema after intracerebral hemorrhage'

Principal Investigator: Julian T. Hoff

Agency: NINDS

Type: R01 (NS 17760; Years 18-22) Period 12/1/99-11/30/04

The aim of this project is to elucidate the potentially harmful factors in blood that cause brain edema formation following a hemorrhage.

'Mechanisms of thrombin-induced tolerance to brain injury'

Principal Investigator: Guohua Xi; Investigator: Julian T. Hoff

Agency: NINDS

Type: R01 (NS 39866; Years 1-7) Period 2/1/00-1/31/07

The aim of this project is to determine the mechanisms by which low doses of thrombin are neuroprotective.

**BIOGRAPHICAL SKETCH**

NAME <b>Richard F. Keep, Ph.D.</b>		POSITION TITLE Crosby/Kahn Collegiate Professor Associate Chair	
INSTITUTION AND LOCATION	DEGREE (if applicable)	YEAR(s)	FIELD OF STUDY
Gonville and Caius College, Cambridge	B.A.	1979	Zoology
University of Aberdeen	Ph.D.	1983	Zoology
University of Hull	Post-doc.	1984-1988	Zoology
King's College, London	Post-doc.	1988	Physiology

**A. Positions and Honors****Professional Positions:**

1989 to 1991	Visiting Research Investigator, Neurosurgery, University of Michigan, Ann Arbor
1991 to 1997	Assistant Research Scientist, , Neurosurgery, University of Michigan, Ann Arbor
1996 to 1999	Acting Director, Crosby Neurosurgical Labs, Neurosurgery, University of Michigan
1997 to 2001	Senior Associate Research Scientist, Neurosurgery, University of Michigan
1999 to present	Director, Crosby Neurosurgical Laboratories, Neurosurgery, University of Michigan
2000 to present	Associate Professor, Physiology, University of Michigan, Ann Arbor
2001 to present	Senior Research Scientist, Neurosurgery, University of Michigan, Ann Arbor
2001 to present	Associate Chair for Research, Neurosurgery, University of Michigan, Ann Arbor
2002 to present	Crosby Kahn Collegiate Professor, Neurosurgery, University of Michigan, Ann Arbor

**Awards and Other Professional Activities:**

1979-1983	Science Research Council Studentship
1998	NIHLB ad hoc reviewer
1998-2002	Wellcome Trust grant reviewer
1999-2004	NINDS ad hoc reviewer
1999 to present	Editorial Board, Journal of Neurochemistry

**B. Selected Peer-Reviewed Publications** (selected of 110)

- Masada, T., Hua, Y., Xi, G., Ennis, S.R. and KEEP, R.F. Attenuation of ischemic brain edema and cerebrovascular injury following ischemic preconditioning in the rat. *J. Cereb. Blood Flow. Metab.* 21: 22-33, 2001.
- Teuscher, N.S., KEEP, R.F. and Smith, D.E. PEPT2-mediated uptake of neuropeptides in rat choroid plexus. *Pharm. Res.* 18: 807-813, 2001
- Masada, T., Hua, Y., Xi, G., Yang, G.-Y., Hoff, J.T. and KEEP, R.F. Overexpression of interleukin-1 receptor antagonist attenuates brain edema induced by intracerebral hemorrhage and thrombin. *J. Neurosurgery* 95:680-686; 2001.
- Xi, G., Hua, Y., Bhasin, R.R., Ennis, S.R., KEEP, R.F. Hoff, J.T. Mechanisms of edema formation following intracerebral hemorrhage: effects of extravasated red blood cells on blood flow and blood-brain barrier integrity. *Stroke* 32: 2932-2938; 2001.
- Hua, Y., Xi, G., KEEP, R.F., Wu, J., Jiang, Y. and Hoff, J.T. Plasminogen activator inhibitor-1 induction after experimental intracerebral hemorrhage. *J. Cereb. Blood Flow Metab* 22: 55-61; 2002.
- Huang, F.-P., Xi, G., Hua, Y., KEEP, R.F., Nemoianu, A. and Hoff, J.T.. Brain edema after experimental intracerebral hemorrhage: Role of hemoglobin breakdown products. *J. Neurosurg.* 96: 287-293, 2002.
- Harrigan, M.R., Ennis, S.R., Masada, T., KEEP, R.F. Intraventricular infusion of vascular endothelial growth factor: a therapeutic window for cerebral angiogenesis. *Neurosurg.* 50: 589-598, 2002.
- Jiang, Y., Hua, Y., KEEP, R.F., Wu, J., Xiang, J., Hoff, J.T. and Xi, G. Thrombin receptor activation and thrombin-induced brain tolerance. *J. Cereb. Blood Flow Metab.* 22: 404-410; 2002
- Shu, C., Shen, H., Teuscher, N.S., Lorenzi, P.J., KEEP, R.F. and Smith, D.E. Role of PEPT2 in peptide/mimetic trafficking at the blood-CSF barrier: studies in rat choroid plexus epithelial cells in primary culture. *J. Pharmacol. Exp. Ther.* 301: 820-829; 2002.

10. Jiang, Y., Xi, G., KEEP, R.F., Hua, Y., Wu, J. and Hoff, J.T. Hypoxia inducible factor-1a accumulation in the brain after intracerebral hemorrhage. *J. Cereb. Blood Flow Metab.* 22:689-696; 2002.
11. Hua Y., Schallert, T., KEEP, R.F., Wu, J., Hoff, J.T. and Xi, G. Behavioral tests after intracerebral hemorrhage in the rat. *Stroke* 33: 2478-2484; 2002.
12. Wu, J., Xi, G., Hua, Y., KEEP, R.F., Schallert, T., and Hoff, J.T. Extravasated erythrocytes and oxidative brain injury after intracerebral hemorrhage. *Brain Res.* 953: 45-52; 2002
13. Kitaoka, T., Hua, Y., Xi, G., Hoff, J.T., KEEP, R.F. Delayed argatroban treatment reduces edema in a rat model of intracerebral hemorrhage. *Stroke* 33: 3012-3018, 2002.
14. Ennis, S.R, Novotny, A., Xiang, J., Shakui, P., Masada, T., Stummer, W., Smith, D.E. and KEEP, R.F. Transport of 5-aminolevulinic acid between blood and brain. *Brain Res.* 959: 226-234; 2003
15. Xi, G., Reiser, G. and KEEP, R.F. The role of thrombin and thrombin receptors in ischemic, hemorrhagic and traumatic brain injury: deleterious or protective? *J. Neurochem.* 84: 3-9; 2003.
16. Harrigan, M, Ennis, S.R., Sullivan, S.E. and KEEP, R.F. Effects of intracerebroventricular infusion of vascular endothelial growth factor on cerebral blood flow, edema and infarct volume. *Acta Neurochir.* 145: 49-53, 2003.
17. Xiang, J., Ennis, S.R., Fujisawa, M., Kawai, N., and KEEP, R.F. Glutamine transport at the blood-brain and blood-cerebrospinal fluid barriers. *Neurochem. Int.* 43: 279-288; 2003
18. Shen, H., Smith, D.E., KEEP, R.F., Xiang, J. and Brosius, F.C. Targeted disruption of the PEPT2 Gene markedly impairs dipeptide uptake in choroid plexus. *J. Biol. Chem.* 278: 4786-4791; 2003.
19. Nakamura, T., Xi, G., Hua, Y., Hoff, J.T. and KEEP, R.F. Nestin expression after experimental intracerebral hemorrhage. *Brain Res.* 981: 108-117; 2003.
20. Sagher, O., Huang, D.-L., KEEP, R.F. Spinal cord stimulation reduces infarct volume in a focal cerebral ischemia model. *J. Neurosurg* 99: 125-131; 2003.
21. Ocheltree, S.M., KEEP, R.F., Shen, H., Yang, D., Hughes, B.A. and Smith, D.E. Preliminary investigation into the expression of proton-coupled oligopeptide transporters in neural retina and retinal pigment epithelium (RPE): Lack of functional activity in RPE plasma membranes *Pharm. Res.* 20: 1364-1372; 2003.
22. Hua, Y., KEEP, R.F., Hoff, J.T. and Xi, G. Thrombin preconditioning attenuates brain edema induced by erythrocytes and iron. *J. Cereb. Blood Flow Metab.* 23: 1448-1454, 2003
23. Stamatovic, KEEP, R.F., Kunkel, S.L. and Andjelkovic, A.V. Potential role of MCP-1 in endothelial cell tight junction "opening": Signaling via Rho and Rho kinase. *J. Cell Sci.* 116: 4615-4628; 2003
24. Andjelkovic, A.V., Stamatovic, S.M. and KEEP, R.F. The protective effect of preconditioning on cerebral endothelial cells in vitro. *J. Cereb. Blood Flow Metab.* 23: 1348-1355; 2003
25. Wu, J., Hua, Y, KEEP, R.F., Nakamura, T., Hoff, J.T., Xi, G. Iron and iron handling proteins in the brain after intracerebral hemorrhage. *Stroke* 34: 2970-2975; 2003
26. Ocheltree, S.M., Shen, H., Hu, Y., Xiang, J., KEEP, R.F. and Smith, D.E. Mechanisms of cefadroxil uptake in the choroid plexus: studies with wild type and PEPT2 knockout mice. *J. Pharmacol. Exp. Ther.* 308:462-467, 2004.
27. Karabiyikoglu, M., Hua, Y, KEEP, R.F., Ennis, S.R., Xi, G. Intracerebral hirudin injection attenuates ischemic tissue damage and neurological deficits without altering local cerebral blood flow. *J. Cereb. Blood Flow Metab.* 24:159-166, 2004.
28. Xi, G., Fewel, M.E., Hua, Y., Thompson, B.G., Hoff, J.T. and KEEP, R.F. Intracerebral hemorrhage: pathophysiology and therapy. *Neurocritical Care* 1: 5-18, 2004.
29. Teuscher, N.S., Shen, H., Shu, C., Xiang, J., KEEP, R.F. and Smith, D.E. Carnosine uptake in rat choroid plexus primary cell cultures and choroid plexus whole tissue from PEPT2 null mice. *J. Neurochem.* (in press).
30. Nakamura, T., KEEP, R.F., Hua, Y., Schallert, T., Hoff, J.T. and Xi, G. Deferoxamine attenuates brain edema and neurological deficits after experimental intracerebral hemorrhage. *J. Neurosurg.* (in press).
31. Nakamura, T., Xi, G., Hua, Y., Schallert, T., Hoff, J.T. and KEEP, R.F. Intracerebral hemorrhage in mice: model characterization and application for genetically modified mice. *J. Cereb. Blood Flow Metab.* (in press)



**C. Research Projects Ongoing or Completed During Last 3 years:  
Ongoing**

“Obesity and hypertension: the role of CNS 5-HT<sub>2c</sub> receptors”

Principal Investigator: Richard F. Keep

Agency: National Heart, Lung, and Blood Institute

Type: P01 (HL 18575) Period July 1999-June 2004

Aim: to examine whether activation of CNS 5-HT<sub>2c</sub> receptors is a link between obesity and hypertension.

“Endothelial preconditioning and ischemic brain injury”

Principal Investigator: Richard F. Keep

Agency: National Institute of Neurological Disorders and Stroke

Type: R01 (NS 34709) Period June 2003-May 2008

Aim: to elucidate the mechanisms involved in the protection of the cerebral endothelium by preconditioning.

“Mechanisms of brain edema formation after intracerebral hemorrhage”

Principal Investigator: Julian T. Hoff, Richard F. Keep Investigator

Agency: National Institute of Neurological Disorders and Stroke

Type: R01 (NS 17760) Period December 1999-November 2004

Aim: to examine the roles of complement activation and erythrocyte lysis in brain edema formation following intracerebral hemorrhage.

"Effects of extravascular thrombin inhibition by melagatran on ischemic brain damage"

Principal Investigator Richard F. Keep

Funding: Astra Zeneca.

Type: Industrial; Period May 2003-April 2004

“Mechanisms of thrombin-induced tolerance to brain injury’

Principal Investigator: Guohua Xi, Richard F. Keep Co-PI

Agency: National Institute of Neurological Disorders and Stroke

Type: R01 (NS 39866) Period February 2000-January 2007

Aim: to determine the mechanisms by which low doses of thrombin are neuroprotective.

“Peptide/mimetic Transport Mechanisms in Choroid Plexus”

Principal Investigator: David E. Smith, Richard F. Keep Investigator

Agency: NIGMS

Type: RO1 (GM35498). Period April 2001- March 2005

Aim: Examine the role of PEPT2 mediated transport of peptides and peptidomimetics between blood and brain.

**Completed**

“Blood-brain-transport and ischemic brain injury”

Principal Investigator: Richard F. Keep

Agency: National Institute of Neurological Disorders and Stroke

Type: R01 (NS 34709) Period September 1999-May 2003

Aim: to elucidate the effects of cerebral ischemia on the clearance of potentially toxic compounds from brain to blood.

“Alcohol and Brain Injury: A Career Development Plan”

Principal Investigator Brian Zink; Mentor Richard F. Keep

Agency: NIAAA

Type: K20 (AA00184) Period July 1999-2001.

Aim: to examine the mechanisms by which alcohol effects traumatic brain injury.

"Effects of argatroban on brain injury following intracerebral hemorrhage"

Principal Investigator Richard F. Keep

Funding: Mitsubishi-Tokyo Pharmaceuticals Inc.

Type: Industrial; Period January 2001-December 2001

"Effects of Radicut on Intracerebral Hemorrhage-Induced Brain Injury"

Principal Investigator Richard F. Keep

Funding: Mitsubishi-Tokyo Pharmaceuticals Inc.

Type: Industrial; Period January 2002-December 2002

**BIOGRAPHICAL SKETCH**

Provide the following information for the key personnel in the order listed for Form Page 2.  
Follow the sample format on preceding page for each person. **DO NOT EXCEED FOUR PAGES.**

NAME		POSITION TITLE	
Ya Hua, M.D.		Research Investigator	
EDUCATION/TRAINING ( <i>Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.</i> )			
INSTITUTION AND LOCATION	DEGREE ( <i>if applicable</i> )	YEAR(s)	FIELD OF STUDY
Zhejiang Medical University, China	MD	1980-1985	Medicine
Zhejiang Mental Diseases and Health Institute	Resident	1985-1988	Psychiatry
University of Cincinnati	Post-doc Assistant	1993-1998	Neurology
University of Michigan	Post-doc Fellow	1998-1999	Neurosurgery

**A. Positions and Honors**Professional Experience:

1992-1993	Lecturer of Neurology, Department of Neurology, Zhejiang Medical University P.R. China
1999-2000	Research Associate I, Department of Surgery (Neurosurgery), University of Michigan Ann Arbor, Michigan
2000-2001	Research Associate II, Department of Neurosurgery, University of Michigan Ann Arbor, Michigan
2001-present	Research Investigator, Department of Neurosurgery, University of Michigan Ann Arbor, Michigan

Professional Societies:

Society for Neuroscience, USA  
American Stroke Association

**B. Selected Peer-Reviewed Publications**

1. Xi G, Wagner KR, Keep RF, **Hua Y**, de-Courten Myers GM, Broderick JP, Brott TG, and Hoff JT. The role of blood clot formation on early edema development following experimental intracerebral hemorrhage. *Stroke* 1998;29:2580-2586.
2. Wagner KR, Xi G, **Hua Y**, Zuccarello M, de-Courten Myers GM, Broderick JP, and Brott TG. Ultra-early clot aspiration after lysis with tissue plasminogen activator in a pig intracerebral hemorrhage model: Edema reduction and blood brain barrier protection. *J Neurosurg* 1999;90:491-498
3. Xi G, Keep RF, **Hua Y**, Xiang J, Hoff JT. Attenuation of thrombin-induced brain edema by cerebral thrombin preconditioning. *Stroke* 1999;30:1247-1255
4. **Hua Y**, Xi G, Keep RF, Hoff JT. Complement activation in the brain after experimental intracerebral hemorrhage. *J Neurosurg* 2000;92:1016-1022
5. Masada T, Xi G, **Hua Y**, Keep RF. The effects of thrombin preconditioning on focal cerebral ischemia in rats. *Brain Res* 2000;867:173-179
6. Wagner KR, **Hua Y**, de Courten-Myers GM, Broderick JP, Nishimura RN, Lu SY and Dwyer BE. Tin-mesoporphyrin, a potent heme oxygenase inhibitor, for treatment of intracerebral hemorrhage: In vivo and in vitro studies. *Cell. Mol. Biol.* 2000; 46(3) pp. 597-608
7. Xi G, **Hua Y**, Keep RF, Hoff JT. Induction of colligins may attenuate brain edema following intracerebral hemorrhage. *Brain Edema XI* pp.501-505.
8. Xi G, Keep RF, **Hua Y**, Hoff JT. Thrombin preconditioning, heat shock proteins and thrombin-induced brain edema. *Brain Edema XI* pp.511-515.

9. Xi G, **Hua Y**, Keep RF, Younger JG, Hoff JT. Systemic complement depletion diminishes perihematomal brain edema. *Stroke* 2001;32:162-167
10. Masada T, **Hua Y**, Xi G, Ennis SR, Keep RF. Attenuation of ischemic brain edema and cerebrovascular injury following ischemic preconditioning in the rat. *J Cereb Blood Flow Metab* 2001;21:22-33
11. Xi G, **Hua Y**, Keep RF, Duong HK, Hoff JT. Activation of p44/42 mitogen activated protein kinases in thrombin-induced brain tolerance. *Brain Res* 2001;895:153-159
12. Masada T, **Hua Y**, Xi G, Yang GY, Hoff JT, Keep RF. Overexpression of interleukin-1 receptor antagonist attenuates brain edema induced by intracerebral hemorrhage and thrombin. *J Neurosurg* 2001;95:680-686
13. Xi G, **Hua Y**, Bhasin RR, Ennis SR, Keep RF, Hoff JT. Mechanisms of edema formation following intracerebral hemorrhage: Effects of extravasated red blood cells on blood flow and blood-brain barrier integrity. *Stroke* 2001;32:2932-2938
14. Huang FP, Xi G, Keep RF, **Hua Y**, Nemoianu A, Hoff JT. Brain Edema After Experimental Intracerebral Hemorrhage: Role of Hemoglobin Degradation Products. *J Neurosurg* 2002;96:287-293
15. **Hua Y**, Xi G, Keep RF, Wu J-M, Jiang Y-J, Hoff JT. Plasminogen Activator Inhibitor-1 Induction after Experimental Intracerebral Hemorrhage. *J Cereb Blood Flood Metab* 2002;22:55-61
16. Jiang Y-J, Wu J-M, **Hua Y**, Keep RF, Xiang JM, Hoff JT, Xi G. Thrombin receptor activation and thrombin-induced brain tolerance. *J Cereb Blood Flood Metab* 2002;22:404-410
17. Jiang Y-J, Wu J-M, Keep RF, **Hua Y**, Hoff JT, Xi G. Hypoxia Inducible Factor-1a Accumulation in the Brain after Experimental Intracerebral Hemorrhage. *J Cereb Blood Flood Metab* 2002;22:689-696
18. Xi G, **Hua Y**, Keep RF, Younger JG, Hoff JT. Brain edema after intracerebral hemorrhage: The effects of systemic complement depletion. *Acta Neurochir(suppl)* 2002;81:253-256.
19. Bhasin RR, Xi G, **Hua Y**, Keep RF, Hoff JT. Experimental intracerebral hemorrhage: Effect of lysed erythrocytes on brain edema and blood-brain barrier permeability. *Acta Neurochir(suppl)* 2002;81:249-251.
20. **Hua Y**, Schallert T, Keep RF, Wu J-M, Hoff JT, Xi G. Behavioral tests after intracerebral hemorrhage in the rat. *Stroke* 2002;33:2478-2484
21. Wu JM, **Hua Y**, Keep RF, Schallert T, Hoff JT, Xi G. Oxidative brain injury from extravasated erythrocytes after intracerebral hemorrhage. *Brain Res* 2002;953:45-52
22. Kitaoka T, **Hua Y**, Xi G, Hoff JT, Keep RF. Delayed argatroban treatment reduces edema in a rat model of intracerebral hemorrhage. *Stroke* 2002;33:3012-3018
23. **Hua Y**, Wu J, Keep RF, Yu ZG, Hoff JT, Xi G. Thrombin exacerbates brain edema in focal cerebral ischemia. *Acta Neurochir* 2003;86[suppl]:163-166
24. Xi G, Wu JM, Jiang YJ, **Hua Y**, Keep RF, Hoff JT. Thrombin preconditioning upregulates transferrin and transferrin receptor and reduces brain edema induced by lysed red blood cells. *Acta Neurochir* 2003;86[suppl]:449-452
25. **Hua Y**, Keep RF, Schallert T, Hoff JT, Xi G. A thrombin inhibitor reduces brain edema, glioma mass and neurological deficits in a rat glioma model. *Acta Neurochir* 2003;86[suppl]:530-506
26. Nakamura T, Xi G, **Hua Y**, Hoff JT, Keep RF. Nestin expression after experimental intracerebral hemorrhage. *Brain Res.* 2003;981:108-117
27. Nakamura T, Keep RF, **Hua Y**, Schallert T, Hoff JT, Xi G. Deferoxamine attenuates brain edema and neurological deficits in a rat model of intracerebral hemorrhage. *Neurosurgical Focus.* 2003;15(4):CP4
28. Xi G, Fewel ME, **Hua Y**, Thompson BG, Hoff JT, Keep RF. Intracerebral hemorrhage: Pathophysiology and therapy. *Neurocritical Care*, 2004;1:5-18
29. **Hua Y**, Keep KF, Hoff JT, Xi G. Thrombin preconditioning attenuates brain edema induced by erythrocyte and iron. *J Cereb Blood Flow Metab*, 2003;23:1448-1454
30. Wu J-M, **Hua Y**, Keep RF, Nakamura T, Hoff JT, Xi G. Iron and iron handling proteins in the brain after intracerebral hemorrhage. *Stroke*, 2003;34:2964-2969.
31. Karabiyikoglu M, **Hua Y**, Keep RF, Ennis SR, Xi G. Intracerebral hirudin injection attenuates ischemic brain damage and neurological deficits without altering local cerebral blood flow. *J Cereb Blood Flow Metab*, 2004;24:159-166.

**C: Research Projects Ongoing or Completed During Last 3 Years;****Ongoing:**

'Mechanisms of brain edema after intracerebral hemorrhage'

Principal Investigator: Julian T. Hoff; Investigator: Ya Hua

Agency: NINDS

Type: R01 (NS 17760; Years 18-22) Period 12/1/99-11/30/04

The aim of this project is to elucidate the potentially harmful factors in blood that cause brain edema formation following a hemorrhage.

'Mechanisms of thrombin-induced tolerance to brain injury'

Principal Investigator: Guohua Xi; Investigator: Ya Hua

Agency: NINDS

Type: R01 (NS 39866; Years 4-7) Period 2/1/03-1/31/07

The aim of this project is to determine the mechanisms by which low doses of thrombin are neuroprotective.

**BIOGRAPHICAL SKETCH**

NAME <b>Guohua Xi, M.D.</b>	POSITION TITLE <b>Assistant Research Scientist; Associate Director</b>		
EDUCATION ( <i>Begin with baccalaureate/other initial professional education, such as nursing, and include postdoc training</i> )			
INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
Zhejiang Medical University, P.R. China	M.D.	1985	Medicine
Zhejiang Medical University, P.R. China	Resident	1987	Neurology
Zhejiang Medical University, P.R. China	M.Sc	1991	Neurology
University of Cincinnati	Post-doc	1996	Neuropathology
University of Michigan	Post-doc	1998	Neurosurgery

**A. Positions and Honors**Professional Experience:

- 1991-1992 Lecturer of Neurology, Department of Neurology, Zhejiang Medical University, China  
 1996 Lecturer of Neurology, Department of Neurology, Zhejiang Medical University, China  
 1998-2001 Research Investigator, Department of Surgery (Neurosurgery), University of Michigan, Ann Arbor, Michigan  
 2000-present Assistant Research Scientist, Department of Neurosurgery, University of Michigan, Ann Arbor, Michigan  
 2002-present Associate Director, Crosby Neurosurgical Laboratories, Department of Neurosurgery, University of Michigan, Ann Arbor, Michigan

Awards and Other Professional Activities:

- 2000 Invited Speaker, Department of Neurosurgery, University of Mississippi  
 2001 Invited Speaker, Beijing Stroke Forum 2001  
 2001-present Editorial Board, Journal of Stroke (China)  
 2002 Invited Speaker, Cellular signaling in neuroprotection and plasticity. Magdeburg, Germany  
 2003 Invited Speaker, Neuroscience, Michigan State University  
 2003 Visiting Professor, Neurosurgery, Huashan Hospital, Fudan University, Shanghai, China  
 2003 Invited Speaker, Great Lakes Glia Meeting, Michigan

**B. Selected Peer-Reviewed Publications:** (selected of 52)

- Xi G**, Keep RF, and Hoff JT: Erythrocytes and delayed brain edema formation following intracerebral hemorrhage in rats. *J. Neurosurg* 1998;89:991-996.
- Wagner KR, **Xi G**, Hua Y, Kleinholz M, deCourten-Myers GM, and Myers RE. Early metabolic alterations in edematous perihematomal brain regions following experimental intracerebral hemorrhage. *J. Neurosurg* 1998;88:1058-1065.
- Xi G**, Wagner KR, Keep RF, Hua Y, deCourten-Myers GM, Broderick JP, Brott TG, and Hoff JT. The role of blood clot formation on early edema development following experimental intracerebral hemorrhage. *Stroke* 1998;29:2580-2586.
- Wagner KR, **Xi G**, Hua Y, Zuccarello M, deCourten-Myers GM, Broderick JP, and Brott TG. Ultra-early clot aspiration after lysis with tissue plasminogen activator in a pig intracerebral hemorrhage model: Edema reduction and blood brain barrier protection. *J Neurosurg* 1999;90:491-498.
- Xi G**, Keep RF, Hua Y, Xiang J, Hoff JT. Attenuation of thrombin-induced brain edema by cerebral thrombin preconditioning. *Stroke* 1999;30 (6):1247-1255
- Xi G**, Hua Y, Keep RF, Hoff JT. Induction of colligin may attenuate brain edema following intracerebral hemorrhage. *Acta Neurochir* 2000;76 (Suppl) :501-505
- deCourten-Myers GM, **Xi G**, Hwang J-H, Dunn RS, Mills A, Holland SK, Wagner KR, Myers RE. Hypoglycemic brain injury: Potentiation from respiratory depression and injury aggravation from hyperglycemic treatment overshoot. *J Cereb Blood Flow Metab* 2000;20:82-92
- Hua Y, **Xi G**, Keep RF, Hoff JT. Complement activation in the brain after experimental intracerebral hemorrhage. *J Neurosurg* 2000;92:1016-1022
- Masada T, **Xi G**, Hua Y, Keep RF. The effects of thrombin preconditioning on focal cerebral ischemia in rats. *Brain Res* 2000;867:173-179

10. **Xi G**, Hua Y, Keep RF, Younger JG, Hoff JT. Systemic complement depletion diminishes perihematomal brain edema. *Stroke* 2001;32(1):162-167
11. Masada T, Hua Y, **Xi G**, Ennis SR, Keep RF. Attenuation of ischemic brain edema and cerebrovascular injury following ischemic preconditioning in the rat. *J Cereb Blood Flow Metab* 2001;21:22-33
12. **Xi G**, Hua Y, Keep RF, Duong HK, Hoff JT. Activation of p44/42 mitogen activated protein kinases in thrombin-induced brain tolerance. *Brain Res* 2001;895:153-159
13. Masada T, Hua Y, **Xi G**, Yang GY, Hoff JT, Keep RF. Overexpression of interleukin-1 receptor antagonist attenuates brain edema induced by intracerebral hemorrhage and thrombin. *J Neurosurg* 2001;95:680-686
14. **Xi G**, Hua Y, Bhasin RR, Ennis SR, Keep RF, Hoff JT. Mechanisms of edema formation following intracerebral hemorrhage: Effects of extravasated red blood cells on blood flow and blood-barrier integrity. *Stroke* 2001;32:2932-2938
15. Huang FP, **Xi G**, Keep RF, Hua Y, Nemoianu A, Hoff JT. Brain Edema After Experimental Intracerebral Hemorrhage: Role of Hemoglobin Degradation Products. *J Neurosurg* 2002;96:287-293
16. Bhasin RR, **Xi G**, Hua Y, Keep RF, Hoff JT. Experimental intracerebral hemorrhage: Effect of lysed erythrocytes on brain edema and blood-brain barrier permibility. *Acta Neurochir(suppl)* 2002;81:249-251.
17. Hua Y, **Xi G**, Keep RF, Wu J-M, Jiang Y-J, Hoff JT. Plasminogen Activator Inhibitor-1 Induction after Experimental Intracerebral Hemorrhage. *J Cereb Blood Flood Metab* 2002;22:55-6128.
18. Jiang Y-J, Hua Y, Keep RF, Xiang J, Wu J-M, Hoff JT, **Xi G**. Thrombin receptor activation and thrombin-induced tolerance. *J Cereb Blood Flood Metab* 2002;22:404-410
19. **Xi G**, Keep RF, Hoff JT. Pathophysiology of Edema. In: *Neurosurgery Clinics of North America*. 2002;13:371-383
20. Jiang Y-J, Wu J-M, Keep RF, Hua Y, Hoff JT, **Xi G**. Hypoxia Inducible Factor-1a Accumulation in the Brain after Experimental Intracerebral Hemorrhage. *J Cereb Blood Flood Metab* 2002;22:689-696
21. **Xi G**, Hua Y, Keep RF, Younger JG, Hoff JT. Brain edema after intracerebral hemorrhage: The effects of systemic complement depletion. *Acta Neurochir(suppl)* 2002;81:253-256.
22. Masada T, **Xi G**, Keep RF. Effect of ischemic preconditioning on edema formation and cerebrovaascular injury following focal cerebral ischemia. *Acta Neurochir(suppl)* 2002;81:265-268
23. Hua Y, Schallert T, Keep RF, Wu J-M, Hoff JT, **Xi G**. Behavioral tests after intracerebral hemorrhage in the rat. *Stroke* 2002;33:2478-2484
24. Wu JM, Hua Y, Keep RF, Schallert T, Hoff JT, **Xi G**. Oxidative brain injury from extravasated erythrocytes after intracerebral hemorrhage. *Brain Res* 2002;953:45-52
25. Kitaoka T, Hua Y, **Xi G**, Hoff JT, Keep RF. Delayed argatroban treatment reduces edema in a rat model of intracerebral hemorrhage. *Stroke* 2002;33:3012-3018
26. **Xi G**, Reiser G, Keep KR. The role of thrombin and thrombin receptors in ischemic, hemorrhagic and traumatic brain injury: deleterious or protective? *J Neurochem* 2003;84:3-9
27. Nakamura T, **Xi G**, Hua Y, Hoff JT, Keep RF. Nestin expression after experimental intracerebral hemorrhage. *Brain Res*. 2003;981:108-117
28. Nakamura T, Keep RF, Hua Y, Schallert T, Hoff JT, **Xi G**. Deferoxamine attenuates brain edema and neurological deficits in a rat model of intracerebral hemorrhage. *Neurosurg Focus*. 2003;15(4):CP4.
29. **Xi G** and Hoff JT. The pathophysiology of hemorrhagic lesions. In: *Diagnosis & Therapy Imaging of the Nervous System*. Latchaw RE, Kucharczyk J, and Moseley ME. eds. In press.
30. Hua Y, Keep KF, Hoff JT, **Xi G**. Thrombin preconditioning attenuates brain edema induced by erythrocyte and iron. *J Cereb Blood Flow Metab*, 2003;23:1448-1454
31. Wu J-M, Hua Y, Keep RF, Nakamura T, Hoff JT, **Xi G**. Iron and iron handling proteins in the brain after intracerebral hemorrhage. *Stroke*, 2003;34:2964-2969.
32. Karabiyikoglu M, Hua Y, Keep RF, Ennis SR, **Xi G**. Intracerebral hirudin injection attenuates ischemic brain damage and neurological deficits without altering local cerebral blood flow. *J Cereb Blood Flow Metab*, 2004;24:159-166.
33. **Xi G**, Fewel ME, Hua Y, Thompson BG, Hoff JT, Keep RF. Intracerebral hemorrhage: Pathophysiology and therapy. *Neurocritical Care*, 2004;1:5-18.

**C: Research Projects Ongoing or Completed During Last 3 Years;****Ongoing:**

'Mechanisms of thrombin-induced tolerance to brain injury'

Principal Investigator: Guohua Xi

Agency: NINDS

Type: R01 (NS 39866; Years 1-7) Period 2/1/00-1/31/07

The aim of this project is to determine the mechanisms by which low doses of thrombin are neuroprotective.

'Thrombin-mediated ischemic brain injury'

Principal Investigator: Guohua Xi

Type: American Heart Association, Grant-in-Aid (0255721N), Period 7/1/02-6/30/05

The aim of this project is to determine the role of thrombin in ischemic brain injury.

'Mechanisms of brain edema after intracerebral hemorrhage'

Principal Investigator: Julian T. Hoff

Investigator: Guohua Xi

Agency: NINDS

Type: R01 (NS 17760; Years 18-22) Period 12/1/99-11/30/04

The aim of this project is to elucidate the potentially harmful factors in blood that cause brain edema formation following a hemorrhage.

**Completed:**

Effects of systemic use of argatroban in gliomas.

Principal Investigator: Guohua Xi

Mitsubishi Pharma Corp. Period 7/1/2002- 6/30/2003

Aim: To examine the effect of anti-thrombin treatment on gliomas.



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## RESOURCES

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FACILITIES: Specify the facilities to be used for the conduct of the proposed research. Indicate the performance sites and describe capacities, pertinent capabilities, relative proximity, and extent of availability to the project. Under "Other," identify support services such as machine shop, electronics shop, and specify the extent to which they will be available to the project. Use continuation pages if necessary.

Laboratory:

The Crosby Neurosurgical Laboratories occupy approximately 3500 sq. ft. of space. Within this space the PI has a 600 sq. ft. laboratory to devote to this project but, in addition, core instrument, storage, and other common use space is available. The laboratory is well equipped for small animal physiology.

Clinical:

NA

Animal:

Animals are housed in University maintained facilities in the same building. The rooms are pathogen free.

Computer:

The Neurosurgery Laboratories have five PowerBook G4, two PowerBook G3, three Power Mac G4, eight iMac connected through a PhoneNet network. This campus wide network provides access to the University mainframe and Internet. The Power Mac G4 is connected to a CCD video camera that runs software for image analysis. We have two Mac laser printers and one Epson scanner that runs software for image and photo management.

Office:

Office space is available within the laboratory for Drs. Hua (5611 Kresge I), Keep (5550 Kresge I), Xi (5605 Kresge 1).

Other:

The Department of Neurosurgery provides a full-time secretary for the laboratory.

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MAJOR EQUIPMENT: List the most important equipment items already available for this project, noting the location and pertinent capabilities of each.

Major equipment includes a Beckman 2-channel scintillation counter, Beckman gamma counter, Zeiss Axiovert 10 inverted microscopes and Olympus CK2, Beckman J2-21 refrigerated centrifuge, Waters HPLC pump, Bio-Rad HPLC Column heater, EG & G Electrochemical detector, Gould 8-channel biophysical recorder, 5 4-channel biophysical recorders, Corning blood gas analyzer, IL943 flame photometer, 2 Wescor vapor pressure osmometers, 3 Kopf stereotaxic frames, 3 Zeiss operating microscopes, 3 Harvard infusion pumps, 2 Gilson Minipulse pumps, rodent ventilators and temperature monitors, Vasamedics Laserflo Blood Perfusion Monitor, minotome, a Lica CM 1800 Cryostat, 3 Bio-Rad Power Pacs for Western and Northern Blots, a hybridization incubator, a BPM<sup>2</sup> laser Doppler flowmeter and a DNA Engine (PRC-200, MJ Research).

## A. SPECIFIC AIMS

Intracerebral hemorrhage (ICH) is a common and often fatal subtype of stroke that produces severe neurologic deficits in survivors<sup>1</sup>. Although death may occur acutely after an ICH, delayed neurologic decline often occurs in patients with a large hematoma. Edema development elevates intracranial pressure and causes herniation and death<sup>2,3</sup>. Perihematomal edema is commonly observed during the acute and subacute stage, and it may be more accountable for the poor neurological outcome than the hematoma mass itself<sup>4</sup>.

Mechanisms of edema formation after intracerebral hemorrhage have been identified during the past decade<sup>5-7</sup>. Now we know that several processes are responsible for edema formation around the clot. These include hydrostatic pressure during the clot formation, clot retraction<sup>8-11</sup>, coagulation cascade activation with thrombin production<sup>12-16</sup>, red blood cell (RBC) lysis with hemoglobin-induced toxicity<sup>17</sup>, and complement cascade activation in the brain parenchyma<sup>18</sup>. Brain edema may result from blood-brain barrier disruption or from cytotoxic effects<sup>5,6,15,19,20</sup>. Most studies now suggest that secondary ischemic/reperfusion injury is not a major component of ICH-induced injury<sup>5-7,21</sup>.

Thrombin, in particular, has been shown to play a major role in early edema formation after ICH. Indeed, a thrombin inhibitor (argatroban) reduced ICH-induced edema formation when given after six hours in a rat ICH model<sup>22</sup> and there is some evidence to support human efficacy<sup>23,24</sup>. However, it would be advantageous to block the effects of thrombin on edema without inhibiting coagulation (which could potentially cause rebleeding). Thus, it is important to understand the downstream events triggered by thrombin and whether the effects of thrombin are mediated by one of the thrombin receptors (protease activated receptors, PARs)<sup>25-27</sup>.

The purpose of this project is to investigate the mechanisms of brain edema formation induced by thrombin. The long term goal of our studies is to inhibit edema formation and limit brain injury after ICH. We propose to test the following hypotheses:

**Hypothesis 1.** Thrombin formation after intracerebral hemorrhage activates the complement cascade in the brain which triggers inflammatory response, forms membrane attack complex and results in blood-brain barrier disruption and brain edema formation.

**Specific Aim 1a.** To determine whether thrombin activates the complement cascade in the brain after ICH.

**Specific Aim 1b.** To determine whether complement depletion or inhibition reduces BBB disruption following ICH.

**Specific Aim 1c.** To examine the effects of complement on acute inflammatory response following ICH.

**Hypothesis 2.** Protease-activated receptors (PARs) play a key role in perihematomal edema formation following ICH.

**Specific Aim 2a.** To examine the time courses of PARs upregulation at gene and protein levels after ICH.

**Specific Aim 2b.** To determine whether brain edema formation and blood-brain barrier disruption after ICH are PAR-mediated.

**Specific Aim 2c.** To determine whether increases of TNF- $\alpha$  levels after ICH and thrombin stimulation are due to activation of PARs.

**Specific Aim 2d.** To determine whether thrombin activates complement through PARs.

**Hypothesis 3.** Thrombin exacerbates brain edema induced by iron. Thrombin inhibition plus iron chelation will be a new treatment for ICH.

**Specific Aim 3a.** To determine whether thrombin exacerbates iron-induced brain edema.

**Specific Aim 3b.** To test the effect of thrombin inhibition plus iron chelation on brain edema formation following ICH.

## B. BACKGROUND AND SIGNIFICANCE

### B.1. Clinical Problem of Intracerebral Hemorrhage

Each year approximately 600,000 people suffer a stroke in our country. The causes of stroke are in general, either hemorrhagic or non-hemorrhagic. Intracerebral hemorrhage is a common and often fatal stroke subtype<sup>1</sup>. About 15% of patients (30,000 annually) die from spontaneous ICH.

Spontaneous intracerebral hemorrhage, from a variety of sources, causes instantaneous mass effect, disruption of surrounding brain, and often an early neurological death. The clinical diagnosis is usually not difficult and CT or MRI confirmation of the lesion, its location and some of its characteristics are readily apparent. If the patient survives the initial ictus the clinical condition often stabilizes for a

number of hours. At times during the course of the illness, particularly in those patients with a deteriorating condition, the issue of clot evacuation is considered by clinicians, patients, and families. The expected quality of life for the patient, with and without removal of the hematoma, is nearly always the ultimate question. Will neurological function improve with clot removal? Will deterioration cease? Will long-term outcome be better with surgical evacuation of the hematoma or with resolution of the hematoma by natural surgical techniques? Are there alternatives to surgery and if so what are they? Surprisingly, answers to the above questions are often vague and poorly founded on factual information.

Sir Wiley McKissock focused attention on intracerebral hemorrhage years ago when he designed a clinical study of the merits of surgical versus non-surgical treatment of intracerebral hematomas<sup>28, 29</sup>. Many clinical studies followed but generally they did not produce conclusive guidelines for the clinician managing individual patient problems. Conservative management has generally been recommended in situations where mass effect has not been significant and neurological function has remained stable from the beginning. Operative intervention has usually been reserved for situations where neurological function deteriorates as a result of expanding mass effect. Treatment decisions are generally pragmatic based upon the quality of life that can be expected with the various treatments available.

Clinical investigations have attempted many different therapeutic regimes for ICH, but specific guidelines for ICH treatment remain elusive<sup>30-33</sup>. Clinical protocols with feasible and reproducible endpoints continue to be problematic despite sophisticated diagnostic tools, and safer, more focused treatments that are currently available<sup>34-36</sup>. Clarification of the mechanisms involved in hemorrhagic brain injury is very helpful for developing new therapeutic strategies.

## **B.2. Perihematomal Brain Edema**

Brain edema develops within minutes after an ICH, peaking several days later<sup>6, 11, 37</sup>. Edema development after ICH can elevate intracranial pressure and cause herniation and death<sup>2</sup>. In experimental models, perihematomal edema peaks around the third or fourth day after the hemorrhage then declines slowly<sup>17, 19, 38, 39</sup>. The volume of edema may actually be larger than that of the hematoma itself<sup>40</sup>. Perihematomal brain edema remains in the rat for more than a week. The most severe edema detected around the clot and mainly in the white matter<sup>39, 41</sup>. Edema formation after ICH is variable in humans, depending on clot size and localization. Early CT scans demonstrate that perihematomal edema develops within three hours of symptom onset in most patients and reaches its maximum between 10 to 20 days after the ictus<sup>42, 43</sup>.

Brain edema around the hematoma is commonly observed during the acute and subacute stage following ICH, contributing to poor outcomes<sup>2, 44, 45</sup>. Although the mechanisms of edema formation following ICH are not fully resolved, several mechanisms are responsible for edema development. These include hydrostatic pressure during hematoma formation and clot retraction, coagulation cascade activation and thrombin production, erythrocyte lysis and hemoglobin toxicity, complement activation, mass effect, secondary perihematomal ischemia, reperfusion brain injury, and blood-brain barrier (BBB) disruption<sup>11, 13, 14, 16-18, 46-48</sup>. Various animal models of ICH have allowed detailed study of these mechanisms and their roles in the pathophysiological events that occur in brain tissue after ICH.

There is a very early phase (first several hours) involving hydrostatic pressure and clot retraction and a second phase (first two days) involving the activation of the coagulation cascade and thrombin production<sup>14, 16, 49</sup>. We found that non-clotting heparinized autologous whole blood fails to produce perihematomal edema within 24 hours in rats and pigs<sup>16</sup>. The same phenomenon also happens in humans<sup>6, 49, 50</sup>. In the Global Utilization of Streptokinase and Tissue Plasminogen Activator for Occluded Coronary Arteries trial, investigators found that brain edema around the clot is diminished in thrombolysis-related ICH compared with spontaneous ICH in patients with normal clotting<sup>49, 50</sup>. Thrombin, an essential component in the clotting cascade, is responsible for early brain edema development after ICH<sup>12-14, 16</sup>. Hirudin, a specific thrombin inhibitor found in leeches, inhibits edema formation in a rat ICH model<sup>13</sup> as does argatroban, another thrombin inhibitor<sup>22</sup>.

Edema around a hematoma reaches its peak several days later<sup>38, 39, 51</sup>. In rats, the edema peak occurs on the third or the fourth day after experimental ICH<sup>17, 19</sup>. In contrast, thrombin-induced brain edema peaks within 48 hours<sup>17</sup>. This difference in time course led us to examine whether there might be a third phase (after three days) of injury involving red blood cell lysis and hemoglobin-induced neuronal toxicity. Infusion of packed erythrocytes causes edema after about three days suggesting that RBCs are associated with delayed edema formation<sup>17</sup>. A clinical study of edema and ICH indicates that delayed brain edema is related to significant midline shift after ICH in humans<sup>45</sup>. This delayed brain edema formation (in the second or third weeks after the onset in human) is probably due to RBC lysis and

hemoglobin degradation products. Our recent studies have demonstrated that iron overload occurs in brain after ICH and iron chelation with deferoxamine reduces perihematomal brain edema<sup>52, 53</sup>.

Although multiple forms of edema are present after ICH, the primary form is probably vasogenic following an increase in permeability of the BBB. BBB is a physical and a physiological barrier to the movement of many molecules between blood and brain<sup>54</sup>. BBB dysfunction occurs following ICH and contributes to brain edema formation. After ICH, the BBB remains intact to large molecules for the first several hours<sup>11</sup>. Eight to twelve hours later, however, BBB permeability in the perihematomal region increases markedly and even more by 48 hours<sup>19</sup>. BBB disruption following ICH may be related to thrombin formation since thrombin, in amounts produced by the hematoma, causes BBB leakage<sup>15</sup>.

Matrix metalloproteinases and plasminogen activators are upregulated following ICH induced by the intracerebral injection of bacterial collagenase. Metalloproteinases can cause disruption of the blood-brain barrier and secondary brain injury. In a rat model, ICH-induced increases in brain water and sodium contents were reduced by a matrix metalloproteinase inhibitor<sup>55</sup>. Fibrinolytic activity also increases for several weeks after ICH and plays an important part in the lysis of the clot<sup>56</sup>.

### **B.3. Thrombin and Protease-Activated Receptors**

Thrombin is a serine protease and an essential component in the coagulation cascade. It is produced immediately in the brain after intracerebral hemorrhage, brain trauma or blood-brain barrier breakdown following many kinds of brain injury<sup>57</sup>. Direct infusion of large doses of thrombin into brain causes inflammatory cell infiltration, mesenchymal cell proliferation, scar formation, brain edema formation and seizures<sup>12-17, 58, 59</sup>. Thrombin-induced brain edema results partly from a direct opening of the blood-brain barrier<sup>15</sup>. Thrombin, at high concentrations also kills neurons and astrocytes *in vitro*<sup>60</sup>. Our laboratory has demonstrated that thrombin is responsible for early brain edema formation following intracerebral hemorrhage (ICH)<sup>13</sup>.

Recent reports indicate that thrombin may regulate a variety of activities in the brain. Thrombin enhances the synthesis and secretion of nerve growth factor in glial cells<sup>63</sup>, modulates neurite outgrowth<sup>64</sup>, reverses process-bearing stellate astrocytes to epithelial-like astrocytes<sup>65</sup>, stimulates astrocyte proliferation<sup>65-67</sup> and modulates the cytoskeleton of endothelial cells<sup>68</sup>. Indeed, thrombin at very low concentration is neuroprotective<sup>60, 61, 69</sup>. On the other hand, thrombin activates potentially harmful pathways. For example, thrombin induces apoptosis in cultured neurons and astrocytes<sup>70</sup>, potentiates NMDA receptor function<sup>71</sup> and activates rodent microglia *in vitro*<sup>72</sup>.

The brain as well as blood may be a site of thrombin production. *In vitro* studies have shown that prothrombin mRNA is expressed in the cells of the nervous system<sup>73</sup>. Prothrombin mRNA is upregulated after spinal cord injury<sup>74</sup>. Our previous study indicated that there is an increase of prothrombin expression in the brain after cerebral ischemia<sup>75</sup> and our preliminary data also found that prothrombin mRNA was detected in the perihematomal zone (Preliminary data, Figure 1). These results suggest that thrombin may be formed and cause brain injury even if the blood-brain barrier is intact.

Although the primary role of thrombin in hemostasis is through cleaving fibrinogen to fibrin and inducing platelet aggregation, other important cellular activities of thrombin may be related to thrombin receptor activation. Cunningham and colleague identified thrombin receptors on cell surfaces in 1978. The thrombin receptor cDNA was cloned in 1991<sup>77, 78</sup>. Three protease-activated receptors (PARs), PAR-1, PAR-3 and PAR-4, have been identified as thrombin receptors<sup>25, 26, 79</sup>. Thrombin receptor mRNA expression is found in neurons and astrocytes<sup>80, 81</sup> and we have detected PAR-1 mRNA in the normal and ICH rat brain. PAR-1 immunoreactivity has been found in human brain tissue<sup>82</sup>. Thrombin receptors are activated by proteolytic cleavage rather than by ligand binding and thrombin receptor-activated peptides are able to mimic many cellular activities of thrombin<sup>83, 84</sup>. Recent studies indicate that PARs mediate some of the pathological effects of thrombin and PARs are involved in pathophysiology of the nervous system<sup>27, 85</sup>. For example, PAR-1 mediates thrombin-induced pulmonary microvascular permeability<sup>86</sup>. In addition, Junge et al. reported that brain infarction is reduced in PAR-1 knockout mice and intracerebroventricular injection of PAR-1 antagonist BMS-200261 reduces infarct volume in a transient (30 minutes middle cerebral artery occlusion) mouse focal cerebral ischemia model<sup>82</sup>.

Thrombin activates gelatinase A (also called matrix metalloproteinase 2, MMP-2) in endothelial cells<sup>87</sup>. MMPs are members of a family of zinc-dependent proteases that can degrade extracellular matrix and cause blood-brain barrier disruption. MMP inhibition reduces thrombolytic-induced hemorrhage after thromboembolic stroke in rabbits<sup>88</sup>.

Our preliminary data indicates that tumor necrosis factor, TNF- $\alpha$ , levels in the brain are increased after thrombin infusion and ICH. TNF- $\alpha$  is one of the major pro-inflammatory cytokines, which is elevated

after many central nervous system diseases such as cerebral ischemia and brain trauma<sup>89</sup>. TNF- $\alpha$  recruits neutrophils by stimulating endothelial cells to produce intercellular adhesion molecule 1 (ICAM) and E-selectin<sup>90</sup>. After tethering, rolling and adhesion, neutrophils can migrate into the brain parenchyma<sup>91</sup>, release proteases and oxidases, and cause secondary brain injury. Barone et al.<sup>92</sup> reported that exogenous TNF- $\alpha$  exacerbates brain injury and that blocking TNF- $\alpha$  activity with anti-TNF monoclonal antibody or soluble TNF receptor I, reduces infarct volume after middle cerebral artery occlusion (MCAO) in the rat. TNF- $\alpha$  itself also increases MMP production and blood-brain barrier permeability<sup>93</sup>.

Thrombin also plays crucial roles in brain injury after ischemic stroke. Intraventricular infusion of hirudin, a specific inhibitor of thrombin, increases the survival of hippocampal CA1 neurons after global cerebral ischemia in gerbils<sup>61</sup>. PARs are markedly upregulated in hippocampus after oxygen-glucose deprivation *in vitro*<sup>94</sup>. Our recent results found that thrombin exacerbates ischemic brain injury<sup>75</sup> and intracerebral hirudin injection attenuates infarct volume and neurological deficits without altering local cerebral blood flow<sup>95</sup>.

Excitotoxicity due to glutamate release is a very important mechanism in brain injury after stroke. For example, MK 801, the N-methyl-D-aspartate (NMDA) antagonist, reduces brain injury in many studies of focal cerebral ischemia in the rat. Activation of PAR-1 potentiates NMDA receptor responses in CA1 pyramidal cells and the effect of thrombin can be mimicked by a thrombin receptor agonist (SFLLRN). Thrombin-induced NMDA receptor potentiation is reduced in PAR-1 knockout mice<sup>71</sup>.

#### **B.4. Activation of Complement Cascade in Central Nervous System**

The complement system is involved in various immune reactions, including cell lysis and the inflammatory response<sup>97,98</sup>. Complement is normally excluded from the brain parenchyma by the BBB but entry can occur after ICH as part of the extravasated blood or later as the result of BBB disruption. There is evidence that the complement cascade is activated in the brain parenchyma after ICH. For example, N-acetylheparin, a heparin congener without anticoagulant properties, inhibits the complement cascade and attenuates perihematomal brain edema<sup>18</sup>.

Complement-related brain injury may be due to membrane attack complex (MAC) formation and the classic inflammatory response. MAC consists of C5b-9 complement forms which is assembled following complement activation<sup>99</sup>. MAC formation can cause the formation of a pore in the cell membrane that leads to cell lysis. Thus, MAC formation may be involved in the lysis of RBC within the clot. However, MAC insertion may occur in neurons, glia and endothelial cells as well, causing neuron death and BBB leakage. Our studies have also shown that MAC is assembled after ICH and clusterin, inhibitor of MAC formation, is upregulated in the brain parenchyma<sup>18</sup>. Recent studies have demonstrated that MAC not only causes cell lysis, but also modulates cellular functions such as the release of cytokines, oxygen radicals, and matrix proteins<sup>100</sup>.

Anaphylatoxin complement C5a is generated following complement activation. C5a is also a potent chemoattractant for polymorphonuclear leukocytes and contributes to inflammatory cell injury<sup>10</sup>. Complement C5a can be detected around the clot<sup>46</sup>. Systemic complement depletion by cobra venom factor, a non-toxic protein in cobra venom, reduces perihematomal edema in the rat ICH model<sup>46</sup>.

Cobra venom factor (CVF) is a non-toxic protein in cobra venom. CVF is able to form a stable C3/C5 convertase which leads to complement depletion. Systemic complement depletion reduces brain edema and TNF- $\alpha$  levels after ICH<sup>46</sup>, and improves blood flow and outcome following cerebral ischemia with reperfusion<sup>102</sup>.

The development of mice deficient in elements of the complement system are starting to enable investigators to study the role of different complement components in systemic injury<sup>103</sup>. Such experiments have suggested that not all components of the complement system may enhance injury but that some may be beneficial<sup>104</sup>. We have found similar evidence with respect to ICH<sup>105</sup>. Thus, more information is necessary in order to devise the most appropriate methods of modulating the complement system in relation to ICH.

#### **B.5. Thrombin and Complement Activation**

Our preliminary data indicates that thrombin can activate the complement cascade in the brain. Intracerebral infusion of thrombin results in a seven-fold increase of complement C9 and a deposition of complement C9 on neuronal membranes. Clusterin, an inhibitor of membrane attack complex (MAC) formation, is upregulated and found in neurons after intracerebral thrombin infusion (preliminary data, Figure 5). In addition, the increase of lung vascular permeability after thrombin-induced pulmonary

microembolism is mediated by the complement system<sup>106, 107</sup>. The effects of coagulation cascade on complement activation are not well-studied. However, studies suggest that there is a very close relationship between thrombin and complement. For example, consumption of complement, which measured by total complement activity (CH50), is extensive. About 50% of C3 is cleaved during clot formation<sup>108</sup>. Thrombin can cleave and activate C3<sup>109</sup>. Thrombin-cleaved C3a-like fragments are chemotactic for leukocytes and to induce enzyme release from neutrophils<sup>110</sup>. Thrombin can also cleave C5 to produce C5a like fragments which are leukotactic<sup>110</sup>.

Much less is known about the interaction between the activation of PARs and the complement system. It is known that thrombin stimulates decay-accelerating factor (DAF) production through PAR-1<sup>111</sup>. DAF is also induced by TNF- $\alpha$ <sup>112</sup>. However, the overall role of PARs in the effects of thrombin on the complement system is uncertain.

## **B.6. Significance/Approach/Innovation**

Intracerebral hemorrhage is a common and often fatal subtype of stroke and produces severe neurologic deficits in survivors. Although death may occur acutely after an ICH, delayed neurologic deterioration often occurs in patients with large hematoma, which may be related to brain edema formation. Although studies indicate that thrombin is a therapeutic target to limit ICH-induced brain edema, concerns over the potential for rebleeding when administering a thrombin inhibitor will limit the use of such a therapeutic approach. It is, therefore, very important to determine the downstream mechanisms by which thrombin exerts its edemagenic effects.

This proposal will use two reproducible rodent models of ICH. Our development of a mouse model will enable us to dissect out the roles of PAR-1, C3 and C5 by using mice deficient in these proteins. If our hypotheses are correct, these experiments may lead to novel treatments for ICH.

## **C. PRELIMINARY STUDIES/PROGRESS REPORT**

### **C1. Publications**

#### **C1.1. Journal Articles** (\* indicates included in Appendix):

- 1\*. Hua Y, Xi G, Keep RF, Hoff JT. Complement activation in the brain after experimental intracerebral hemorrhage. *J Neurosurg* 2000;92:1016-1022
2. Gong C, Hoff JH, Keep RF: Acute inflammatory reaction following experimental intracerebral hemorrhage. *Brain Res* 871:57-65, 2000.
- 3\*. Xi G, Hua Y, Keep RF, Younger JG, Hoff JT. Systemic complement depletion diminishes perihematomal brain edema. *Stroke* 2001;32:162-167
4. Xi G, Hua Y, Keep RF, Duong HK, Hoff JT. Activation of p44/42 mitogen activated protein kinase in thrombin-induced brain tolerance. *Brain Res* 2001;895:153-159
- 5\*. Masada T, Hua Y, Xi G, Yang GY, Hoff JT, Keep RF. Overexpression of interleukin-1 receptor antagonist attenuates brain edema induced by intracerebral hemorrhage and thrombin. *J Neurosurg* 2001;95:680-686
6. Xi G, Hua Y, Bhasin RR, Ennis SR, Keep RF, Hoff JT. Mechanisms of edema formation following intracerebral hemorrhage: Effects of extravasated red blood cells on blood flow and blood-brain barrier integrity. *Stroke* 2001;32:2932-2938
7. Gong C, Boulis N, Qian J, Turner DE, Hoff JT, Keep RF: Intracerebral hemorrhage induced neuronal death. *Neurosurgery* 48:875-883, 2001.
8. Gong C, Ennis SR, Hoff JT, Keep RF: Inducible cyclo-oxygenase-2 expression after experimental intracerebral hemorrhage. *Brain Res* 901:38-46, 2001.
9. Huang FP, Xi G, Keep RF, Hua Y, Nemoianu A, Hoff JT. Brain edema after experimental intracerebral hemorrhage: Role of hemoglobin degradation products. *J Neurosurg* 2002;96:287-293
10. Hua Y, Xi G, Keep RF, Wu J-M, Jiang Y-J, Hoff JT. Plasminogen Activator Inhibitor-1 Induction after Experimental Intracerebral Hemorrhage. *J Cereb Blood Flow Metab* 2002;22:55-61
11. Jiang Y-J, Wu J-M, Y Hua, Keep RF, Xiang JM, Hoff JT, Xi G. Thrombin receptor activation and thrombin-induced brain tolerance. *J Cereb Blood Flow Metab* 2002;22:404-410
12. Jiang Y-J, Wu J-M, Keep RF, Y Hua, Hoff JT, Xi G. Hypoxia inducible factor-1 $\alpha$  accumulation in the brain after experimental intracerebral hemorrhage. *J Cereb Blood Flow Metab* 2002;22:689-696
- 13\*. Hua Y, Schallert T, Keep RF, Wu J-M, Hoff JT, Xi G. Behavioral tests after intracerebral hemorrhage in the rat. *Stroke* 2002;33:2478-2484

14. Wu J-M, Hua Y, Keep RF, Schallert T, Hoff JT, Xi G. Oxidative brain injury from extravasated erythrocytes after intracerebral hemorrhage. *Brain Res* 2002;953:45-52
15. Xi G, Keep RF and Hoff JT. Pathophysiology of edema. *Neurosurgery Clinics of North America*. 2002;13:371-383
16. Xi G, Reiser G, Keep KF. The role of thrombin and thrombin receptors in ischemic, hemorrhagic and traumatic brain injury: deleterious or protective? *J Neurochem* 2003;84:3-9
17. Nakamura T, Xi G, Hua Y, Hoff JT, Keep RF. Nestin expression after experimental intracerebral hemorrhage. *Brain Res*. 2003;981:108-117
18. Hua Y, Keep KF, Hoff JT, Xi G. Thrombin preconditioning attenuates brain edema induced by erythrocyte and iron. *J Cereb Blood Flow Metab* 2003;23:1448-1454
19. Wu J, Hua Y, Keep RF, Nakamura T, Hoff JT, Xi G. Iron and iron handling proteins in the brain after intracerebral hemorrhage. *Stroke* 2003;34:2964-2969
20. Xi G, Fewel ME, Hua Y, Thompson BG, Hoff JT, Keep RF. Intracerebral hemorrhage: Pathophysiology and therapy. *Neurocritical Care*, 2004;1:5-18
21. Nakamura T, Keep RF, Hua Y, Schallert T, Hoff JT, Xi G. Deferoxamine-induced attenuation of brain edema and neurological deficits in a rat model of intracerebral hemorrhage. *J Neurosurg* 2004 in press.
- 22\*. Nakamura T, Xi G, Hua Y, Schallert T, Hoff JT, Keep RF. Intracerebral hemorrhage in mice: Mo characterization and application for genetically modified mice. *J Cereb Blood Flow Metab*, 2004 press

### **C1.2. Books:**

1. Crockard A, Hayward R, Hoff J: *Neurosurgery: Scientific Basis of Clinical Practice*, 3rd Edition. London: Blackwell, 2000, pp 1301.
2. Mendelow AD, Baethmann A, Czernicki Z, Hoff JT, et al: *Brain Edema XI*. Springer, Wien, New York, 2000, pp. 569.
3. Kuroiwa T, Baethmann A, Czernicki Z, Hoff JT, et al: *Brain Edema XII*. Springer, Wien, New York, 2003, pp. 609.

### **C1.3. Book Chapters:**

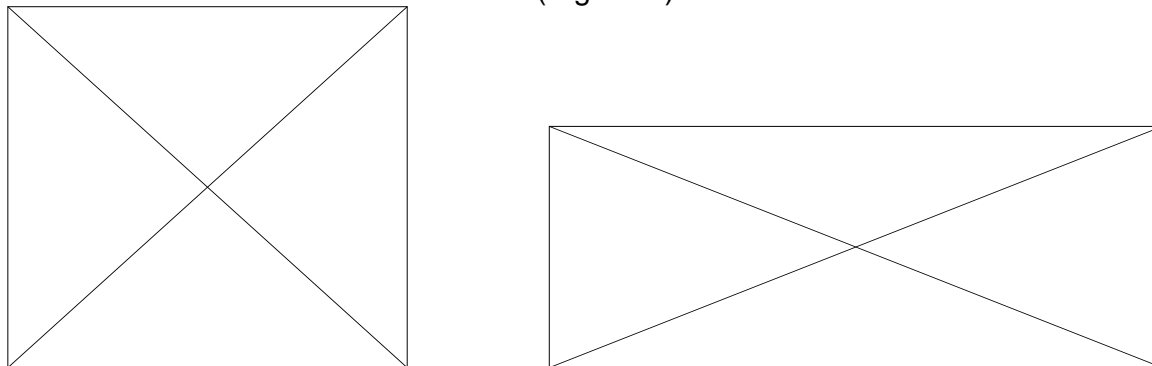
1. Xi G, Hua Y, Keep RF, Hoff JT. Induction of colligin may attenuate brain edema following intracerebral hemorrhage. *Brain Edema XI* pp.501-505, 2000
2. Xi G, Keep RF, Hua Y, Hoff JT. Thrombin preconditioning, heat shock proteins and thrombin-induced brain edema. *Brain Edema XI* pp.511-515, 2000
3. Xi G, Hua Y, Keep RF, Younger JG, Hoff JT. Brain edema after intracerebral hemorrhage: The effects of systemic complement depletion. *Intracranial Pressure and Brain Biochemical Monitoring* pp.253-256, 2002
4. Bhasin RR, Xi G, Hua Y, Keep RF, Hoff JT. Experimental intracerebral hemorrhage: Effect of lysed erythrocytes on brain edema and blood-brain barrier permeability. *Intracranial Pressure and Brain Biochemical Monitoring* pp.249-251, 2002
5. Masada T, Hua Y, Xi G, Ennis SR, Keep RF. Effect of ischemic preconditioning on edema formation in cerebral vasculature following focal cerebral ischemia. *Intracranial Pressure and Brain Biochemical Monitoring* pp.265-268, 2002
6. Xi G and Hoff JT. The pathophysiology of hemorrhagic lesions. In: *Diagnosis & Therapy Imaging of the Nervous System*. Latchaw RE, Kucharczyk J, and Moseley ME. eds. In press
7. Kitaoka T, Hua Y, Xi G, Hoff JT, Keep RF. Effect of delayed argatroban treatment on intracerebral hemorrhage-induced edema in the rat. *Brain Edema XII*, pp.457-461, 2003
8. Hua Y, Wu J, Keep RF, Hoff JT, Xi G. Thrombin exacerbates brain edema in focal cerebral ischemia. *Brain Edema XII*, pp.163-166, 2003
9. Xi G, Wu JM, Jiang YJ, Hua Y, Keep RF, Hoff JT. Thrombin preconditioning upregulates transferrin and transferrin receptor and reduces brain edema induced by lysed red blood cells. *Brain Edema XII*, pp.449-452, 2003
10. Hua Y, Keep RF, Schallert T, Hoff JT, Xi G. A thrombin inhibitor reduces brain edema, glioma mass and neurological deficits in a rat glioma model. *Brain Edema XII*, pp.503-506, 2003
11. Hoff JT and Xi G. Brain edema from intracerebral hemorrhage. *Brain Edema XII*, pp.11-15, 2003

## **C2. Progress Report and Preliminary Data:**

### **C2.1. Thrombin and Brain Injury**

**C2.1.1. Thrombin activity after ICH.** For thrombin activity measurements, rat brain samples

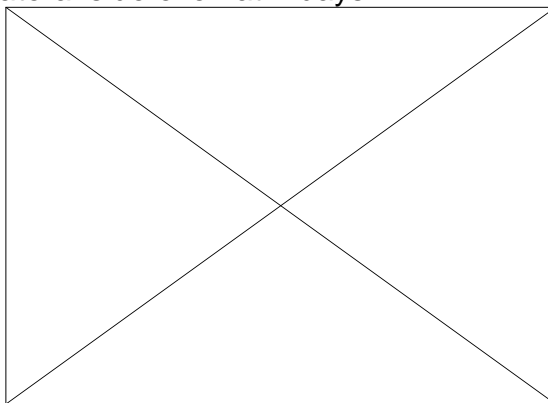
were homogenized and thrombin activities were measured using the thrombin-specific chromogenic substrate S2238 (Chromogenix). The final concentration of S2238 was 0.3 mmol/L in PBS and the absorption at 405 nm of the supernatant is measured one hour later. A calibration curve of thrombin activity was made for the thrombin activity quantitation<sup>113</sup>. Thrombin activities in the ipsilateral basal ganglia were increased significantly one hour after ICH (Figure 1). To test whether brain itself can produce thrombin, we measured brain prothrombin mRNA levels by RT-PCR. Prothrombin mRNA was detected in the brain of either sham or ICH (Figure 1).



**Figure 1.** **Left:** Thrombin activity in the ipsilateral and contralateral basal ganglia one hour after ICH. Value are mean  $\pm$  SD, n=6. \*p<0.01 vs. sham and contralateral side. **Right:** Prothrombin mRNA was detected in perihematomal zone 1 h (lanes 1 & 2) and 4 h (lanes 3 & 4) after ICH.

**C2.1.2. Thrombin-induced brain injury *in vivo*.** We used five or ten units of thrombin for infus into the caudate because a 50- $\mu$ L blood clot contains approximately 30  $\mu$ l plasma; the potential amount thrombin produced in that size clot was calculated to be ten units<sup>15</sup>. Thrombin was infused stereotactically into the right basal ganglia of rat. Thrombin infusion resulted in significant increase in brain water content. Thrombin-induced brain edema was inhibited by a specific and potent thrombin inhibitor, hirudin<sup>12</sup>. This edema was accompanied by increases in brain sodium and chloride contents and a decrease in brain potassium content. The time course of brain water content following infusion of rat thrombin (5 Units) is shown in **Figure 2**. Edema formation starts as early as 4 hours after thrombin infusion. The peak in edema is at 24 and 48 hours. Although water content begins to decrease after 4 hours, it is still higher than the contralateral side even at 7 days.

**Figure 2.** Brain water content 4h, 12h, and 1, 2, 3, 7 days after intracerebral infusion of rat thrombin (5 Units, 50  $\mu$ l) into the right basal ganglia of rats. Values are means  $\pm$  SEM (n=5-6). \*P<0.001 versus contralateral side.



Terminal deoxynucleotidyl transferase biotin-d-UTP nick end labeling (TUNEL) and DNA polymerase I-mediated biotin-dATP nick-translation (PANT) were used for DNA damage detection. Both TUNEL and PANT positive cells were found in the ipsilateral basal ganglia 24 hours after infusion of 5 thrombin (data not shown).

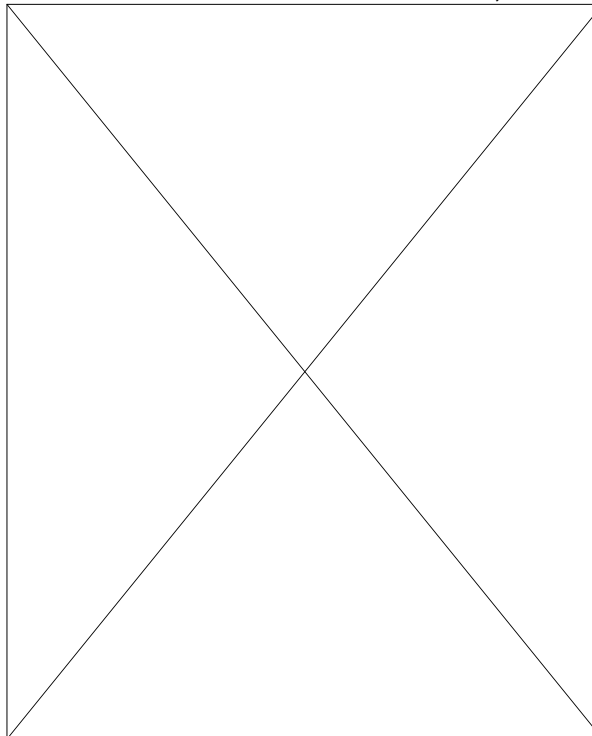
Intracerebral injection of thrombin dose not cause ischemic brain damage. After infusion of ten units of thrombin, rCBF declines over the first hour, rises to baseline or above by two hours, and then returns to baseline by 24 hours. The lowest blood flow recorded is 44.6 $\pm$ 6.7 ml/100g/minute in the ipsilateral hemisphere at one hour (i.e. well above the values expected to cause ischemic damage). There are no significant differences in CBF between the control and thrombin-infused rats<sup>15</sup>.

In addition, we have found that intracerebral thrombin infusions cause blood-brain barrier



disruption<sup>15</sup> and induce focal motor seizures in all animals examined<sup>58</sup>. Thrombin-induced seizures were blocked by co-infusion with a-NAPAP, a thrombin inhibitor.

To examine whether delayed and systemic administration of a thrombin inhibitor could reduce ICH-induced injury, experiments were performed with argatroban. Intracerebral infusion of blood caused a marked increase in perihematomal water content. Intracerebral injection of argatroban 3 hours after ICH caused a significant reduction in edema measured at 48 hours (80.9±1.0% versus 82.6±0.8%; P<0.01). The systemic administration of high-dose argatroban (0.9 mg/h) starting 6 hours after ICH also significantly reduced edema (80.3±1.1% versus 82.0±1.3% in vehicle controls; P<0.05, Figure 3).



**Figure 3.** Brain water content (top) and brain ion contents (bottom) 48 hours after intra-cerebral infusion of 100 µL of blood. Starting 6 hours after ICH, rats received an intra-peritoneal infusion of the higher dose of argatroban (0.9 mg/h per rat) or saline. Values are mean±SD. #P<0.05 compared with vehicle. Contra indicates contralateral; Ipsi, ipsilateral; and BG, basal ganglia.

**C2.1.3. Thrombin-induced cell injury *in vitro*.** Cell culture experiments were performed to determine whether thrombin has a direct toxic effect on brain cells. To ascertain the effect of thrombin cell viability in mixed rat neuron/astrocyte cultures, different doses of thrombin (1, 2, 5, 10, 20, 50 or 100 U/ml) were added to the cell cultures and media lactate dehydrogenase (LDH) concentrations were determined twenty-four hours later, as an indicator of cell viability. Low doses of thrombin (1 and 2 U/ml) did not induce cell death. However, doses greater than 5 U/ml resulted in dose-dependent LDH release. For example, the LDH release was higher in the thrombin (5 U/ml) group compared to the control group (242 ± 47 vs. 127 ± 59 BBU/mg protein, p<0.01)<sup>62</sup>.

**C2.2. Complement activation after ICH and intracerebral infusion of thrombin.**

**C2.2.1. Complement activation after ICH.** Complement activation plays an important role in brain edema formation after ICH. N-acetylheparin, a complement activation inhibitor, reduced brain edema formation in the ipsilateral basal ganglia at 24 hours (78.5 ± 0.5 % versus 81.6 ± 0.8 % in control, p<0.001, Fig. 4) and at 72 hours (80.9 ± 2.2 % versus 83.6 ± 0.9 % in control, p<0.05) after ICH<sup>18</sup>.

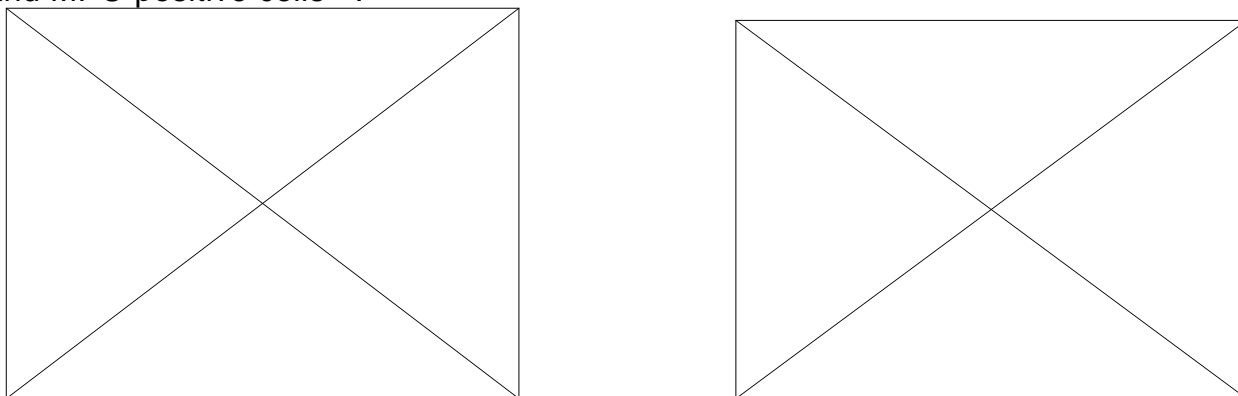
**Table 1. TNF-α levels (pg/g) in the basal ganglia two hours after intracerebral hemorrhage in control and complement depleted (CVF treated) rats**

Group	Contralateral	Ipsilateral
Control	98 ± 39	471 ± 237
CVF-treated	135 ± 21	243 ± 68
p	>0.05	<0.05

Values are mean ± SD; n=6.

In another experiment, rats were depleted of complement with cobra venom factor (CVF). Perihemato

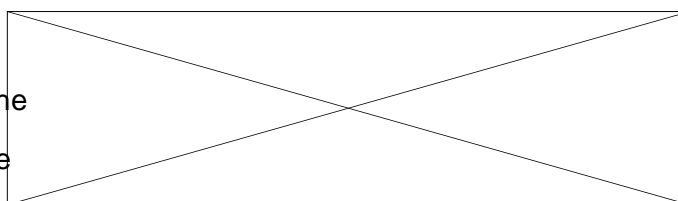
brain edema was reduced by systemic complement depletion at 24 hours ( $78.8 \pm 0.6\%$  vs.  $81.5 \pm 0.8\%$  in control,  $p < 0.01$ , Fig. 4) and 72 hours ( $81.5 \pm 1.5\%$  vs.  $83.5 \pm 0.9\%$  in control,  $p < 0.05$ ), while cerebellar water content was unaffected ( $78.2 \pm 0.3$  vs.  $78.0 \pm 0.1\%$ ). Complement depletion reduced TNF- $\alpha$  production 2 hours post ICH (Table 1). Immunohistochemistry showed that complement depletion significantly reduced perihematomal C9 deposition, C3d production and the number of C and MPO positive cells <sup>46</sup>.



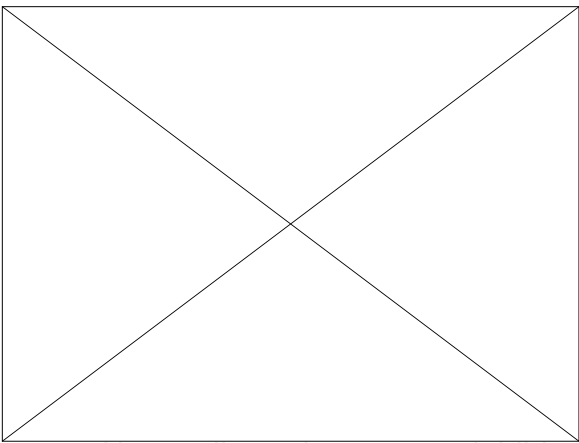
**Figure 4.** Brain water contents 24 hours after intracerebral infusion of 100  $\mu$ L autologous blood with CVF treatment (Left) or N-acetylheparin (Right) treatment. Values are expressed as mean  $\pm$  SD;  $n=5$ . \* $p < 0.05$ , # $p < 0.01$  vs. control group.

**C2.2.2 Thrombin and complement.** Western blot analysis demonstrated that C9 had a seven-fold increase 24 hours after intracerebral infusion of five-unit thrombin ( $3329 \pm 433$  vs.  $458 \pm 395$  pixels in saline control,  $p < 0.01$ , Fig. 5). Immunohistochemistry showed that C9 was deposited on neuronal membranes, indicating activation of the complement cascade and the formation of membrane attack complex. Clusterin, an inhibitor of membrane attack complex formation, also increased after thrombin infusion ( $4925 \pm 686$  vs.  $2453 \pm 264$  pixels in saline control,  $p < 0.01$ , Fig. 5) and was expressed in neurons. Rabbit anti-rat C9 polyclonal antibody and rabbit anti-clusterin polyclonal antibody were received as gifts from Dr. P. Morgan (University of Wales, U.K.) and Dr. M. Griswold (Washington State University). In addition, complement C3 mRNA levels were significantly increased after thrombin injection ( $3025 \pm 664$  pixels vs.  $683 \pm 467$  pixels in sham,  $P < 0.01$ ) at 24 hours.

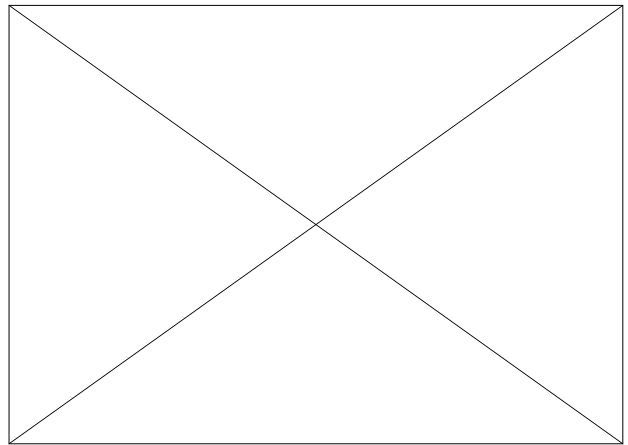
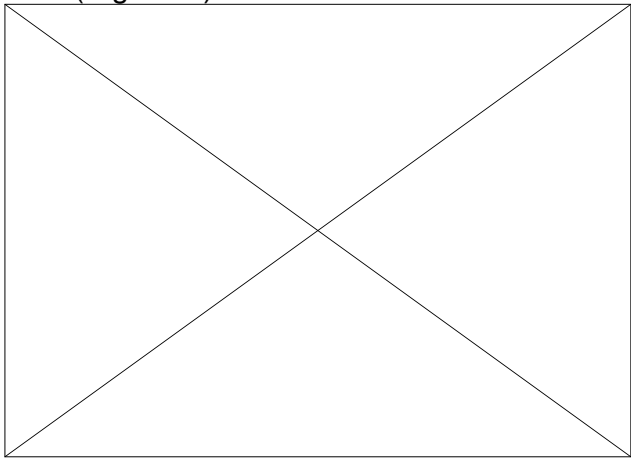
**Figure 5.** Complement C9 and clusterin immunoreactivities (Western blotting) in the ipsilateral basal ganglia 24 h after 5 U thrombin infusion (lanes 1 to 3) and saline infusion (lanes 4 to 6).



**Figure 6.** Brain water contents at 24 hours after intracerebral injection of thrombin 5U + vehicle, thrombin 5U + N-acetylheparin 25  $\mu$ g and thrombin 5U + N-acetylheparin 100  $\mu$ g. Values are means  $\pm$  S.D.,  $n=6$ , \* $p < 0.05$  vs. vehicle.



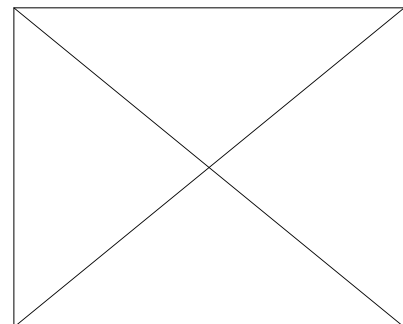
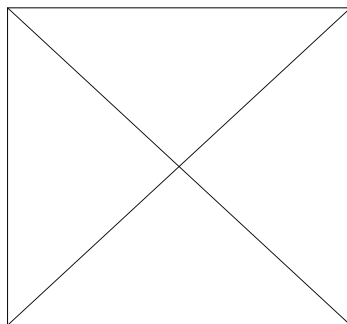
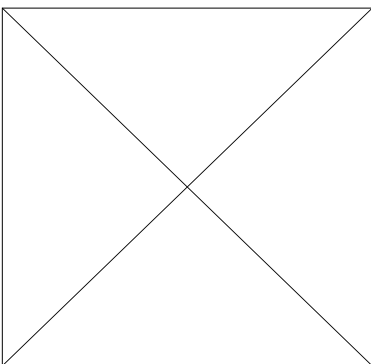
N-acetylheparin, a potent inhibitor of complement activation, attenuates thrombin-induced brain edema (Figure 6) and reduces thrombin-induced neurological deficits (Figure 7).



**Figure 7.** Neurological deficits after intracerebral injection of thrombin 5U+vehicle (control) and thrombin 5 N-acetylheparin (100  $\mu$ g). Values are means  $\pm$  S.D., n=6, \*p<0.05, #p<0.01 vs. control. Normal rats have scores of 100 and 0% for the forelimb placing and the forelimb asymmetry tests respectively.

### **C2.3. The role of PAR-1 in thrombin-induced activation of mitogen activated protein kinases.**

By Western blot analysis, PAR-1, -3 and -4 protein can be detected in the normal brain but at very low levels. PAR-1, -3, and -4 mRNAs were also detected in the brain. PAR-1,-3 and -4 protein levels were upregulated in the ipsilateral basal ganglia one day after 1 U thrombin infusion (Fig 8).



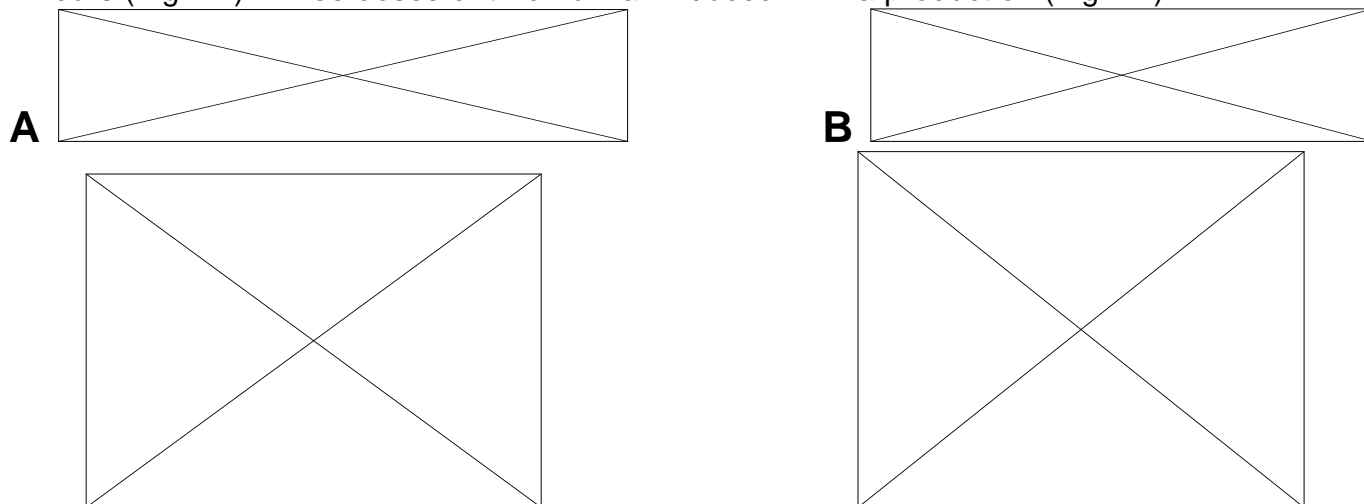
**Figure 8.** PAR-1(A), PAR-3 (B) and PAR-4 (C) protein levels measured by Western blot analysis in the ipsilateral basal ganglia 1, 3 and 7 days after 1U thrombin infusion. Values are mean  $\pm$  SD, n=3, \*p<0.05 vs. day 1, \*\*p<0.05 vs. day 3.

Mitogen activated protein kinases (MAPKs) are well-known cytoplasmic signal transducers with

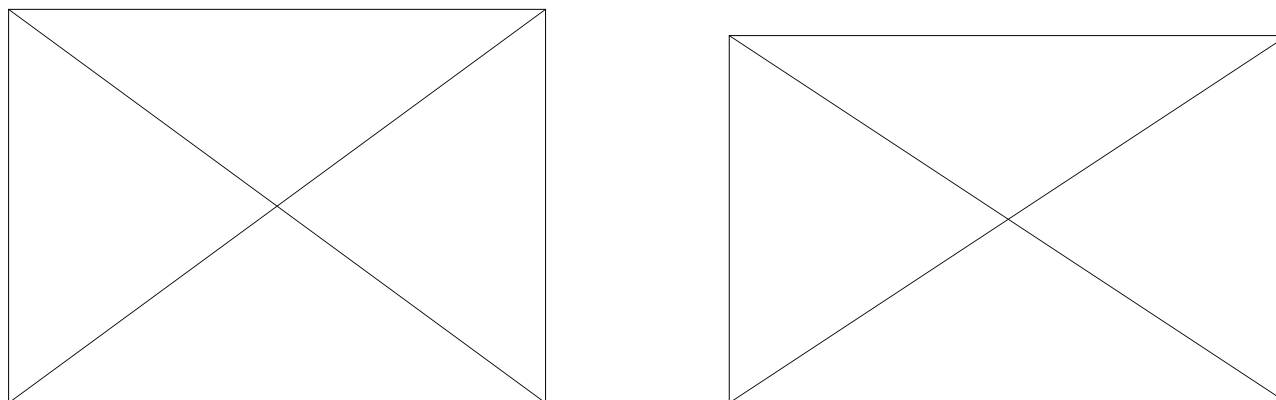
an important role in both ischemic and hemorrhagic stroke<sup>114</sup>. To test whether thrombin activates MAP through PAR-1, we infused one unit thrombin into the basal ganglia of mice (C57BL/6 wild type or PAR knockout). Thrombin activates both p44/42- and p38-MAPK in the wild type mice. There was no activation in PAR-1 knockout mice (Fig. 9).

**C2.4. Thrombin and cytokines (TNF-a and interleukin 1).**

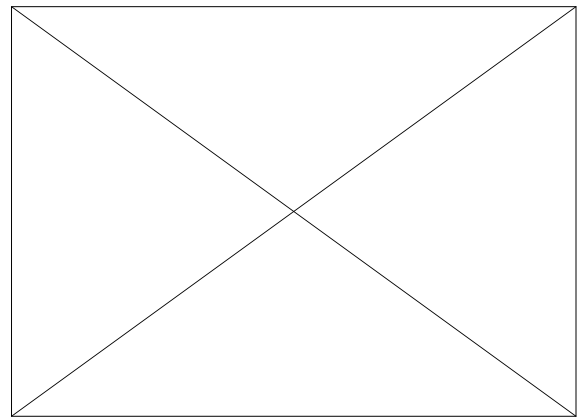
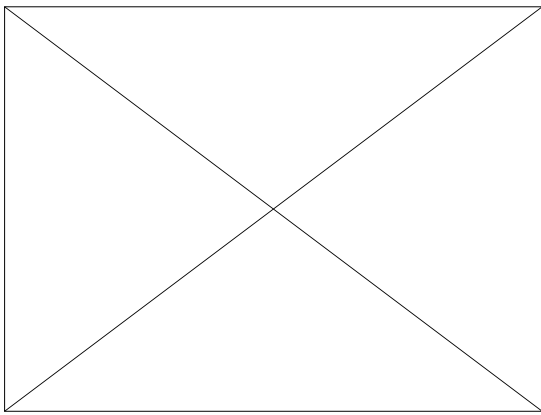
Recent studies indicate that an inflammatory reaction occurs around the clot<sup>91, 115-117</sup>. Cytokines including TNF-a and IL-1, play an important role in both ischemic and hemorrhagic stroke<sup>46, 92, 118-120</sup>. Brain TNF-a levels were increased after ICH (Figure 10). We also examined brain TNF-a levels after intracerebral infusion of thrombin by ELISA. Thrombin was infused into the right basal ganglia. A time course study indicated that TNF-a increased within 2 hours and returned to normal levels between 4 a 24 hours (Fig. 11). Three doses of thrombin all induced TNF-a production (Fig. 11).



**Figure 9.** Phospho-p44/42 MAP kinases (A) and phospho-p38 MAP kinase (B) levels (Western blot analysis 24 hours after intracerebral infusion of 1 U thrombin in 10 µl saline into the right basal ganglia of either C57BL/6 wild type or PAR-1 knockout mice. Values are mean ± SD, n=3, #p<0.01 vs. wild type.

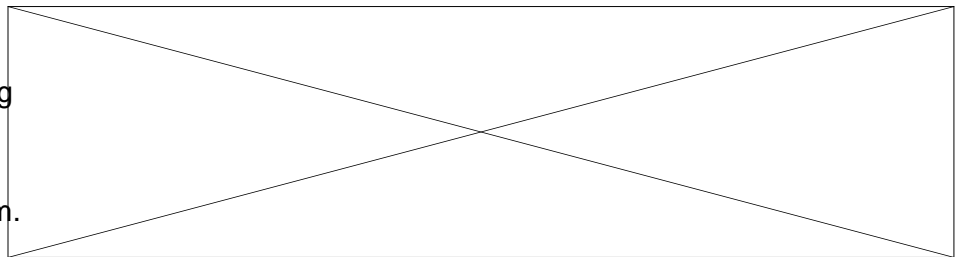


**Figure 10.** Left: Time course of TNF-a in the ipsi- and contralateral basal ganglia after 100 µl autologous whole blood infusion. Values are mean ± SD. n=3. \*p<0.05 vs. contralateral side. Right: TNF-a in the basal ganglia after ICH or sham. Values are mean ± SD. n=6. #p<0.01 vs. sham.



**Figure 11.** Left: Time course of TNF-a in the ipsi- and contralateral basal ganglia after one unit thrombin infusion. Values are mean  $\pm$  SD. n=4-5. \*p<0.01 vs. contralateral side. Right: Dose-response of TNF-a in ipsilateral basal ganglia after thrombin infusion. Values are mean  $\pm$  SD. n=5. \*p<0.01 vs. saline; \*\*p<0.05 0.1 U and 5 U.

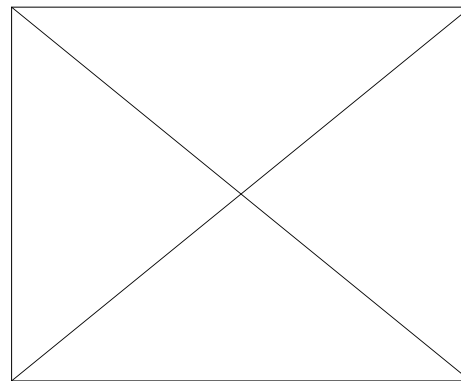
**Figure 12.** Double labeling showing TNF-a positive cells (A), NSE positive cells (B) and the merged image (C) 2 hours after one unit thrombin injection, scale bar=20  $\mu$ m.



We also found that overexpression of IL-1 receptor antagonist attenuates brain edema formation and thrombin-induced intracerebral inflammation following ICH<sup>120</sup>.

### C2.5. Iron toxicity and brain injury after ICH.

By enhanced Perl's reaction, iron positive cells were found in the perihematomal zone as early the first day. Perl's positive cells were neurons at the first day and neuron-like and glia-like cells sever days later (Figure 13).



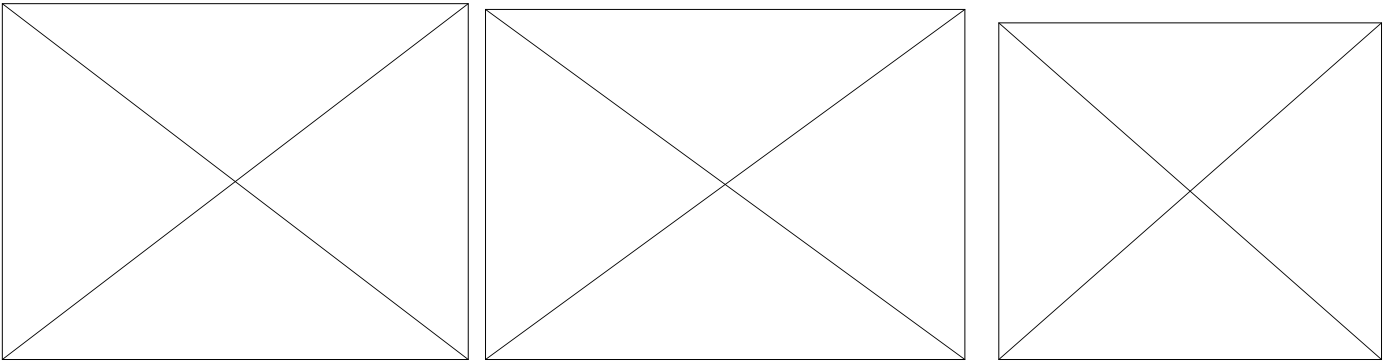
**Figure 13.** Iron histochemistry in the contralateral (B, E, H) and the ipsilateral basal ganglia (C, F, I) at day 1 (A, B, C), day 3 (D, E, F) and day 28 (G, H, I) after ICH. (A, D, G) are coronal gross sections. Bar= 20  $\mu$ m.

Male Sprague-Dawley rats received an infusion of 100- $\mu$ l autologous whole blood into the right basal ganglia and were sacrificed 1, 3 or 7 days later. The effects of deferoxamine (100 mg/kg, starting 2 hrs after ICH, every 12 hrs, up to 7 days) on ICH-induced brain injury were examined by measuring brain edema and neurological deficits. Apurinic/aprimidinic endonuclease/redox effector factor-1 (APE/Ref-1), a repair mechanism for DNA oxidative damage that is reduced following ICH, was quantitated by Western blot analysis. Deferoxamine attenuated brain edema and reduced neurological deficits. In addition, in deferoxamine treated rats, the reduction in APE/Ref-1 levels found in controls was absent (Fig 14).

**A**

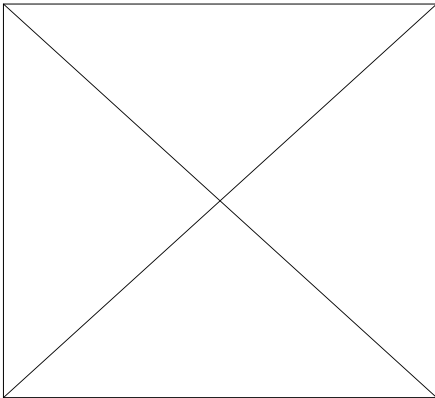
**B**

**C**



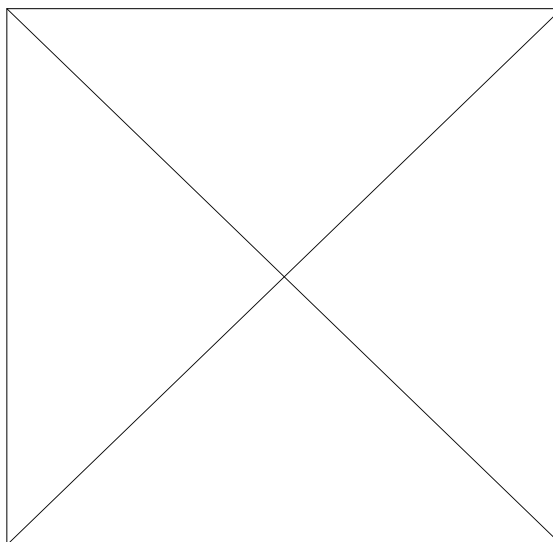
**Fig 14.** The effect of deferoxamine treatment on brain water content 3 days (A), forelimb placing score (B) and APE/Ref-1(C) after ICH. (A) Measurements were made on brains from rats treated with deferoxamine vehicle 2 hrs after ICH. Cont-CX: contralateral cortex. Ipsi-CX: ipsilateral cortex. Cont-BG: contralateral basal ganglia. Ipsi-BG: ipsilateral basal ganglia. Cerebel: cerebellum. (B) Bar graph showing the effect of deferoxamine treatment on forelimb placing scores following ICH. (C) Results of Western blot analysis showing APE/Ref-1 content in the vehicle-treated contralateral (lanes 1-3), vehicle-treated ipsilateral (lane 6) and deferoxamine-treated ipsilateral (lanes 7-9) basal ganglia 3 days after ICH. Values are expressed as means  $\pm$  SD; \* and # indicate differences from vehicle groups at the  $p < 0.05$  and  $p < 0.01$  levels, respectively.

**C2.6. Thrombin exacerbates iron-induced brain edema.** In control rats, intracerebral caudate injections of high dose of thrombin or ferrous iron cause marked edema formation in the ipsilateral basal ganglia<sup>12, 16, 17, 121-123</sup>. A dose of one unit thrombin and low dose of ferrous iron (0.1 mM, 50  $\mu$ l) fail to cause significant edema. However, co-injection of one unit of thrombin and low dose of iron produce a significant increase of brain water content. Thus, it appears that the toxic effects of thrombin are enhanced by low-dose of iron (Figure 15).



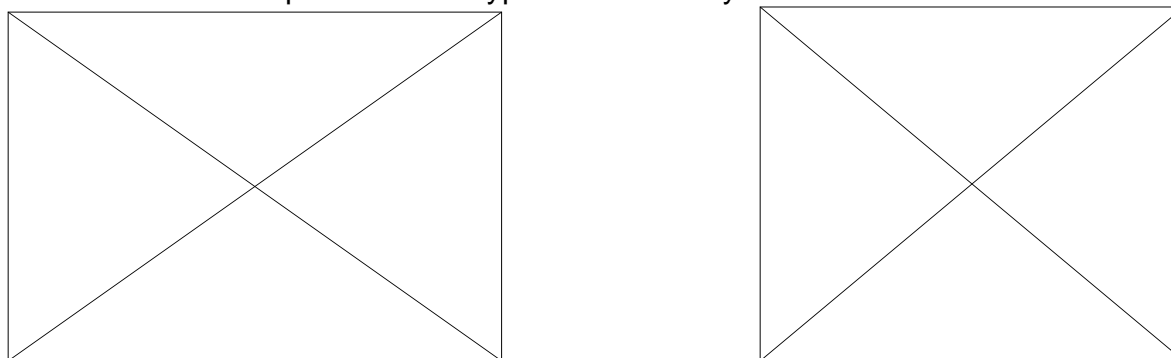
**Figure 15.** Brain water contents in the basal ganglia 24 hours after intra-caudate injection of 30  $\mu$ l either saline, thrombin(1U), ferrous iron(0.1mM) or thrombin(1U)+ferrous iron(0.1mM). Values are mean $\pm$ S.D., n=5, \* $p < 0.05$  vs. the other groups.

**C2.7. Brain edema and behavioral changes after ICH.** There was a temporal relationship between ICH-induced forelimb placing deficits and ICH-induced edema. Forelimb placing deficits peaked at day 3 after ICH and almost recovered by day 14. Perihematomal brain edema also peaked at day 3 and resolved by day 14 (Figure 16). There was a negative correlation between forelimb placing score and brain edema ( $y = -9.6x + 817$ ,  $R^2 = 0.96$ ,  $p < 0.05$ ). Although there was a tendency towards there being correlation between forelimb use asymmetry, corner turn score and brain edema, it did not reach a significant level.



**Figure 16.** The relationship between forelimb placing score and brain water content in the caudate after infusion of 100- $\mu$ l autologous whole blood. Values are mean  $\pm$  SEM. n=5. #p<0.01 vs. non-impaired or contralateral side.

**C2.8. ICH model in mice.** Gene knockout or transgenic animals may assist in elucidating the mechanisms of brain injury after intracerebral hemorrhage. However, almost all commercial available transgenic or knockout animals are mice. TNF- $\alpha$  knockout, wild type or C57BL/6 mice received intracerebral infusions of either 30  $\mu$ L autologous blood. A control group received 30  $\mu$ l saline infusion. The C57BL/6 mice were killed 24 hours later and the brains were used for water content measurement. Brain edema was quantitated by wet/dry weight method. An increase of brain water content was found ipsilateral basal ganglia and cortex 24 hours after ICH (Fig 17). Perihematomal edema was much less TNF- $\alpha$  knockout mice compared to wild type mice at 3 days after ICH.



**Fig 17.** Left: Brain water content 24 hours after ICH in C57BL/6 mice. Values are mean  $\pm$  SD, n=6, \*p<0.01 vs. saline. Right: Brain water content 72 hours after ICH in TNF- $\alpha$  knockout or wild type mice. Values are mean $\pm$ SD, n=6, \*p<0.05 vs. wild type.

## D. RESEARCH DESIGN AND METHODS

### D1. Rational

Our overall goal is to understand the mechanisms of edema formation after ICH in order to design effective treatments to prevent edema formation. Our preliminary data have demonstrated that thrombin plays a key role in edema formation following ICH. This grant proposal focuses on (1) whether thrombin formation after intracerebral hemorrhage results in activation of the complement cascade in the brain which triggers inflammatory response, forms membrane attack complex and results in blood-brain barrier disruption and brain edema formation; (2) whether thrombin-induced brain edema is mediated by PAR and (3) whether thrombin exacerbates brain edema induced by iron and whether thrombin inhibition plus iron chelation will be a new treatment for ICH.

The study will mostly use a rat model of ICH. Experimental models of ICH have been available since the 1960's and commonly involve the injection of autologous blood into the frontal lobe of dogs,

cats, pigs or monkeys<sup>11, 124-126</sup>. Now, rodents have been found to provide equally convenient and suitable models<sup>16-19, 46</sup>. They have the advantages of a lower cost (for animals and procedures), a relative homogeneity within strains owing to inbreeding, a close resemblance of the cerebrovascular anatomy and physiology to that of higher species, and a small brain size well suited to immunohistochemical and biochemical studies. There are other models of ICH. In particular a collagenase model has been used extensively. Indeed, we have used it in a preliminary study of whether argatroban might induce rebleeding. However, the collagenase-induced widespread disruption of the extracellular matrix (including the endothelial basement membrane) is not found in human ICH. It also appears to induce areas of ischemia that we do not find after injection of blood. Recent human studies suggest that ischemia is not a major component of ICH-induced injury<sup>127</sup>.

There will also be selective use of transgenic mice (PAR-1 knockout, complement C3 and C5 deficient mice; all commercially available from the Jackson Laboratory). These mice will provide another method for examining the role of complement factor and PAR-1 in brain edema following ICH. We have recently developed a mouse ICH model (preliminary data,<sup>105</sup>). The corner turn test and forelimb use asymmetry test, which we have previously used in the rat<sup>128</sup>, can also be used in the mouse<sup>105, 129</sup>.

The three major endpoints in our study are brain edema, blood-brain barrier permeability and neurological scores.

Perihematomal edema is thought by many, but not all, to be a major cause of death and disability following ICH, particularly in relation to herniation<sup>2, 6, 45, 130</sup>. In combination with the presence of the hematoma, further mass effect due to edema formation can result in a midline shift and herniation. Perihematomal edema, as seen on CT scan, can result from clot retraction. However, that represents redistribution of fluid between hematoma and brain. A progressive mass effect can only result from a movement of fluid from the blood to brain, either in the form of hematoma enlargement and/or progressive perihematomal edema. Although all agree that the latter does occur, its extent/prevalence has been debated<sup>45</sup>. It is easier to examine in animal models where perihematomal tissue can be sampled and water content determined directly. In animal models there is substantial evidence for progressive edema formation<sup>11, 17, 19, 38, 39</sup>. It should be noted that apart from its direct potential importance as a clinical endpoint, brain edema is also an easily quantifiable marker of brain injury. Therapeutic agents that reduce perihematomal cellular injury will almost certainly reduce edema formation (although the converse may not necessarily be true). Our brain edema measurements are done in concert with Na<sup>+</sup> and K<sup>+</sup> content measurements. ICH-induced edema is associated with an increase in brain Na<sup>+</sup>, while (a predominantly intracellular cation) loss from the brain is an indirect measure of brain injury.

To assess blood-brain barrier permeability, we will measure the influx rate constant ( $K_i$ ) for movement of  $^*-[^3\text{H}]$ aminoisobutyric acid (AIB) from blood to brain. This amino acid is not actively transported from blood to brain<sup>131</sup> and thus has a low  $K_i$  ( $\approx 1.5 \mu\text{l/g/min}$ ). The  $K_i$  is increased in a number of disease states where the blood-brain barrier is disrupted (e.g. cerebral ischemia<sup>132</sup>). It has the advantage over other commonly used markers of BBB disruption, such as Evans blue, of being quantifiable, and of having a very large volume of distribution within the brain so that efflux is not a concern<sup>131</sup>. Currently we plan to use tissue sampling to determine changes in AIB permeability although we have expertise in autoradiography if greater spatial resolution is required. We have used this technique previously to detect changes following ICH and cerebral ischemia<sup>20, 132</sup>.

Behavioral outcome is also examined in this application. We propose to use tests that evaluate both motor and sensory function, based on a set of tests developed by T. Schallert and others<sup>133, 134</sup>. A three sensorimotor behavioral tests were sensitive enough to detect behavioral deficits in the first day after ICH<sup>128</sup>. We found that forelimb placing and forelimb use asymmetry tests can detect even mild neurological deficits such as that found after 15 minutes of middle cerebral artery occlusion with reperfusion. All three tests appear to be well suited to models of unilateral brain injury because they



measure asymmetry. Thus, they can factor out confounding variables for behavioral tests such as decreased overall activity following surgery. These sensorimotor tests are also not altered by repeated testing and they do not require special training or food deprivation <sup>133</sup>.

Specific Aim 1 has three parts. The first (1a) examines whether thrombin activates complement cascade in the brain following ICH. The second (1b) examines the effects of complement activation on BBB disruption. The third (1c) examines the effects of complement on acute inflammatory response after ICH.

The effects of thrombin on the complement cascade will first be examined using RT-PCR to assess changes in brain C3, C5 and C9 mRNA along with changes in the complement regulators clusterin and DAF. To examine whether changes in mRNA correspond to changes in protein, Western blots and immunohistochemistry will be performed for C5, C9 and clusterin. Immunohistochemistry for C9 will provide evidence for whether this protein is present in cell membranes, an indication of MAC formation as well as the cell types affected.

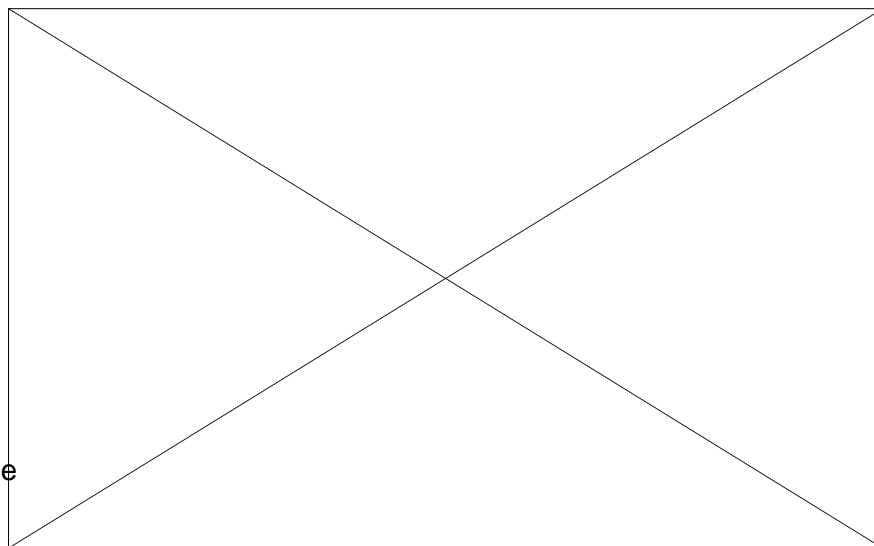
Four methods will be used to modulate the complement system. Complement depletion with cobra venom factor, complement inhibition with N-acetylheparin, and C3 and C5 deficient mice. Each of these methods has different effects on the complement system and will provide different information. Our data suggests that different elements of the complement system may have differential effects on ICH-induced brain injury <sup>18, 46, 105</sup>. Thus, cobra venom factor produces a stable C3 convertase that consumes all C3 in the plasma effectively obliterating functional complement in the bloodstream <sup>46, 135</sup>. By depleting rats of both C3 and C5, it prevents the generation of opsonic C3b and chemotactic C5a fragments and MAC complex formation. It will, therefore, inactivate a number of the elements that might be involved in complement-induced brain injury following ICH. It does have the disadvantage of depleting the rats of complement, making them more prone to infection. We will monitor animals closely for this possibility since such infections might modulate brain injury (e.g. by increasing body temperature). There are also concerns over other effects that cobra venom factor might have on the brain. Since CVF is a protein it is unlikely to cross the intact blood-brain barrier. It is also only useful for short-term experiments (<1 week) because of antibody reactions to CVF.

Heparin interferes with the complement system at multiple levels. It inactivates C1, blocks the assembly of C3 convertases and interferes with MAC assembly <sup>136</sup>. N-acetylheparin has similar properties to heparin but does not have the anticoagulant properties of heparin <sup>137, 138</sup>. Unlike CVF, which will be given three times at 36, 24 and 12 hours before ICH or intracerebral thrombin injection, N-acetylheparin can be given concomitantly with the intracerebral injection or after a time delay. By giving the drug intracerebrally we will avoid questions of drug access to the site of injury. This is a proof of concept study.

The use of commercially available C3 and C5 deficient mice will enable us to further dissect the role of different elements of the complement system. Thus, C5 deficient mice will prevent the formation of C5a fragments and MAC complex formation, but will not prevent the formation of the opsonic C3b. In contrast the C3 deficient mice will prevent the formation of C3b, C5a and MAC.

Specific Aim 2 will examine whether PARs play a key role in perihematomal edema formation. We will examine whether PAR-1, -3 or -4 activation induces brain edema following intracerebral hemorrhage. Three different ways will be used to test the activation of PAR-1 in this proposal. First, PAR-1-activating peptide (TFLLR); Second, a specific PAR-1 antagonist (3-mercaptopropionyl-Phe-Cha-C-Arg-Lys-Pro-Asn-Asp-Lys-amide, Mpa-peptide)<sup>139</sup> will be applied; Third, PAR-1 knockout mice. It is difficult to choose a PAR-1 antagonist because of deficiencies such as weak potency and mixed agonist/antagonist activity. For example, the peptide YFLLRNP was reported as a PAR-1 antagonist <sup>1</sup> but it could stimulate platelet aggregation as a partial PAR-1 agonist <sup>139</sup>. Mpa-peptide, however, not only can prevent thrombin- and TRAP-induced platelet aggregation and calcium mobilization, but also can inhibit thrombin- and TRAP-induced platelet phospholipase A<sub>2</sub> and Na<sup>+</sup>/K<sup>+</sup> exchange activation <sup>139</sup>. For PAR-3 studies, two PAR-3 agonists (TFRGAP and TFRGAPPNS) will be tested <sup>141</sup>, so the results of TFRGAP can be confirmed by the results of TFRGAPPNS. PAR-3 blocking antibody also will be used. In the PAR-4 study, peptide GYPGKF will be used as a PAR-4 agonist <sup>143</sup>. PAR-4 blocking antibody <sup>14</sup> and P4pal-10 pepducin, a PAR-4 antagonist, will also be used <sup>144</sup>. It should be noted that PAR-3 is a cofactor for PAR-4 activation <sup>145</sup>.

**Fig 18.** An overview of the three hypotheses.



Thrombin and hemoglobin are two major factors that responsible for brain edema formation<sup>6, 27</sup>. Our preliminary data have found that thrombin exacerbates iron-induced brain edema (Figure 15). In Specific Aim 3, we will test the effects of thrombin on RBC-induced brain edema and BBB disruption and the effects of thrombin inhibition plus iron chelation on brain edema formation, BBB disruption and ear neurological deficits.

Deferoxamine, the only iron chelator available for clinical use which can cross the blood-brain barrier easily<sup>146</sup>, will be used for this study. Deferoxamine can chelate free iron when it exists in excess and hence reduce its toxicity. In vitro studies have shown that deferoxamine can remove iron from transferrin, hemosiderin and ferritin, and also reduces hemoglobin-induced brain  $\text{Na}^+/\text{K}^+$  ATPase inhibition and neuronal toxicity<sup>146-148</sup>. However, they may become toxic in the absence of excess iron by forming toxic metabolites. Deferoxamine will be used alone or in combination with the thrombin inhibitor argatroban. We have shown that delayed (6 hr) treatment with argatroban or deferoxamine also can reduce ICH-induced brain edema. These experiments will determine whether the effects are addendum.

## D2. Research Plan

**Hypothesis 1.** Thrombin formation after intracerebral hemorrhage activates the complement cascade in the brain which triggers inflammatory response, forms membrane attack complex and results in blood-brain barrier disruption and brain edema formation.

**Specific Aim 1a.** To determine whether thrombin activates the complement cascade in the brain after ICH.

Both thrombin formation and complement activation are related to brain injury following many central nervous system disorders<sup>27, 97, 149</sup>. Activation of complement cascade in the brain contributes to brain edema formation and very early TNF- $\alpha$  production after ICH<sup>18, 46</sup>. In Specific Aim 1a, we will test whether thrombin is responsible for complement activation following ICH. Rats will receive intracaudal infusion of 50  $\mu\text{l}$  thrombin (5U). Control rats will receive intracerebral infusion of either 50  $\mu\text{l}$  saline or 5 thrombin plus 5U hirudin.

Specific Aim 1a.1. The rats will be killed at 1, 12, 24 and 48 hours. Brain will be harvested for RT-PCR to detect C3, C5, C9, clusterin and DAF mRNAs. In situ hybridization will be performed for the localization. Three groups, 2 endpoints, 4 time points of 4 rats each = 96 rats. We expect that C3, C5, C9, clusterin and DAF mRNA levels will be upregulated within 24 hours.

Specific Aim 1a.2. Rats will be killed at 1, 3 and 5 days. Brain will be perfused for Western blot and immunohistochemistry. We will use Western blot analysis, immunohistochemistry and immunofluorescent double labeling to test when, where and what kind of cells upregulate C5, C9 and clusterin. Six groups, 3 time points of 4 rats each = 72 rats. We expect complement C5, C9 and clusterin protein levels will be increased at the first day.

If this hypothesis is correct, we will test whether thrombin inhibition can block complement activation following intracerebral hemorrhage.

**Specific Aim 1b.** To examine the effects of complement activation on BBB disruption following ICH.

Our previous studies found that complement depletion and inhibition reduce perihematomal edema in the rat<sup>18, 46</sup>. In Specific Aim 1b, we will test the effects of complement activation on BBB permeability. To test this hypothesis, three sets of experiments are necessary. In the first set, we will use complement depleted rats to examine the role of complement on BBB permeability after ICH or thrombin injection. In the second set, the effects of complement inhibition with N-acetylheparin on BBB disruption will be tested. In the third set, brain edema and BBB permeability will be tested in C3 and C5 deficient mice.

Specific Aim 1b.1. Rats will be treated with cobra venom factor (CVF, 60 units/kg, i.p., three times at 36, 24 and 12 hours before ICH). Control rats will receive an equal volume of saline injection. All rats will receive intracerebral infusion of 100- $\mu$ l autologous whole blood or 5 U thrombin into the right basal ganglia and be killed 1, 3 and 5 days later for BBB permeability measurement. With 4 groups, 3 time points and 8 rats each, this study requires 96 rats. We expect complement depletion will reduce BBB disruption after ICH and injection of thrombin.

To confirm complement depletion in CVF treated animals, total serum hemolytic activity is measured prior to injury using the CH<sub>50</sub> technique<sup>150</sup>. Serial dilutions of serum are incubated for one hour at 37° C with sheep erythrocytes (Colorado Serum Company) which have been sensitized with rabbit anti-sheep hemolysin (Colorado Serum Company). The reciprocal of the serum dilution that results in 50% erythrocyte hemolysis is recorded as the CH<sub>50</sub> value. To assess the degree of depletion further, C3 titers are measured by double immunodiffusion using rabbit anti-rat C3 IgG<sup>46</sup>.

Specific Aim 1b.2. Rats will receive intracerebral infusion of 100- $\mu$ l autologous whole blood or 5 thrombin into the right basal ganglia. The rats will receive another intracerebral injection of N-acetylheparin (10  $\mu$ g in 10  $\mu$ l saline) into the right basal ganglia 3 hours after ICH. Control rats will have an equal volume of saline injection. Rats will be killed at 1, 3 and 5 days later for BBB permeability measurement (12 groups of 8 rats = 96 rats). We expect complement inhibition will reduce BBB disruption after ICH and thrombin injection. If our hypothesis is correct, we will test whether delayed systemic complement inhibition will reduce BBB disruption after ICH.

Specific Aim 1b.3. Complement C3 and C5 sufficient and deficient mice will receive injection of 30  $\mu$ l autologous whole blood. Brain edema and BBB permeability will be measured at 1, 3 and 5 days later. With 2 groups, 2 endpoints, 3 time points, 8 mice each group = 96 mice.

**Specific Aim 1c.** To examine the effects of complement on acute inflammatory response following ICH

Inflammatory response contributes to hemorrhagic brain injury<sup>120</sup>. We have found that TNF- $\alpha$  and intercellular adhesion molecule-1 (ICAM-1) levels are increased after ICH<sup>46, 115</sup>, and perihematomal edema is attenuated by overexpression of interleukin-1 (IL-1) receptor antagonist<sup>120</sup>. In Specific Aim 1c we will test the effects of complement C3 and C5 on potent inflammatory mediators such as TNF- $\alpha$ , IL-1 and ICAM-1. Complement C3 and C5 sufficient and deficient mice will receive injection of 30  $\mu$ l autologous whole blood. The mice will be killed at 1, 2, 4 and 24 hours later to examine brain TNF- $\alpha$ , IL-1 and ICAM-1 levels (ELISA). With 4 groups, 4 time points, 3 endpoints 8 mice per group, this study needs 384 mice.

In relation to Hypothesis 1, all proposed studies are well supported by our preliminary data. We believe these experiments can be carried out without significant difficulty. Brain injury after ICH is very complex, however. We might see some negative results. For example, in Specific Aim 1b2, we will test whether complement inhibition with N-acetylheparin can reduce BBB disruption. If N-acetylheparin (10  $\mu$ g) does not work, we will increase the dose to 20  $\mu$ g or test another complement inhibitor, pentosan polysulfate.

**Hypothesis 2.** Protease-activated receptors (PARs) play a key role in perihematomal edema formation

**Specific Aim 2a.** To examine the natural time courses of PARs at gene and protein levels after ICH.

PARs are involved in many neurological disorders<sup>27, 85</sup>. Our preliminary data demonstrated that brain PAR-1, -3 and -4 levels are upregulated after thrombin stimulation. In Specific Aim 2a, we will examine brain PARs after ICH. All rats will have intra-caudate infusion of 100- $\mu$ l autologous whole blood. Control rats will receive same volume of saline.

Specific Aim 2a.1. Rats will be killed at 1, 4, 24 and 48 hours. Brain will be sampled for RT-PCR to detect PAR-1, -3 and -4 mRNAs. The localization of PARs will be examined by in situ hybridization. With 4 groups of 4 rats each = 64 rats will be used.

Specific Aim 2a.2. Rats will be killed at 1, 3, 5 and 7 days and the rat brains will be perfused for Western blot analysis and immunohistochemistry for PARs. 16 groups of 4 rats each = 64 rats will be tested.

**Specific Aim 2b.** To determine whether brain edema formation and blood-brain barrier disruption after ICH are PAR-mediated.

Specific Aim 2b.1. Rats will receive an intracerebral injection of 50- $\mu$ l of saline, thrombin (5U), PAR-1 agonist (TFLLR, synthesized by University of Michigan Peptide Core; 0.1, 1 and 10 mM), PAR-3 agonist (TFRGAP, Bachem; 0.1, 1 and 10 mM)<sup>141</sup> or PAR-4 agonist (GYPGKF, Bachem; 0.1, 1 and 10 mM)<sup>143</sup>. All rats will be killed at 24 hours for brain water content and BBB permeability measurements. total 22 groups of 8 rats each = 176 rats. We expect that high dose of PAR-1 and PAR-4 agonists but PAR-3 agonist will cause brain edema and BBB disruption.

Specific Aim 2b.2. Rats will receive 100- $\mu$ l of autologous whole blood+vehicle, blood+10 nmol PAR-1 antagonist (3-mercapto-propionyl-Phe-Cha-Cha-Arg-Lys-Pro-Asn-Asp-Lys-amide, Bachem), blood+10 nmol PAR-4 antagonist (P4pal-10, Bachem) or blood+PAR1 antagonist (10 nmol)+PAR4 antagonist(10 nmol). The rats will be killed at 1 and 3 days later for brain edema and BBB permeability measurements. A total 16 groups of 8 rats each = 128 rats. Table 2 shows the expected results.

**Table 2. Expected results after ICH**

		ICH+Vehicle	ICH+PAR1 antagonist	ICH+PAR4 antagonist	ICH+PAR1 & PAR4 Antagonists
<b>Edema</b>	Day 1	++++	+++	++ or +++	+ or ++
	Day 3	+++++	+++	+++	++
<b>BBB</b>	Day 1	++++	++ or +++	++ or +++	+ or ++
	Day 3	+++++	+++	+++	++

+++++ = maximal; + = minimal.

Specific Aim 2b.3. PAR-1 knockout and wild type mice will receive an intracerebral injection of  $\mu$ l saline or autologous whole blood into the right caudate. The mice will be killed at 1 and 3 days later for brain edema and BBB permeability measurements. A total 4 groups, 2 endpoints, 2 time points and 8 mice each = 128 mice. We expect both brain edema and BBB disruption will be less in PAR-1 knockout mice.

**Specific Aim 2c.** To determine whether increases of TNF- $\alpha$  levels after ICH and thrombin stimulation are due to activation of PARs.

Our preliminary data showed that brain edema after ICH is less in TNF- $\alpha$  knockout mice. In Specific Aim 2c, we will test whether PARs have a key role in TNF- $\alpha$  increases after ICH or thrombin injection.

Specific Aim 2c.1. Rats will receive an intracerebral injection of 50  $\mu$ l of saline, PAR-1 agonist (TFLLR, 1 mM), PAR-3 agonist (TFRGAP, 1 mM), PAR-4 agonist (GYPGKF, 1 mM). Rats will be killed 2 hours after injection for TNF- $\alpha$  ELISA. A total 4 groups of 8 rats each = 32 rats. We expect the PAR-4 agonist will increase brain TNF- $\alpha$  levels markedly.

Specific Aim 2c.2. Rats will receive 100- $\mu$ l of autologous whole blood+vehicle, blood+10 nmol PAR-1 antagonist (3-mercapto-propionyl-Phe-Cha-Cha-Arg-Lys-Pro-Asn-Asp-Lys-amide, Bachem), blood+10 nmol PAR-4 antagonist (P4pal-10, Bachem) or blood+PAR1 antagonist (10 nmol)+PAR4 antagonist(10 nmol). All rats will be killed at 2 hours for TNF- $\alpha$  ELISA. A total 4 groups of 8 rats each = 32 rats. We expect that the PAR-4 antagonist will reduce perihematomal TNF- $\alpha$  levels.

**Specific Aim 2d.** To determine whether thrombin activates complement through PARs.

The role of PARs in thrombin and ICH-induced complement activation has not been examined. This specific aim will, therefore, examine whether PAR agonists can induce elements of the complement system (mRNA and protein). If those initial experiments indicate that PAR activation can activate the complement system, we will examine whether PAR antagonists can inhibit ICH-induced complement activation.

Specific Aim 2d.1. Rats will receive an intracerebral injection of 50  $\mu$ l of saline, PAR-1 agonist

(TFLLR, 1 mM), PAR-3 agonist (TFRGAP, 1 mM), PAR-4 agonist (GYPGKF, 1 mM). The rats will be killed at 1, 12, 24 and 48 hours. Brain will be harvested for RT-PCR to detect C3, C5, C9, clusterin and DAF mRNAs. In situ hybridization will be performed for the localization. Four groups, 2 endpoints, 4 time points of 4 rats each = 96 rats. We expect that C3, C5, C9, clusterin and DAF mRNA levels will be upregulated within 24 hours by the PAR-1 and perhaps the PAR-4 agonists.

Specific Aim 2d.2. Rats will receive an intracerebral injection of 50  $\mu$ l of saline, PAR-1 agonist (TFLLR, 1 mM), PAR-3 agonist (TFRGAP, 1 mM), PAR-4 agonist (GYPGKF, 1 mM). Rats will be killed 1, 3 and 5 days. Brain will be perfused for Western blotting and immunohistochemistry. We will use Western blot analysis, immunohistochemistry and immunofluorescent double labeling to test when, where and what kind of cells upregulate C5, C9 and clusterin. Eight groups, 3 time points of 4 rats each = 96 rats. We expect complement C5, C9 and clusterin protein levels will be increased at the first day by at least one of the PAR agonists.

Should the PAR agonists activate the complement system, we will examine whether PAR antagonists can prevent ICH-induced complement activation. The dosing for the antagonists will be as described in Specific 2b.2. Complement activation will be assessed as described in Specific Aim 2d.1 and 2d.2.

We understand that thrombin-induced brain injury may through one of the PARs, especially PAR 1. We have found that activation of p38 and p44/42 MAPKs are PAR-1 mediated (preliminary data, Fig 1). If PAR-4 is not the major receptor which mediates thrombin-induced injury, we will test the effects of MAPKs inhibition on brain injury after ICH and thrombin injection.

**Hypothesis 3.** Thrombin exacerbates brain edema induced by iron. Thrombin inhibition plus iron chelation will be a new treatment for ICH.

**Specific Aim 3a.** To determine whether thrombin exacerbates RBC-induced brain edema and BBB disruption.

Lysed RBCs but not packed RBCs produce marked BBB disruption and brain edema in the ipsilateral basal ganglia and cortex 24 hours after intracerebral infusion<sup>17, 20</sup>. We also examined the time course of brain water content following injection of packed RBCs into the caudate. We found no edema formation 24 hours post packed RBCs injection. There was, however, marked edema after three days<sup>1</sup>. Our preliminary data showed that thrombin exacerbates iron-induced brain edema. In Specific Aim 3a, we will examine whether thrombin potentiates erythrocyte-induced BBB disruption and brain edema.

Specific Aim 3a.1. Rats will receive intracaudate infusion of 30- $\mu$ l saline, thrombin (1 U), packed RBCs, lysed RBCs, thrombin (1 U) plus packed RBCs or thrombin (1 U) plus lysed RBCs. All rats will be killed at 24 hours later for 1) BBB permeability measurement; 2) Brain edema measurement. Packed RBCs (hematocrit=87 $\pm$ 1%) are obtained by centrifuging unclotted blood. The plasma and buffy coat are discarded. The RBCs are washed with five volumes of saline three times. For lysed RBCs, the packed RBCs are lysed by freezing in liquid nitrogen for five minutes followed by thawing at 37  $^{\circ}$ C. Six groups endpoints of 8 rats each = 96 rats will be tested. We expect thrombin will exacerbate BBB disruption and brain edema formation induced by lysed RBCs.

Specific Aim 3a.2. Rats will receive intracaudate infusion of 30- $\mu$ l saline or packed RBCs. All rats will receive one unit thrombin (30- $\mu$ l) 48 hours later. Rats will be killed at another 24 hours for 1) BBB permeability measurement; 2) Brain edema measurement. Two groups, 2 endpoints of 8 rats each = 3 rats will be tested. We expect that thrombin will exacerbate BBB disruption and brain edema induced by packed RBCs at day 3.

**Specific Aim 3b.** To test the effects of thrombin inhibition plus iron chelation on brain edema formation, BBB disruption and early neurological deficits.

We have previously found that systemic argatroban treatment initiated at 6 hours can reduce perihematomal edema<sup>22</sup>. We also showed that iron contributes to brain edema formation and an iron chelator, deferoxamine, reduces brain edema after ICH<sup>53, 122</sup>. In Specific Aim 3b, we will test whether combining thrombin inhibition with iron chelation will provide further brain protection.

Specific Aim 3b.1. Rats will receive a 100- $\mu$ l intracaudate injection of autologous whole blood and will be treated either vehicle, deferoxamine (100 mg/kg in 1 ml intraperitoneally every 12 hours), argatroban (0.9 mg/h/rat, i.p.) or deferoxamine plus argatroban. Treatments will be commenced 3, 6 or hours post ICH. All rats will be sacrificed at 1, 3 or 7 days for brain water content measurements. With groups, 3 treatment started time points, 3 sacrifice time points and 8 rats per group, this will require 28 rats. Behavior will be assessed at 1, 3 and 7 days. Table 3 shows expected results from edema study.

**Table 3. Expected brain water contents after ICH**

		<b>1 day</b>	<b>3 days</b>	<b>7 days</b>
<b>3 h delayed:</b>	Vehicle	++++	+++++	++ or +++
	Argatroban	++	+++	+ or ++
	Deferoxamine	++ or +++	+++	+ or ++
	Argatroban+Deferoxamine	+	+ or ++	+
<b>6 h delayed:</b>	Vehicle	++++	+++++	++ or +++
	Argatroban	++ or +++	+++	++
	Deferoxamine	++ or +++	+++	++
	Argatroban+Deferoxamine	+	+ or ++	+
<b>12 h delayed:</b>	Vehicle	++++	+++++	++ or +++
	Argatroban	+++ or ++++	++++ or ++++	++ or +++
	Deferoxamine	+++ or ++++	++++ or ++++	++ or +++
	Argatroban+Deferoxamine	++	++	+

+++++ = maximal; + = minimal.

Specific Aim 3b.2. Rats will have ICH and will be treated as Specific Aim 3b.1. Then rats will be sacrificed at 24 and 48 hours after ICH for BBB permeability measurement. With 4 groups, 3 treatment initiated time points, 2 sacrifice time points and 8 rats per group, this will require 192 rats. We expect argatroban and deferoxamine will reduce BBB disruption. However, if deferoxamine does not work, this may indicate BBB disruption after ICH results in accumulation of ferrous iron rather than ferric iron. We would then test the effects of ferrous iron chelator, 2,2'-dipyridyl, on BBB disruption. In addition, if results from Specific Aim 2 demonstrate that PAR antagonists can reduce ICH-induced edema, we will plan to examine a combination of deferoxamine plus the selected PAR antagonists.

### D3. Time Table

	<b>Year 1</b>	<b>Year 2</b>	<b>Year 3</b>	<b>Year 4</b>	<b>Year 5</b>
<b>S.A. 1a</b>	X				
<b>S.A. 1b</b>	X	X			
<b>S.A. 1c</b>		X			
<b>S.A. 2a</b>		X	X		
<b>S.A. 2b</b>			X		
<b>S.A. 2c</b>			X	X	
<b>S.A. 2d</b>				X	
<b>S.A. 3a</b>				X	X
<b>S.A. 3b</b>				X	X

### D4. Specific Methods

**1) Animal Preparation.** Adult male Sprague-Dawley rats (275-325 g) are anesthetized with pentobarbital (40 mg/kg, i.p.). Mice (wild type or transgenic) are anesthetized with 4% chloral hydrate (0.1 ml/10 g). Aseptic precautions will be utilized in all surgical procedures. After anesthesia is achieved

a polyethylene catheter (PE-50 or PE 10) is inserted into the right femoral artery in order to monitor arterial blood pressure and to obtain blood for analysis of blood gases, blood pH, hematocrit, blood glucose concentration. Body temperature is maintained at 37.5 °C by using a feedback-controlled heating pad.

**2) Intracerebral Infusion.** Before intracerebral infusion, the rats or mice are positioned in a stereotactic frame (Kopf Instrument, Tujunga, CA), the scalp is incised along the sagittal midline using sterile technique. A cranial burr hole (1 mm) is drilled near the right coronal suture 4.0 mm (rats) or 2.5 mm (mice) lateral to the midline. A 26-gauge needle is inserted stereotaxically into the right basal ganglia (coordinates for rats: 0.2 mm anterior, 5.5 mm ventral, and 4.0 mm lateral to the bregma; coordinates for mice: 0 mm anterior, 3.5 mm ventral and 2.5 mm lateral to the bregma). Whole blood, thrombin or saline are infused into right basal ganglia at a rate of 10  $\mu$ l/minute using a microinfusion pump. After infusion the needle is removed and the skin incisions are closed with sutures. Animals are allowed to recover.

**3) Brain Water, Sodium and Potassium Contents.** Animals are sacrificed by decapitation under deep pentobarbital anesthesia (60 mg/kg i.p.). The brains are removed immediately and a 3 mm thick coronal brain slice with hematoma is cut. That slice is divided into four samples, ipsilateral and contralateral basal ganglia and ipsilateral and contralateral cortex. Cerebellum is obtained as a control. Tissue samples are weighed on an electronic analytical balance to the nearest 0.1 mg to obtain the wet weight (WW). The tissue is then dried in a gravity oven at 100 °C for more than 24 hours to determine the dry weight (DW). Tissue water contents (%) are calculated as  $((WW-DW)/WW)*100$ . The dehydrated brain samples are digested in 1 ml of 1 N nitric acid for 1 week. The sodium and potassium ion contents in this solution are measured by flame photometry. Ion contents are expressed in micro equivalents per gram of dehydrated brain tissue ( $\mu$ Eq/gm dry wt).

**4) Blood-Brain Barrier Permeability.** Blood-brain barrier integrity will be assessed using  $^3$ H-aminoisobutyric acid (AIB). The permeability of the BBB to  $^3$ H-AIB will be determined as described previously in our studies on cerebral ischemia<sup>132</sup> and cerebral hemorrhage<sup>19</sup>. After an i.v. bolus injection, the amount of tracer that moves into the extravascular compartment of the brain (Ab) is divided by the integral of the plasma concentration of the tracer determined between the time of injection and killing of the animal ( $\int C_p dt$ ). Ab is determined by subtracting the amount of tracer in the plasma compartment (PV x Ct) from the total radioactivity in the brain (At), where PV is the plasma volume estimated with  $^{14}$ C-inulin and Ct is the terminal plasma concentration. The integral of the plasma concentration is obtained from a continuous withdrawal of arterial blood (0.1ml/min) during the course of isotope circulation, and calculated by dividing by the total counts withdrawn.

$^3$ H-AIB (25  $\mu$ Ci in rat, 5  $\mu$ Ci in mouse) is injected 10 minutes before the end of the experiment, while  $^{14}$ C-inulin (10  $\mu$ Ci in rat and 2 $\mu$ Ci in mouse) is given as a second injection 2 min before the end. Simultaneous with the  $^3$ H-AIB injection, a peristaltic pump is started and blood is withdrawn from an arterial cannula. At the end of the experiment, a terminal plasma sample is obtained and the animal is killed by decapitation. The entire contents of the arterial cannula are emptied and an aliquot pipetted for counting. Blood samples are digested in methylbenzethonium hydroxide, bleached with H<sub>2</sub>O<sub>2</sub>, and counted in an aqueous-based liquid scintillation cocktail. Brain tissue is also digested in methylbenzethonium hydroxide and counted in a Beckman 3801 two-channel liquid scintillation counter. The results are expressed as a rate constant for brain uptake, which is equal to the product of the capillary permeability and the surface area of the exposed vascular bed (PS product) for a compound of low BBB permeability such as AIB.

**5) Western Blot Analysis.** Animals are anesthetized and decapitated at different time points. Brain is perfused transcardially with saline and brain tissues are sampled as described in the water content paragraph. The brain tissues are immersed in 0.5 mL Western sample buffer (62.5 mM Tris-HCl pH 6.8, 2.3% sodium dodecyl sulfate, 10% glycerol, and 5%  $\beta$ -mercaptoethanol) and then are sonicated for 10 seconds. Ten- $\mu$ L of the sample solution are taken for protein assay (Bio-Rad), while the rest is frozen at -20 °C for Western blot. Western blot analysis is performed as described previously<sup>69</sup>. Brief

50 µg protein is run on polyacrylamide gels with a 4% stacking gel (SDS-PAGE) after 5 minutes boiling 95 °C. The protein is transferred to hybond-C pure nitrocellulose membrane (Amersham). The membranes are blocked in 5% Carnation non-fat dry milk in TBST (150 mM NaCl, 100 mM Tris-base, 0.1% Tween 20, pH 7.6) buffer for 1 hour at 37 °C. After washing in TBST buffer 3 times, membranes probed with the primary antibody for 1.5 hours at room temperature. After washing the membranes with TBST buffer 3 times, the membranes are immunoprobed again with the second antibody for one hour at room temperature. Finally, membranes are washed three times in TBST buffer and the antigen-antibody complexes are visualized with the ECL chemiluminescence system (Amersham) and exposed to Kodak X-OMAT film. The relative densities of the protein bands are analyzed with a public domain NIH Image program (NIH Image Version 1.61).

**6) Immunohistochemistry and Immunofluorescent Double Labeling.** Animals are anesthetized with pentobarbital (60 mg/kg, i.p.) and perfused with 4% paraformaldehyde in 0.1 M pH 7 phosphate-buffered saline (PBS). Removed brains are kept in 4% paraformaldehyde for six hours, then immersed in 25% sucrose for three to four days at 4 °C. The brains are embedded in O.C.T. compound (Sakura Finetek U.S.A. Inc.) and sectioned on a cryostat (18 µm thick). Sections are incubated overnight with the primary antibody. After three washes in PBS, sections are incubated for 90 min with biotinylated second antibody. After another three PBS washes, brain sections are incubated with avidin-biotinylated horseradish peroxidase (Vector Laboratories) for 90 min. Brain sections are re-washed three times in PBS and then incubated with diaminobenzidine and hydrogen peroxide (Stable DAB, Research Genetics Inc.). The sections are then washed in water for five minutes, dehydrated and covered with a coverslip for microphotography. Normal IgG (from the same species as that which produces primary antibody) or omission of the primary antibody or an antibody preabsorbed with the purified antigens will be used as negative control.

Different antibody combinations will be used for immunofluorescent double labeling. Each primary antibody (1:100 dilution) is incubated overnight at 4 °C. Fluorescein isothiocyanate (FITC) or rhodamine labeled second antibody is incubated with sections for 2 hours at room temperature. The double label is analyzed by a fluorescence microscope (Nikon Microphoto-SA) using a rhodamine filter and a FITC filter<sup>69</sup>.

**7) Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis**<sup>151, 152</sup>. Brain total RNA will be extracted with Trizol reagent and cDNA will be synthesized by reverse transcription (RT). PCR product will be electrophoretically separated in a 1% agarose gel, stained with ethidium bromide, visualized under UV light, and photographed. PCR products will be normalized to its respective GAPDH PCR product. Data are expressed as pixels.

**8) *In situ* hybridization.** Rat brains will be immediately frozen in isoamylalcohol. Coronal cryostat (18 µm) of the rat brain at the level of the caudate will be mounted on poly-L-lysine-coated slides and will be kept in -80 °C until needed. *In situ* hybridization with digoxigenin-labelled RNA probes for complement factors and PARs will be carried out as described by Niclou et al.<sup>80</sup>

**9) Behavioral Tests.** For the behavioral tests, all animals will be tested before and after surgery and scored by experimenters who will be blind to both neurological and treatment conditions<sup>128, 134</sup>. **(A) Measurement of Forelimb Placing.** Forelimb placing is scored using the vibrissae-elicited forelimb-placing test. Animals are held by their torsos allowing forelimb to hang free. While holding the animal the experimenter makes gentle up and down movement in space prior to placing testing, which facilitates muscle relaxation and eliminates any struggling movement. Independent testing of each forelimb is induced by brushing the respective vibrissae on the edge of a table top once per trial for 10 trials. Intact animals place the forelimb of both sides quickly onto the countertop. Percent successful placing responses were determined. **(B) Forelimb Limb-Use Asymmetry Test.** Forelimb use during exploratory activity is analyzed by videotaping rats in a transparent cylinder (20cm diameter and 30 cm height) for



10min depending on the degree of activity during the trial. A mirror is placed to the side of the cylinder an angle to enable the recording of forelimb movements even when the animal is turned away from the camera. Scoring is done by an experimenter blinded to the condition of the animal using a VCR with slow motion and clear stop-frame capabilities. The behavior is scored using the following criteria: (A) independent use of the left or right forelimb for contacting the wall during a full rear to initiate a weight shifting movement or to regain center of gravity while moving laterally in a vertical posture. (B) Simultaneous use of both the left and right forelimb for contacting the cylinder wall during a full rear or for alternating lateral stepping movements along the wall. Behavior is quantified by determining the occasions when the non-impaired (Ipsilateral) forelimb is used as a percentage of total number of limb use observations on the wall (I). The occasions when the impaired forelimb (Contralateral to the blood injection site) is used as a percentage of total number of limb use observations on the wall (C), and the occasions when "both" forelimbs are used simultaneously (or nearly simultaneously during lateral side stepping movements) as a percentage of total number of limb use observations on the wall (B). A single overall limb use asymmetry score is calculated as:  $\text{Limb use asymmetry score} = (I/(I+C+B)) - (C/(I+C+B))$

**(C) Corner Turn Test.** The rat is allowed to proceed into a corner, the angle of which is 30 degrees. At the exit the corner, the rat can turn either to the left or the right and this is recorded. This is repeated 1 to 15 times, with at least 30 seconds between trial, and the percentage of right turns calculated<sup>128, 153</sup>. Only turns involving full rearing along either wall are included (i.e. ventral tucks or horizontal turns are excluded). The rats are not picked up immediately following each turn so that they do not develop an aversion for their prepotent turning response.

**10) Thrombin Preparation.** Thrombin will be prepared as follows: Prothrombin (40 mg in 10 ml 25 mM Bis-Tris buffer, pH 6.5/40 mM NaCl) will be activated with 0.5 mg of factor Xa in the presence of 10 mM CaCl<sub>2</sub> for 2 hr at 37 C. The reaction is terminated by adding 10 mM EDTA. Thrombin is eluted in the non-absorbed fraction and appears as a single band on nonreducing SDS/PAGE<sup>154</sup>.

**11) Alzet Osmotic Mini-pump Implantation.** The pump is preloaded with or without drug and delivers 10 µl/h. For thrombin inhibitor argatroban, an osmotic minipump will be implanted intraperitoneally.

**12) Systemic Complement Depletion.** Circulating complement will be depleted using cobra venom factor (CVF) as previously described<sup>155</sup>. Briefly, whole venom (*Naja naja atra*, Sigma Co.) will be dialyzed for 24 h against 40 mM PBS, then fractionated by anion exchange chromatography on a diethylaminoethyl cellulose column (DE52, Whatman International)<sup>156</sup>. Animals will be given intraperitoneal injections of 25 units of CVF in 1.0 ml of saline 36, 24, and 12 hours prior to induction of cerebral ischemia. To confirm complement depletion in CVF treated animals, total serum hemolytic activity will be measured prior to injury using the CH<sub>50</sub> technique<sup>150</sup>. Serial dilutions of serum will be incubated for one hour at 37° C with sheep erythrocytes (Colorado Serum Company) which have been sensitized with rabbit anti-sheep hemolysin (Colorado Serum Company). The reciprocal of the serum dilution that results in 50% erythrocyte hemolysis will be recorded as the CH<sub>50</sub> value. To assess the degree of depletion further, C3 titers will be measured by double immunodiffusion using rabbit anti-rat IgG (United States Biochemical Corp.).

**13) Enzyme-Linked Immunosorbent Assays (ELISA).** IL-1b, TNF-a and ICAM-1 levels will be measured by ELISA (R&D Systems). The colorimetric reaction will be analyzed in a microplate reader (Biotek, EL311). Protein levels are normalized by protein content. The concentration of protein is measured using the Bio-Rad protein assay.

**14) Statistical Analyses.** The analyses generally will characterize the parameters measured. Statistical analyses will examine the assumptions of normal distribution, homogeneity of variance, etc. If any are found in violation of these assumptions, the data transformations will be examined to determine their appropriateness. When necessary, we will use non-parametric procedures.

For all three hypotheses, the endpoints (brain water content, BBB permeability, forelimb placing

score, forelimb use asymmetry score, corner turn score, ion contents, ELISA data, Western blotting data, rtPCR data, etc.) will be compared with control groups and among different time points.

Brain water content data and BBB permeability data will be analyzed by Student t test (two groups) or ANOVA with a Scheffe's multiple comparisons test (more than two groups). Behavioral outcome and Western blot analysis data will be analyzed by Mann-Whitney U rank test (two groups) or Kruskal-Wallis rank test (more than two groups). Differences will be considered significant at the  $p < 0.05$  level.

#### **E. HUMAN SUBJECTS**

None

#### **F. VERTEBRATE ANIMALS**

1. These experiments require rats. Approximately 340 male Sprague-Dawley rats weighing 275-325 grams (60-70 days old) and 120 mice weighing 25-30 grams (60-70 days old) will be used annually.

2. In vitro systems can not be used to study the complex interactions involving blood clot, mass effect, brain cells, and the microvasculature that follow brain hemorrhage. The rat brain is large enough to permit the injection of a reproducible volume of blood and the measurements that we will make (i.e. brain edema, BBB permeability, RT-PCR, *in situ* hybridization, Western blot analysis and immunocytochemistry). Based upon our preliminary studies with these models, group sizes of 8 animals are required for brain edema, BBB disruption and behavioral tests. Four animals each group will be used for RT-PCR, *in situ* hybridization, Western blot analysis and immunocytochemistry. Results will be monitored as they are collected and if larger changes are seen, then it may be possible to reduce the number of animals used.

3. Animal husbandry will be provided by the Unit for Laboratory Animal Medicine (ULAM) under the guidance of supervisors who are certified Animal Technologists. Veterinary care will be provided by ULAM faculty members and veterinary residents. The University of Michigan has an Animal Welfare Assurance (A3114-01) on file with the Office for Protection from Research Risks and is fully accredited by the American Association for the Accreditation of Laboratory Animal Care.

4. All invasive procedures will be conducted under anesthesia using sterile technique. Animals that are allowed to recover after the injection of whole blood or saline are kept warm until awake and then watched carefully every 6-12 hours. Our model of intracerebral hemorrhage allows the rats and the mice to survive for at least six months with less than 5% mortality. The majority of the rats begin eating and drinking water within 6 hours of the operation. Some have a mild unilateral weakness that does not interfere with their ability to move around the cage and to feed and clean themselves. Any animal which appears sick, has obvious infection at the site of surgery, or does not drink following the procedure will be euthanized.

5. All animals will be sacrificed within 10 days of the operation. They will be reanesthetized and euthanized by decapitation without awakening from the anesthetic.

## G. LITERATURE CITED

1. Kase CS, Caplan LR. *Intracerebral hemorrhage*. Boston: Butterworth-Heinemann; 1994.
2. Ropper AH. Lateral displacement of the brain and level of consciousness in patients with an acute hemispherical mass. *New England Journal of Medicine*. 1986;314:953-958
3. Xi G, Fewel ME, Hua Y, Thompson BG, Hoff J, Keep R. Intracerebral hemorrhage: Pathophysiology and therapy. *Neurocritical Care*. 2004;1:5-18
4. Broderick J, Brott T, Tomsick T, Tew J, Duldner J, Huster G. Management of intracerebral hemorrhage in a large metropolitan population. *Neurosurgery*. 1994;34:882-887; discussion 887
5. Hoff J, Xi G. Brain edema from intracerebral hemorrhage. *Acta Neurochirurgica*. 2003;86[suppl]:11-15
6. Xi G, Keep RF, Hoff JT. Pathophysiology of brain edema formation. *Neurosurgery Clinics of North America*. 2002;13:371-383
7. Qureshi AI, Tuhim S, Broderick JP, Batjer HH, Hondo H, Hanley DF. Spontaneous intracerebral hemorrhage. *New England Journal of Medicine*. 2001;344:1450-1460
8. Kendall BE, Radue EW. Computed tomography in spontaneous intracerebral haematomas. *Br J Radiol*. 1978;51:563-573
9. Bergstrom M, Ericson K, Levander B, Svendsen P, Larsson S. Variation with time of the attenuation values of intracranial hematomas. *J Comput Assist Tomogr*. 1977;1:57-63
10. Brott T, Broderick J, Barsan K, Kothari R, Tomsick T, Spilker J, Huster G. Hyperacute clot retraction in spontaneous intracerebral hemorrhage. *Stroke*. 1992;23:141
11. Wagner KR, Xi G, Hua Y, Kleinholz M, de Courten-Myers GM, Myers RE, Broderick JP, Brott TG. Lobar intracerebral hemorrhage model in pigs: Rapid edema development in perihematomal white matter. *Stroke*. 1996;27:490-497
12. Lee KR, Betz AL, Keep RF, Chenevert TL, Kim S, Hoff JT. Intracerebral infusion of thrombin as cause of brain edema. *J Neurosurg*. 1995;83:1045-1050
13. Lee KR, Colon GP, Betz AL, Keep RF, Kim S, Hoff JT. Edema from intracerebral hemorrhage: The role of thrombin. *J Neurosurg*. 1996;84:91-96
14. Lee KR, Betz AL, Kim S, Keep RF, Hoff JT. The role of the coagulation cascade in brain edema formation after intracerebral hemorrhage. *Acta Neurochir*. 1996;138:396-400
15. Lee KR, Kawai N, Kim S, Sagher O, Hoff JT. Mechanisms of edema formation after intracerebral hemorrhage: Effects of thrombin on cerebral blood flow, blood-brain barrier permeability, and cell survival in a rat model. *J Neurosurg*. 1997;86:272-278
16. Xi G, Wagner KR, Keep RF, Hua Y, de Courten-Myers GM, Broderick JP, Brott TG, Hoff JT. The role of blood clot formation on early edema development following experimental intracerebral hemorrhage. *Stroke*. 1998;29:2580-2586
17. Xi G, Keep RF, Hoff JT. Erythrocytes and delayed brain edema formation following intracerebral hemorrhage in rats. *J Neurosurg*. 1998;89:991-996
18. Hua Y, Xi G, Keep RF, Hoff JT. Complement activation in the brain after experimental intracerebral hemorrhage. *J Neurosurg*. 2000;92:1016-1022
19. Yang GY, Betz AL, Chenevert TL, Brunberg JA, Hoff JT. Experimental intracerebral hemorrhage: Relationship between brain edema, blood flow, and blood-brain barrier permeability in rats. *J Neurosurg*. 1994;81:93-102
20. Xi G, Hua Y, Bhasin RR, Ennis SR, Keep RF, Hoff JT. Mechanisms of edema formation after intracerebral hemorrhage: Effects of extravasated red blood cells on blood flow and blood-brain barrier integrity. *Stroke*. 2001;32:2932-2938
21. Qureshi AI, Wilson DA, Hanley DF, Traystman RJ. No evidence for an ischemic penumbra in massive experimental intracerebral hemorrhage. *Neurology*. 1999;52:266-272
22. Kitaoka T, Hua Y, Xi G, Hoff JT, Keep RF. Delayed argatroban treatment reduces edema in a rat model of intracerebral hemorrhage. *Stroke*. 2002;33:3012-3018
23. Hamada R, Matsuoka H, Wagner KR, Broderick JP, deCourten-Myers GM, Xi G, Keep RF, Hoff JT. Antithrombin therapy for intracerebral hemorrhage. *Stroke*. 2000;31:794-795
24. Matsuoka H, Hamada R. Role of thrombin in CNS damage associated with intracerebral hemorrhage. *CNS Drugs*. 2002;16:509-516
25. Coughlin SR. How the protease thrombin talks to cells. *Proceedings of the National Academy of Sciences of the United States of America*. 1999;96:11023-11027
26. Coughlin SR. Thrombin signalling and protease-activated receptors. *Nature*. 2000;407:258-264

27. Xi G, Reiser G, Keep RF. The role of thrombin and thrombin receptors in ischemic, hemorrhagic and traumatic brain injury: Deleterious or protective? *Journal of Neurochemistry*. 2003;84:3-9
28. McKissock W, Richardson A, Walsh L. Primary intracerebral haemorrhage: Results of surgical treatment in 244 consecutive cases. *Lancet*. 1959;1:683
29. McKissock W, Richardson A, Taylor J. Primary intracerebral haemorrhage: A controlled trial of surgical and conservative treatment in 180 unselected cases. *Lancet*. 1961;2:221-226
30. Kaneko M, Tanaka K, Shimada T, Sato K, Uemura K. Long-term evaluation of ultra-early operation for hypertensive intracerebral hemorrhage in 100 cases. *J Neurosurg*. 1983;58:838-842
31. Kanno T, Sano H, Shinomiya Y, Katada K, Nagata J, Hoshino M, Mitsuyama F. Role of surgery in hypertensive intracerebral hematoma. A comparative study of 305 nonsurgical and 154 surgical cases. *J Neurosurg*. 1984;61:1091-1099
32. Juvela S, Heiskanen O, Poranen A, Valtonen S, Kuurne T, Kaste M, Troupp H. The treatment of spontaneous intracerebral hemorrhage. A prospective randomized trial of surgical and conservative treatment [see comments]. *J Neurosurg*. 1989;70:755-758
33. Morgenstern LB, Frankowski RF, Shedden P, Pasterur W, Grotta JC. Surgical treatment for intracerebral hemorrhage(stich): A single-center, randomized clinical trial. *Neurology*. 1998;51:1359-1363
34. Lisk DR, Pasteur W, Rhoades H, Putnam RD, Grotta JC. Early presentation of hemispheric intracerebral hemorrhage: Prediction of outcome and guidelines for treatment allocation. *Neurology*. 1994;44:133-139
35. Hankey GJ, Hon C. Surgery for primary intracerebral hemorrhage: Is it safe and effective? A systematic review of case series and randomized trials. *Stroke*. 1997;28:2126-2132
36. Prasad K, Browman G, Srivastava A, Menon G. Surgery in primary supratentorial intracerebral hematoma: A meta-analysis of randomized trials. *Acta Neurol Scand*. 1997;95:103-110
37. Suzuki J, Ebina T. Sequential changes in tissue surrounding ich. In: Pia HW, Longmaid C, Ziers J, eds. *Spontaneous intracerebral hematomas*. Berlin: Springer; 1980:121-128.
38. Enzmann DR, Britt RH, Lyons BE, Buxton JL, Wilson DA. Natural history of experimental intracerebral hemorrhage: Sonography, computed tomography and neuropathology. *Ajnr: Am J Neuroradiol*. 1981;2:517-526
39. Tomita H, Ito U, Ohno K, Hirakawa K. Chronological changes in brain edema induced by experimental intracerebral hematoma in cats. *Acta Neurochir - Suppl*. 1994;60:558-560
40. Jenkins A, Maxwell W, Graham D. Experimental intracerebral hematoma in the rat: Sequential light microscopic changes. *Neuropathol Appl Neurobiol*. 1989;15:477-486
41. Garcia JH, Ho KL, Caccamo D. Intracerebral hemorrhage: Pathology of selected topics. In: Kase CS, Caplan LR, eds. *Intracerebral hemorrhage*. Boston: Butterworths; 1994:45-72.
42. Broderick J, Brott T, Kothari R. Very early edema growth with ich. *Stroke*. 1995;26:184
43. Suzuki R, Ohno K, Hiratsuka H, Inaba Y. Chronological changes in brain edema in hypertensive intracerebral hemorrhage observed by ct and xenon-enhanced ct. In: Inaba Y, Klatzo I, Spatz M eds. *Brain edema*. Berlin Heidelberg, New York, Tokyo: Springer; 1985:613-620.
44. Ropper AH, King RB. Intracranial pressure monitoring in comatose patients with cerebral hemorrhage. *Arch Neurol*. 1984;41:725-728
45. Zazulia AR, Diringner MN, Derdeyn CP, Powers WJ. Progression of mass effect after intracerebral hemorrhage. *Stroke*. 1999;30:1167-1173
46. Xi G, Hua Y, Keep RF, Younger JG, Hoff JT. Systemic complement depletion diminishes perihematomal brain edema. *Stroke*. 2001;32:162-167
47. Mendelow AD. Mechanisms of ischemic brain damage with intracerebral hemorrhage. *Stroke*. 1993;24:1115-1117; discussion 1118-1119
48. Mayer SA, Lignelli A, Fink ME, Kessler DB, Thomas CE, Swarup R, Van Heertum RL. Perilesion blood flow and edema formation in acute intracerebral hemorrhage: A spect study. *Stroke*. 1998;29:1791-1798
49. Gebel JM, Brott TG, Sila CA, Tomsick TA, Jauch E, Salisbury S, Khoury J, Miller R, Pancioli A, Duldner JE, Topol EJ, Broderick JP. Decreased perihematomal edema in thrombolysis-related intracerebral hemorrhage compared with spontaneous intracerebral hemorrhage. *Stroke*. 2000;31:596-600
50. Gebel JM, Sila CA, Sloan MA, Granger CB, Mahaffey KW, Weisenberger J, Green CL, White HD, Gore JM, Weaver WD, Califf RM, Topol EJ. Thrombolysis-related intracranial hemorrhage: A radiographic analysis of 244 cases from the gusto-1 trial with clinical correlation. Global utilization

- of streptokinase and tissue plasminogen activator for occluded coronary arteries. *Stroke*. 1998;29:563-569
51. Mun-Bryce S, Kroh FO, White J, Rosenberg GA. Brain lactate and ph dissociation in edema: 1h and 31p-nmr in collagenase-induced hemorrhage in rats. *Am J Physiol*. 1993;265:R697-702
  52. Wu J, Hua Y, Keep RF, Nakamura T, Hoff JT, Xi G. Iron and iron-handling proteins in the brain after intracerebral hemorrhage. *Stroke*. 2003;34:2964-2969
  53. Nakamura T, Keep R, Hua Y, Schallert T, Hoff J, Xi G. Deferoxamine-induced attenuation of brain edema and neurological deficits in a rat model of intracerebral hemorrhage. *Neurosurg Focus*. 2003;15: Clinical Pearl 4
  54. Betz AL, Iannotti F, Hoff JT. Brain edema: A classification based on blood-brain barrier integrity *Cereb Brain Metab Rev*. 1989;1:133-154
  55. Rosenberg GA, Navratil M. Metalloproteinase inhibition blocks edema in intracerebral hemorrhage in the rat. *Neurology*. 1997;48:921-926
  56. Masuda T, Dohrmann GJ, Kwaan HC, Erickson RK, Wollman RL. Fibrinolytic activity in experimental intracerebral hematoma. *Journal of Neurosurgery*. 1988;68:274-278
  57. Gingrich MB, Traynelis SF. Serine proteases and brain damage - is there a link?. *Trends in Neurosciences*. 2000;23:399-407
  58. Lee KR, Drury I, Vitarbo E, Hoff JT. Seizures induced by intracerebral injection of thrombin: A model of intracerebral hemorrhage. *J Neurosurg*. 1997;87:73-78
  59. Nishino A, Suzuki M, Ohtani H, Motohashi O, Umezawa K, Nagura H, Yoshimoto T. Thrombin contributes to the pathophysiology of central nervous system injury. *J Neurotrauma*. 1993;10:167-179
  60. Vaughan PJ, Pike CJ, Cotman CW, Cunningham DD. Thrombin receptor activation protects neurons and astrocytes from cell death produced by environmental insults. *Journal of Neuroscience*. 1995;15:5389-5401
  61. Striggow F, Riek M, Breder J, Henrich-Noack P, Reymann KG, Reiser G. The protease thrombin: an endogenous mediator of hippocampal neuroprotection against ischemia at low concentration but causes degeneration at high concentrations. *Proceedings of the National Academy of Sciences of the United States of America*. 2000;97:2264-2269
  62. Jiang Y, Wu J, Hua Y, Keep RF, Xiang J, Hoff JT, Xi G. Thrombin-receptor activation and thrombin-induced brain tolerance. *J Cereb Blood Flow Metab*. 2002;22:404-410
  63. Neveu I, Jehan F, Jandrot-Perrus M, Wion D, Brachet P. Enhancement of the synthesis and secretion of nerve growth factor in primary cultures of glial cells by proteases: A possible involvement of thrombin. *Journal of Neurochemistry*. 1993;60:858-867
  64. Gurwitz D, Cunningham DD. Thrombin modulates and reverses neuroblastoma neurite outgrowth. *Proceedings of the National Academy of Sciences of the United States of America*. 1988;85:344-3444
  65. Cavanaugh KP, Gurwitz D, Cunningham DD, Bradshaw RA. Reciprocal modulation of astrocyte stellation by thrombin and protease nexin-1. *Journal of Neurochemistry*. 1990;54:1735-1743
  66. Loret C, Sensenbrenner M, Labourdette G. Differential phenotypic expression induced in cultured rat astroblasts by acidic fibroblast growth factor, epidermal growth factor, and thrombin. *Journal of Biological Chemistry*. 1989;264:8319-8327
  67. Perraud F, Besnard F, Sensenbrenner M, Labourdette G. Thrombin is a potent mitogen for rat astroblasts but not for oligodendroblasts and neuroblasts in primary culture. *International Journal of Developmental Neuroscience*. 1987;5:181-188
  68. Vouret-Craviari V, Boquet P, Pouyssegur J, Van Obberghen-Schilling E. Regulation of the actin cytoskeleton by thrombin in human endothelial cells: Role of rho proteins in endothelial barrier function. *Molecular Biology of the Cell*. 1998;9:2639-2653
  69. Xi G, Keep RF, Hua Y, Xiang JM, Hoff JT. Attenuation of thrombin-induced brain edema by cerebral thrombin preconditioning. *Stroke*. 1999;30:1247-1255
  70. Donovan FM, Pike CJ, Cotman CW, Cunningham DD. Thrombin induces apoptosis in cultured neurons and astrocytes via a pathway requiring tyrosine kinase and rhoa activities. *Journal of Neuroscience*. 1997;17:5316-5326
  71. Gingrich MB, Junge CE, Lyuboslavsky P, Traynelis SF. Potentiation of nmda receptor function by the serine protease thrombin. *Journal of Neuroscience*. 2000;20:4582-4595
  72. Moller T, Hanisch UK, Ransom BR. Thrombin-induced activation of cultured rodent microglia. *Journal of Neurochemistry*. 2000;75:1539-1547

73. Dihanich M, Kaser M, Reinhard E, Cunningham D, Monard D. Prothrombin mrna is expressed b cells of the nervous system. *Neuron*. 1991;6:575-581
74. Citron BA, Smirnova IV, Arnold PM, Festoff BW. Upregulation of neurotoxic serine proteases, prothrombin, and protease-activated receptor 1 early after spinal cord injury. *J Neurotrauma*. 2000;17:1191-1203
75. Hua Y, Wu J, Keep RF, Hoff JT, Xi G. Thrombin exacerbates brain edema in focal cerebral ischemia. *Acta Neurochirurgica Supplement*. 2003;86:163-166
76. Carney DH, Cunningham DD. Role of specific cell surface receptors in thrombin-stimulated cell division. *Cell*. 1978;15:1341-1349
77. Rasmussen UB, Vouret-Craviari V, Jallat S, Schlesinger Y, Pages G, Pavirani A, Lecocq JP, Pouyssegur J, Van Obberghen-Schilling E. Cdna cloning and expression of a hamster alpha-thrombin receptor coupled to ca<sup>2+</sup> mobilization. *FEBS Letters*. 1991;288:123-128
78. Vu TK, Hung DT, Wheaton VI, Coughlin SR. Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. *Cell*. 1991;64:1057-1068
79. Dery O, Corvera CU, Steinhoff M, Bunnett NW. Proteinase-activated receptors: Novel mechanisms of signaling by serine proteases. *American Journal of Physiology*. 1998;274:C1429-1452
80. Niclou S, Suidan HS, Brown-Luedi M, Monard D. Expression of the thrombin receptor mrna in rat brain. *Cellular & Molecular Biology*. 1994;40:421-428
81. Weinstein JR, Gold SJ, Cunningham DD, Gall CM. Cellular localization of thrombin receptor mrn in rat brain: Expression by mesencephalic dopaminergic neurons and codistribution with prothrombin mrna. *Journal of Neuroscience*. 1995;15:2906-2919
82. Junge CE, Sugawara T, Mannaioni G, Alagarsamy S, Conn PJ, Brat DJ, Chan PH, Traynelis SF. The contribution of protease-activated receptor 1 to neuronal damage caused by transient focal cerebral ischemia. *Proceedings of the National Academy of Sciences of the United States of America*. 2003;100:13019-13024
83. Suidan HS, Stone SR, Hemmings BA, Monard D. Thrombin causes neurite retraction in neuronal cells through activation of cell surface receptors. *Neuron*. 1992;8:363-375
84. Debeir T, Benavides J, Vige X. Dual effects of thrombin and a 14-amino acid peptide agonist of the thrombin receptor on septal cholinergic neurons. *Brain Research*. 1996;708:159-166
85. Noorbakhsh F, Vergnolle N, Hollenberg MD, Power C. Proteinase-activated receptors in the nervous system. *Nature Reviews Neuroscience*. 2003;4:981-990
86. Vogel SM, Gao X, Mehta D, Ye RD, John TA, Andrade-Gordon P, Tiruppathi C, Malik AB. Abrogation of thrombin-induced increase in pulmonary microvascular permeability in par-1 knockout mice. *Physiological Genomics*. 2000;4:137-145
87. Nguyen M, Arkell J, Jackson CJ. Thrombin rapidly and efficiently activates gelatinase a in human microvascular endothelial cells via a mechanism independent of active mt1 matrix metalloproteinase. *Laboratory Investigation*. 1999;79:467-475
88. Lapchak PA, Chapman DF, Zivin JA. Metalloproteinase inhibition reduces thrombolytic (tissue plasminogen activator)-induced hemorrhage after thromboembolic stroke. *Stroke*. 2000;31:3034-3040
89. Barone FC, Feuerstein GZ. Inflammatory mediators and stroke: New opportunities for novel therapeutics. *Journal of Cerebral Blood Flow & Metabolism*. 1999;19:819-834
90. Thorp KM, Southern C, Bird IN, Matthews N. Tumour necrosis factor induction of elam-1 and ica-1 on human umbilical vein endothelial cells--analysis of tumour necrosis factor-receptor interactions. *Cytokine*. 1992;4:313-319
91. Del Bigio MR, Yan HJ, Buist R, Peeling J. Experimental intracerebral hemorrhage in rats. Magnetic resonance imaging and histopathological correlates. *Stroke*. 1996;27:2312-2319
92. Barone FC, Arvin B, White RF, Miller A, Webb CL, Willette RN, Lysko PG, Feuerstein GZ. Tumour necrosis factor-alpha. A mediator of focal ischemic brain injury. *Stroke*. 1997;28:1233-1244
93. Rosenberg GA, Estrada EY, Dencoff JE, Stetler-Stevenson WG. Tumor necrosis factor-alpha-induced gelatinase b causes delayed opening of the blood-brain barrier: An expanded therapeutic window. *Brain Research*. 1995;703:151-155
94. Striggow F, Riek-Burchardt M, Kiesel A, Schmidt W, Henrich-Noack P, Breder J, Krug M, Reymann KG, Reiser G. Four different types of protease-activated receptors are widely expressed in the brain and are up-regulated in hippocampus by severe ischemia. *Eur J Neurosci*. 2001;14:595-608

95. Karabiyikoglu M, Hua Y, Keep R, Ennis SR, Xi G. Intracerebral hirudin injection attenuates ischemic damage and neurologic deficits without altering local cerebral blood flow. *J Cereb Blood Flow Metab.* 2004;24:159-166
96. Choi DW. The excitotoxic concept. In: Welch KMA, Caplan LR, Reis DJ, Siesjo BK, Weir B, eds. *Primer on cerebrovascular diseases.* San Diego: Academic Press; 1997:187-190.
97. Morgan BP, Gasque P, Singhrao S, Piddlesden SJ. The role of complement in disorders of the nervous system. *Immunopharmacology.* 1997;38:43-50
98. Cole DS, Morgan BP. Beyond lysis: How complement influences cell fate. *Clinical Science.* 2003;104:455-466
99. Esser AF. Big mac attack: Complement proteins cause leaky patches. *Immunol Today.* 1991;12:316-318
100. Hansch GM, Shin MI. Complement attack phase. In: Rother K, Till GO, Hansch GM, eds. *The complement system.* Heidelberg, Berlin: Springer-Verlag; 1998:pp115-145.
101. Lucchesi BR. Complement activation, neutrophils, and oxygen radicals in reperfusion injury. *Stroke.* 1993;24:141-47
102. Vasthare US, Barone FC, Sarau HM, Rosenwasser RH, DiMartino M, Young WF, Tuma RF. Complement depletion improves neurological function in cerebral ischemia. *Brain Res Bull.* 1998;45:413-419
103. Holers VM. Phenotypes of complement knockouts. *Immunopharmacology.* 2000;49:125-131
104. Mukherjee P, Pasinetti GM. The role of complement anaphylatoxin c5a in neurodegeneration: Implications in alzheimer's disease. *J Neuroimmunol.* 2000;105:124-130
105. Nakamura T, Xi G, Hua Y, Schallert T, Hoff J, Keep R. Intracerebral hemorrhage in mice: Model characterization and application for genetically modified mice. *J Cereb Blood Flow Metab.* 2004 press
106. Johnson A, Blumenstock FA, Malik AB. Effect of complement depletion on lung fluid balance aft thrombin. *Journal of Applied Physiology: Respiratory, Environmental & Exercise Physiology.* 1983;55:1480-1485
107. Johnson A, Lo SK, Blumenstock FB, Malik AB. Cvf-induced decomplementation: Effect on lung transvascular protein flux after thrombin. *Journal of Applied Physiology.* 1987;62:863-869
108. Fareed J, Messmore HL, Fenton JW, Brinkhous KM. *Perspectively in hemostasis.* New York: Pergamon Press; 1981.
109. Bokisch VA, Muller-Eberhard HJ, Cochrane CG. Isolation of a fragment (c3a) of the third component of human complement containing anaphylatoxin and chemotactic activity and description of an anaphylatoxin inactivator of human serum. *Journal of Experimental Medicine.* 1969;129:1109-1130
110. Hugli TE. Complement factors and inflammation: Effects of thrombin on components of c3 and c In: Lundblad RL, Fenton JW, Mann KG, eds. *Chemistry and biology of thrombin.* Ann Arbor: Ann Arbor Science Publishers; 1977:345-360.
111. Lidington EA, Haskard DO, Mason JC. Induction of decay-accelerating factor by thrombin through a protease-activated receptor 1 and protein kinase c-dependent pathway protects vascular endothelial cells from complement-mediated injury. *Blood.* 2000;96:2784-2792
112. Ahmad SR, Lidington EA, Ohta R, Okada N, Robson MG, Davies KA, Leitges M, Harris CL, Haskard DO, Mason JC. Decay-accelerating factor induction by tumour necrosis factor-alpha, through a phosphatidylinositol-3 kinase and protein kinase c-dependent pathway, protects murin vascular endothelial cells against complement deposition. *Immunology.* 2003;110:258-268
113. Stoop AA, Lupu F, Pannekoek H. Colocalization of thrombin, pai-1, and vitronectin in the atherosclerotic vessel wall: A potential regulatory mechanism of thrombin activity by pai-1/vitronectin complexes. *Arteriosclerosis, Thrombosis & Vascular Biology.* 2000;20:1143-1149
114. Irving EA, Bamford M. Role of mitogen- and stress-activated kinases in ischemic injury. *Journal Cerebral Blood Flow & Metabolism.* 2002;22:631-647
115. Gong C, Hoff JT, Keep RF. Acute inflammatory reaction following experimental intracerebral hemorrhage. *Brain Res.* 2000;871
116. Xue M, Del Bigio MR. Intracerebral injection of autologous whole blood in rats: Time course of inflammation and cell death. *Neurosci Lett.* 2000;283:230-232
117. Xue M, Del Bigio MR. Intracortical hemorrhage injury in rats : Relationship between blood fractio and brain cell death. *Stroke.* 2000;31:1721-1727
118. Yang GY, Liu XH, Kadoya C, Zhao YJ, Mao Y, Davidson BL, Betz AL. Attenuation of ischemic

- inflammatory response in mouse brain using an adenoviral vector to induce overexpression of interleukin-1 receptor antagonist. *Journal of Cerebral Blood Flow & Metabolism*. 1998;18:840-84
119. Betz AL, Yang GY, Davidson BL. Attenuation of stroke size in rats using an adenoviral vector to induce overexpression of interleukin-1 receptor antagonist in brain. *Journal of Cerebral Blood Flow & Metabolism*. 1995;15:547-551
  120. Masada T, Hua Y, Xi G, Yang GY, Hoff JT, Keep RF. Attenuation of intracerebral hemorrhage a thrombin-induced brain edema by overexpression of interleukin-1 receptor antagonist. *Journal of Neurosurgery*. 2001;95:680-686
  121. Figueroa BE, Keep RF, Betz AL, Hoff JT. Plasminogen activators potentiate thrombin-induced brain injury. *Stroke*. 1998;29:1202-1207
  122. Huang F, Xi G, Keep RF, Hua Y, Nemoianu A, Hoff JT. Brain edema after experimental intracerebral hemorrhage: Role of hemoglobin degradation products. *J Neurosurg*. 2002;96:287-293
  123. Hua Y, Keep RF, Hoff JT, Xi G. Thrombin preconditioning attenuates brain edema induced by erythrocytes and iron. *Journal of Cerebral Blood Flow & Metabolism*. 2003;23:1448-1454
  124. Whisnant JP, Sayer GP, Millikan CH. Experimental intracerebral hematoma. *Arch Neurol*. 1963;9:586-592
  125. Sussman BJ, Barber JB, Goald H. Experimental intracerebral hematoma. Reduction of oxygen tension in brain and cerebrospinal fluid. *J Neurosurg*. 1974;41:177-186
  126. Takasugi S, Ueda S, Matsumoto K. Chronological changes in spontaneous intracerebral hematoma--an experimental and clinical study. *Stroke*. 1985;16:651-658
  127. Zazulia AR, Diringer MN, Videen TO, Adams RE, Tundt K, Aiyagari V, Grubb Jr RL, Powers WJ. Hypoperfusion without ischemia surrounding acute intracerebral hemorrhage. *J Cereb Blood Flo Metab*. 2001;21:804-810
  128. Hua Y, Schallert T, Keep RF, Wu J, Hoff JT, Xi G. Behavioral tests after intracerebral hemoorrhage in the rat. *Stroke*. 2002;33:2478-2484
  129. Zhang L, Schallert T, Zhang ZG, Jiang Q, Arniago P, Li Q, Lu M, Chopp M. A test for detecting long-term sensorimotor dysfunction in the mouse after focal cerebral ischemia. *Journal of Neuroscience Methods*. 2002;117:207-214
  130. Clasen RA, Huckman MS, Von Roenn KA, Pandolfi S, Laing I, Clasen JR. Time course of cereb swelling in stroke: A correlative autopsy and ct study. *Advances in Neurology*. 1980;28:395-412
  131. Blasberg RG, Fenstermacher JD, Patlak CS. Transport of alpha-aminoisobutyric acid across bra capillary and cellular membranes. *J Cereb Blood Flow Metab*. 1983;3:8-32
  132. Menzies SA, Betz AL, Hoff JT. Contributions of ions and albumin to the formation and resolution ischemic brain edema. *J Neurosurg*. 1993;78:257-266
  133. Schallert T, Fleming SM, Leasure JL, Tillerson JL, Bland ST. Cns plasticity and assessment of forelimb sensorimotor outcome in unilateral rat models of stroke, cortical ablation, parkinsonism and spinal cord injury. *Neuropharmacology*. 2000;39:777-787
  134. Schallert T, Leasure JL, Kolb B. Experience-associated structural events, subependymal cellula proliferative activity, and functional recovery after injury to the central nervous system. *Journal of Cerebral Blood Flow & Metabolism*. 2000;20:1513-1528
  135. Mulligan MS, Schmid E, Beck-Schimmer B, Till GO, Friedl HP, Brauer RB, Hugli TE, Miyasaka M, Warner RL, Johnson KJ, Ward PA. Requirement and role of c5a in acute lung inflammatory inju in rats. *J Clin Invest*. 1996;98:503-512
  136. Edens RE, Linhardt RJ, Bell CS, Weiler JM. Heparin and derivatized heparin inhibit zymosan an cobra venom factor activation of complement in serum. *Immunopharmacology*. 1994;27:145-153
  137. Black SC, Gralinski MR, Friedrichs GS, Kilgore KS, Driscoll EM, Lucchesi BR. Cardioprotective effects of heparin or n-acetylheparin in an in vivo model of myocardial ischaemic and reperfusio injury. *Cardiovascular Research*. 1995;29:629-636
  138. Park JL, Kilgore KS, Naylor KB, Booth EA, Murphy KL, Lucchesi BR. N-acetylheparin pretreatm reduces infarct size in the rabbit. *Pharmacology*. 1999;58:120-131
  139. Seiler SM, Peluso M, Michel IM, Goldenberg H, Fenton JW, 2nd, Riexinger D, Natarajan S. Inhibition of thrombin and sflr-peptide stimulation of platelet aggregation, phospholipase a2 and na+/h+ exchange by a thrombin receptor antagonist. *Biochemical Pharmacology*. 1995;49:519-528
  140. Rasmussen UB, Gachet C, Schlesinger Y, Hanau D, Ohlmann P, Van Obberghen-Schilling E, Pouyssegur J, Cazenave JP, Pavirani A. A peptide ligand of the human thrombin receptor



- antagonizes alpha-thrombin and partially activates platelets. *Journal of Biological Chemistry*. 1993;268:14322-14328
141. Ishihara H, Connolly AJ, Zeng D, Kahn ML, Zheng YW, Timmons C, Tram T, Coughlin SR. Protease-activated receptor 3 is a second thrombin receptor in humans. *Nature*. 1997;386:502-506
  142. Kahn ML, Nakanishi-Matsui M, Shapiro MJ, Ishihara H, Coughlin SR. Protease-activated receptor 1 and 4 mediate activation of human platelets by thrombin. *Journal of Clinical Investigation*. 1999;103:879-887
  143. Kahn ML, Zheng YW, Huang W, Bigornia V, Zeng D, Moff S, Farese RV, Jr., Tam C, Coughlin SR. A dual thrombin receptor system for platelet activation. *Nature*. 1998;394:690-694
  144. Covic L, Misra M, Badar J, Singh C, Kuliopulos A. Pepducin-based intervention of thrombin-receptor signaling and systemic platelet activation. *Nature Medicine*. 2002;8:1161-1165
  145. Nakanishi-Matsui M, Zheng YW, Sulciner DJ, Weiss EJ, Ludeman MJ, Coughlin SR. Par3 is a cofactor for par4 activation by thrombin. *Nature*. 2000;404:609-613
  146. Keberle H. The biochemistry of desferrioxamine and its relation to iron metabolism. *Ann NY Acad Sci*. 1974;119:758-768
  147. Sadrzadeh SM, Anderson DK, Panter SS, Hallaway PE, Eaton JW. Hemoglobin potentiates central nervous system damage. *J Clin Invest*. 1987;79:662-664
  148. Regan RF, Panter SS. Neurotoxicity of hemoglobin in cortical cell culture. *Neurosci Lett*. 1993;153:219-222
  149. del Zoppo GJ. In stroke, complement will get you nowhere. *Nature Medicine*. 1999;5:995-996
  150. Schur PH. Complement studies of sera and other biologic fluids. *Human Pathology*. 1983;14:333-342
  151. Ninomia TN, Wang L, Kumar SR, Kim A, Zlokovic BV. Brain injury and cerebrovascular fibrin deposition correlate with reduced antithrombotic brain capillary functions in a hypertension stroke model. *J Cereb Blood Flow Metab*. 2000;20:998-1009
  152. Hua Y, Xi G, Keep RF, Wu J, Jiang Y, Hoff JT. Plasminogen activator inhibitor-1 induction after experimental intracerebral hemorrhage. *J Cereb Blood Flow Metab*. 2002;22:55-61
  153. Schallert T, Upchurch M, Wilcox RE, Vaughn DM. Posture-independent sensorimotor analysis of inter-hemispheric receptor asymmetries in neostriatum. *Pharmacology, Biochemistry & Behavior*. 1983;18:753-759
  154. Andersen H, Greenberg DL, Fujikawa K, Xu W, Chung DW, Davie EW. Protease-activated receptor 1 is the primary mediator of thrombin-stimulated platelet procoagulant activity. *Proceedings of the National Academy of Sciences of the United States of America*. 1999;96:11189-11193
  155. Mulligan MS, Schmid E, Till GO, Hugli TE, Friedl HP, Roth RA, Ward PA. C5a-dependent up-regulation in vivo of lung vascular p-selectin. *J Immunol*. 1997;158:1857-1861
  156. Ballou M, Cochrane CG. Two anticomplementary factors in cobra venom: Hemolysis of guinea erythrocytes by one of them. *Journal of Immunology*. 1969;103:944-952

## H. CONSORTIUM/CONTRACTUAL ARRANGEMENTS

None.

## I. CONSULTANTS

None.