Improvement in recovery after experimental intracerebral hemorrhage using a selective cathepsin B and L inhibitor

Laboratory investigation

DONGMEI YANG, M.D.,1 YUXIA HAN, B.S.,1 JIANFENG ZHANG, M.S.,1 CHRISTOPHER DING,1 JOHN ANAGLI, PH.D.,2,3 AND DONALD M. SEYFRIED, M.D.1

Departments of 1Neurosurgery and 2Pathology, and 3Proteomics Core Facility, Henry Ford Hospital, Detroit, Michigan

Object. This study investigates a potential novel application of a selective cathepsin B and L inhibitor in experimental intracerebral hemorrhage (ICH) in rats.

Methods. Forty adult male Wistar rats received an ICH by stereotactic injection of 100 μl of autologous blood or sham via needle insertion into the right striatum. The rats were treated with a selective cathepsin B and L inhibitor (CP-1) or 1% dimethyl sulfoxide sterile saline intravenously at 2 and 4 hours after injury. Modified neurological severity scores were obtained and corner turn tests were performed at 1, 4, 7, and 14 days after ICH. The rats were sacrificed at 3 and 14 days after ICH for immunohistological analysis of tissue loss, neurogenesis, angiogenesis, and apoptosis.

Results. The animals treated with CP-1 demonstrated significantly reduced apoptosis as well as tissue loss compared with controls (p < 0.05 for each). Neurological function as assessed by modified neurological severity score and corner turn tests showed improvement after CP-1 treatment at 7 and 14 days (p < 0.05). Angiogenesis and neurogenesis parameters demonstrated improvement after CP-1 treatment compared with controls (p < 0.05) at 14 days.

Conclusions. This study is the first report of treatment of ICH with a selective cathepsin B and L inhibitor. Cathepsin B and L inhibition has been shown to be beneficial after cerebral ischemia, likely because of its upstream regulation of the other prominent cysteine proteases, calpains, and caspases. While ICH may not induce a major component of ischemia, the cellular stress in the border zone may activate these proteolytic pathways. The observation that cathepsin B and L blockade is efficacious in this model is provocative for further investigation.

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Key Words • intracerebral hemorrhage • cysteine protease inhibitor • cathepsin B • cathepsin L

S PONTANEOUS ICH represents 10–15% of all strokes, and it leads to a much worse prognosis than injury caused by ischemic stroke.9 The size of the hematoma formed in the brain parenchyma from the rupture of a cerebral vessel is a significant predictor of poor patient outcome.40 Inflammation and toxicity triggered by extravascular blood products increase the cerebral damage observed in the early phases after hematoma formation and also lead to worsening edema around the clot.41 Effective surgical and drug approaches for clinical practice have been elusive.9,10,14

Cathepsins B and L are intracellular/lysosomal and secreted cysteine proteases that have been implicated in the process of neuronal cell death after experimental global and focal cerebral ischemia and after experimental spinal cord injury.1,6,11,16,17,33,38 The levels of these cathepsins are significantly increased, and they relocate from the lysosomes to the cytoplasm following global cerebral ischemia. Cathepsin B is one of the causative factors of microglia-induced neuronal apoptosis.32,33 In our previous study, CP-1, a nontoxic cysteine protease inhibitor that is selective for cathepsins B and L, but not the calpains or caspases, was shown to be effective at reducing infarct volume and improving functional scores when administered intravenously to rats after 2 hours of middle cerebral artery occlusion and reperfusion.75

The role of cathepsins in ICH has not been studied. However, results from models of cerebral ischemia suggest that inhibition of cathepsin B and L may be efficacious in preventing neuronal cell death. In this study, we investigated the ability of CP-1 to promote functional and histological recovery after ICH.
Neurological recovery by a cysteine protease inhibitor after ICH

Methods

Animal Surgical Procedures

All studies were approved by the Institutional Animal Care and Use Committee. Forty male Wistar rats (Charles River) weighing between 270 and 320 grams were selected for this study. An ICH lesion was induced by injecting 100 µl of nonheparinized autologous blood, which was obtained from the femoral vein and placed into a 1-ml syringe with a 26 1/2-gauge needle, into the right striatum (3.5 mm lateral to midline, 0.5 mm anterior to bregma, at a depth 5.5 mm below the surface to midline) at a steady infusion rate of 10 µl per minute. Sham-injured animals were subjected to the same manipulations as ICH rats, but no blood was injected.

Cysteine Protease Inhibitor

Carbobenzoxy-phenylalanine-serine(O-benzyl)-di-azomethyl ketone (Cbz-Phe-Ser(OBzl)-CHN₂; carbobenzoxy phenylalanine serine O-benzyl carboxyl terminus), is lipophilic and its molecular mass is 500.5. This inhibitor was chosen based on its relative selectivity for cathepsins B and L. The compound was synthesized and purified according to the method of Shaw et al.36 After synthesis, CP-1 was chromatographed on silica gel with chloroform/methanol (49:1, v/v) and recrystallized from boiling ethyl acetate (melting point 125–126°C). The purity of the compound was checked by high-performance liquid chromatography, thin-layer chromatography, elemental analysis, infrared spectroscopy, proton nuclear MR, and mass spectrometry.4 The effectiveness of CP-1 in vitro was determined by protease inhibition kinetic measurements on purified cathepsin B, cathepsin L, calpain 1, and calpain 2, as described previously.2,3,8

The dosing of CP-1 after experimental ICH was based on in vivo dose-response studies that we had previously performed in Wistar rats subjected to focal cerebral ischemia.3,35 Cysteine protease inhibitor-1 at 0.35 mg/kg significantly reduced cathepsin B activity in the brain in vivo.1 In the current study, CP-1 was initially dissolved in 100% DMSO, and was blended into a serial dilution of 0.1 mM in 1% DMSO in sterile saline. Cysteine protease inhibitor-1 was given intravenously (0.175 mg/kg) at 2 and 4 hours after surgery (total dosage 0.35 mg/kg), while control animals received a same volume infusion of 1% DMSO in sterile saline.

Experimental Groups

This study was randomly divided into 4 groups: 1) CP-1 group, in which 16 rats subjected to ICH were treated with the selective cathepsin B and L inhibitor; 2) control group, in which 16 rats subjected to ICH were treated with 1% DMSO in sterile saline; 3) sham-CP-1 group, in which 4 sham rats were treated with CP-1; and 4) sham-control group, in which 4 sham rats were treated with sterile saline. Six rats in the first group and 6 rats in the second group were killed at Day 3 for TUNEL staining. The rest of the animals received daily injections of BrdU 100 mg/kg (Sigma) intraperitoneally starting at 24 hours after injury and subsequently for 13 consecutive days. These rats were evaluated for neurological function as described below. At 14 days after ICH or the sham procedure, the rats from Groups 1–4 were killed for immunohistological analysis.

Neurological Functional Studies

Functional outcomes were measured using both the mNSS12 and the corner turn test43 by an observer blinded to the individual treatment status of the animals. Functional outcomes for each rat were tested at Days 1, 7, and 14 after ICH. The mNSS is used to assess motor, sensory, balance, and reflex skills, with higher scores implying greater neurological injury, while the corner turn test measures the number of times that an animal turns to the right or left when placed in a corner.

Histology and Immunohistochemistry

At 14 days postoperatively, the animals were anesthetized with an intraperitoneal injection of ketamine and xylazine and subsequently perfused transcardially with phosphate-buffered saline followed by 4% paraformaldehyde. Brain tissues were excised, fixed in formalin, and sliced into a series of 2-mm-thick blocks. Each block was processed and embedded in paraffin. A series of adjacent 6-µm-thick coronal sections were cut with a microtome from each block. A total of 7 sections from each animal were processed and stained with H & E. These sections were tracked using an image analysis system (Data Translation). The intact striatal area of the ipsilateral hemisphere was subtracted from the striatal area of the contralateral hemisphere, and the tissue loss volume was presented as a percentage of the lesion compared with the contralateral striatum.

The brain tissue residing between +0.1 mm and −0.86 mm of the bregma on the third block was the most severely injured, and therefore every 40th coronal section from the third block (for a total of 6 sections) was specifically selected for immunohistochemical staining with the same antibody. Immunohistochemical staining was used for BrdU (1:100, Boehringer Mannheim), TUJ1 (1:5,000, BABC0), synaptophysin (1:1000, Millipore), and vWF (1:400, Dako), as previously described.13,27 All immunostaining was performed at the same time with the omission of primary antibody and the use of normal non-immunized serum (Santa Cruz Biotechnology) for quality control of the immunoassay procedure. To identify cellular apoptosis, TUNEL was performed using an ApopTag peroxidase in situ apoptosis detection kit (Millipore).

Quantification

For semiquantitative measurements, all slides were digitized under a 20-magnification objective lens (Olympus BX40, Olympus Optical Co.) by using a 3D-CCD color video camera (model DXC-970MD, Sony Corp.) interfaced with an image analysis system (Imaging Research, Inc.). The number of BrdU-positive cells and the percentage of TUJ1-positive signals were counted in the subventricular zone; the percentage of synaptophysin-
positive signals compared with the corresponding contralateral side and the numbers of apoptosis cells were calculated in the subcortical striatum; the peripherals of vWF positive vessels were measured in the perihemorrhagic striatum. The cells with BrdU (brown stained) that colocalized to the nucleus (hematoxylin stained) were counted as BrdU-positive cells, and the cells stained with TUNEL that showed dark brown, rounded, or oval apoptotic bodies were counted as TUNEL-positive cells.

Statistical Analysis
Statistical analysis of neurological functional scores, areas of ICH-related tissue losses, and immunohistochemical results were all performed or obtained using the independent Student t-test. Data were presented as the mean ± SEM, and probability values < 0.05 were considered significant. All measurements were performed by observers blinded to individual treatments.

Results

Neurobehavioral Tests
The results for the cornering and mNSS tests are presented in Fig. 1. All ICH rats displayed similar and marked neurological impairments at Day 1 after injury compared with the sham-operated groups. Before the first week after ICH there were still no significant differences observed between the control group and CP-1 group. At 1 and 2 weeks after ictus, cornering scores demonstrated that the CP-1 group improved significantly compared with the control group. Similarly, improvement of neurological function assessed using mNSS was significant at 1 and 2 weeks after ICH compared with the control group; however, no significant differences were observed in mNSS and corner turn test results between the sham-control group and the sham CP-1 group during the 2 weeks of experiments.

Tissue Loss Measurements
Using this model of ICH, we have demonstrated consistent results measured by MR imaging and immunohistochemical methods. After breakdown or resorption of the hematoma, there is an area of tissue loss in this model.24,34 The areas of striatal tissue loss as a percentage of the normal contralateral side obtained from H & E histology were as follows: sham control group, 1.56 ± 0.85%; sham CP-1 group, 1.48 ± 1.09%; control group, 30.67 ± 2.46%; and CP-1 group, 23.22 ± 1.61% (Fig. 2). Statistically, the CP-1 group showed significantly reduced tissue loss compared with the control group at 2 weeks after ICH.

Cell Proliferation and Differentiation
Endogenous cell proliferation and differentiation are believed to contribute to neurological functional recovery after ICH. To test if post-ICH treatment with CP-1 may have an effect on this process, BrdU and TUJ1 immunostainings were performed. The ipsilateral subventricular zone demonstrated a significant increase in the number of newly formed cells in the CP-1-treated groups at 2 weeks after ICH, compared with the control group (p < 0.05; Fig. 3). The CP-1 treatment also significantly enhanced the TUJ1 expression compared with the control group. A small number of BrdU-positive cells were colabeled with TUJ1, suggesting that only limited, newly formed cells represent neurogenesis. In each hemispheric section of the sham-operated rats and in the contralateral hemisphere of ICH rats, there were limited positive signals for BrdU and TUJ1 stainings, and no significant difference between the 2 sham groups.

Angiogenesis
Angiogenesis is described as the sprouting of new blood vessels from preexisting vessels. Cerebral vascular parameters (vWF) were significantly increased in the CP-1 treatment group compared with the control group (p < 0.05; Fig. 4). Double immunostaining revealed a subpopulation of cells that express a vascular marker while still proliferating, suggesting that this subpopulation of cells positive for vascular phenotype is newly formed during the recovery stage.

Aptoptosis
The TUNEL staining showed apoptotic cells with typical dark brown, rounded, or oval apoptotic bodies (Fig. 2). Scattered apoptotic cells were present throughout the damaged tissue, the vast majority of which were located along the boundary zone of the hematoma. The number of apoptotic cells was significantly reduced in the ipsilateral hemisphere in the CP-1 treatment groups compared with the control group (p < 0.05).

Discussion
Accumulating evidence has suggested a prominent role for cathepsins B and L in the pathogenesis of brain ischemia.1,12,25,30,35 Cathepsins B and L have been shown to be upregulated in microvessels and adjacent glial cells or neurons after brain ischemia.19 Broad specificity cysteine protease inhibitors and selective cathepsin B and L inhibitors have been shown to promote neurological recovery following stroke.18,38 While ICH may not have a major component of ischemia, the cellular stress along the border zone may activate these proteolytic pathways. The findings from our current study suggest that early treatment with CP-1 after ICH improves neurological function and reduces tissue loss, supporting the hypothesis that cathepsins B and L play an important role in ICH.
Neurological recovery by a cysteine protease inhibitor after ICH

The underlying damaging effects of cathepsins following brain injury are partially associated with apoptosis and necrotic cell death. Microglial-secreted cathepsin B induces neuronal apoptosis and activates the proinflammatory caspases 11 and 1. Furthermore, cathepsin B can cleave the Bcl-2 family member Bid, which may lead to cytochrome c release from the mitochondria and subsequent caspase activation. Cathepsin L is also implicated in apoptosis. In line with these findings, the selective inhibitor for cathepsins B and L reduced the number of apoptotic cells at 2 weeks after ICH, which may contribute to neurological recovery. Regarding necrotic cell death, we described previously that cathepsin B and L inhibition reduces loss of tissue after cerebral ischemia; one of the postulated mechanisms of this effect was reduction of the lysosome-mediated cell destruction. In the cerebral hemorrhage model 10%–20% of cell death is due to necrosis from direct injury from the clot. This suggests that CP-1 may improve post-ICH recovery by interfering with the cathepsin B- and L-induced apoptosis as well as the necrotic cell death pathway.

Neurogenesis has been demonstrated in animal models and the adult human brain after ICH, and this may contribute to brain repair and functional recovery, either by cell replacement or by indirect mechanisms such as cytoprotective or trophic factors. Inflammatory processes, which also play a vital role in secondary brain injury following the initial hematoma after ICH, may block neurogenesis. Cathepsins represent one of the important factors involved in necrosis and the inflammatory cascade, and inhibition of cathepsins B and L by CP-1 in the current study enhanced endogenous neurogenesis after ICH. Therefore, we postulate that CP-1 may facilitate endogenous neurogenesis because it interferes with the inflammatory enzymatic cascades. Cystatin C, a potent endogenous inhibitor of lysosomal proteinases including cathepsins B and L, has also been shown to stimulate self-renewing, multipotent neural stromal cells in vitro and neurogenesis in vivo. Cysteine protease inhibitor-1 may be functioning with a similar mechanism as cystatin C in regards to neurogenesis.

Neurological functional recovery has been attributed to synaptic reorganization after stroke and brain trauma. Synaptophysin expression is an indicator of synaptogenesis. In our study, synaptophysin density is significantly increased in the boundary zone around the
hematoma region 2 weeks after CP-1 treatment, which suggests that CP-1 treatment may either directly protect the synapses from damage or enhance new synaptic formation.

An interesting finding is that CP-1 treatment at the acute period promotes endogenous angiogenesis following ICH. This may relate to the neuroprotective effect of CP-1, whereby cell survival and recovery are promoted by a cathepsin B and L inhibitor during the first day after injury. Cathepsin cysteine proteases, however, have been reported to be effectors of invasive growth and angiogenesis during multistage tumorigenesis. Moreover, deficiency of these cysteine proteases can impair microvessel growth. The main effect of cathepsins is to penetrate the extracellular matrix to form vessel sprouts. After middle cerebral artery occlusion, new capillary buds start to appear in the ischemic bed 3 days postinjury, and angiogenesis is induced after 7 days of focal cerebral ischemia. Following acute treatment of ICH in our study, CP-1 is expected to limit the initial cathepsin surge after injury.

However, cathepsin activity is restored subacutely by the creation of new enzymes, which are thereby available to promote neurological recovery with angiogenesis at the later time points. Whether CP-1 has a direct effect on angiogenesis after ICH cannot be determined by the current study.

Conclusions

This is the first study demonstrating that post-ICH administration of a cysteine protease inhibitor selective for cathepsins B and L significantly reduces tissue loss and improves neurological function in the rat. One likely mechanism for this reduction is neuroprotection, as demonstrated by decreased neuronal apoptosis following CP-1 treatment, substantiating the involvement of cathepsins B and/or L in neuronal cell death after ICH. This suggests that the selective inhibitor improves cellular survival along the ICH border independently from direct effects on the calpain or caspase enzyme system. In addition to
the observed neuroprotective effect of CP-1, the ICH region also shows posttreatment evidence of neuronal recovery with neuroplasticity and angiogenesis.

**Disclosure**

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