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Hemin-induced Apoptosis in PC12 and Neuroblastoma Cells: Implications for Local Neuronal Death Associated with Intracerebral Hemorrhage

YOSSEF S. LEVY, JONATHAN Y. STREIFLER, HANNA PANET, ELDAD MELAMED and DANIEL OFFEN*

Department of Neurology, Rabin Medical Center and the Laboratory for Neurosciences, Felsenstein Medical Research Center, Petach Tikva, The Sackler School of Medicine, Tel Aviv University, Tel Aviv, Israel

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The exact pathogenesis of neuronal death following bleeding in brain parenchyma is still unknown. Hemoglobin (Hb) toxicity has been postulated to be one of the underlying mechanisms. The purpose of this study was to examine the possible contribution to neurotoxicity of each of the Hb compounds and to characterize the death pathway. Pheochromocytoma (PC12) and neuroblastoma (SH-SY5Y) cell lines were exposed to Hb, globin, hemin, protoporphyrin IX, and iron for 1.5–24 h. We found that Hb and hemin are highly toxic (LD_{50} of 8 and 20 μmol/l, respectively) as compared to globin that was not toxic. In addition, protoporphyrin IX and iron, compounds of hemin, were less toxic than hemin itself (LD_{50} of 962 and 2070 μmol/l, respectively). We also demonstrated that non-specific protein digestion with proteinase-K, markedly increased Hb toxicity. Hemin-treated cells caused a typical apoptotic cell-death pattern as indicated by DNA fragmentation, caspase activation and reduction in the mitochondrial membrane potential. Treatment with the antioxidant *N*-acetyl-L-cysteine or iron chelator, deferoxamine, diminished hemin-induced cell death, indicating a role of oxidative stress in this deleterious process. Thus, therapeutic strategies, based on antioxidant, iron chelator and anti-apoptotic agents may be effective in counteracting Hb neurotoxicity.

Keywords: Apoptosis; Heme; Hemoglobin; Intracerebral hemorrhage

INTRODUCTION

In normal conditions, brain tissue is not exposed to blood products. However, in several pathological situations such as head trauma, intracerebral hemorrhage (ICH) and hemorrhagic infarctions, there may be interaction between neurons and blood constituents. Following ICH there is lysis of the extravasated red blood cells resulting in the release of blood breakdown products into the extravascular space. The pathological picture is characterized by the presence of edema and infiltration of macrophages and neutrophils intermingled with normal neuronal tissue. It was generally believed that most of the tissue injury results from primary local compression and secondary ischemia. However, recent studies have indicated the role of additional mediators such a blood and plasma products that mediate most secondary processes initiated after an ICH (Qureshi *et al.*, 2001). Several studies in animal models and in patients have demonstrated that brain tissue exposure to blood causes peroxidation of polyunsaturated fatty acids, generation of prostaglandins and leukotrienes and an accumulation of polymorphonuclear neutrophils (PMN) (Means and Anderson, 1983). Moreover, the inflammatory response is associated with the release of cytokines such as tumor necrosis factor, interleukin 6, interferon-gamma or inducible nitric oxide synthase that enhance the immune system, but might also potentiate neuronal damage (Munoz-Fernandez and Fresno, 1998). In an experimental model of subarachonid hemorrhage (SAH) in mice, injection of autologous hemolysate into the cerebrospinal

*Corresponding author. Address: Felsenstein Medical Research Center, Rabin Medical Center, Petach Tikva 49100, Israel. Tel.: +972-3-9376130. Fax: +972-3-9211478. E-mail: doffen@post.tau.ac.il

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fluid (CSF), induces expression of stress proteins and mediates apoptotic cell-death, as indicated by DNA fragmentation (Turner *et al.*, 1999). Similarly, induction of ICH by stereotactic infusion of collagenase into the rat striatum causes apoptosis of neurons and astrocytes (Matsushita *et al.*, 2000).

The contribution of the various blood compounds to the induction of cell death has not yet been verified. Hemoglobin (Hb), the main constituent of red blood cells, is composed of four polypeptide chains, which are held together by noncovalent attractions. The capacity of Hb to bind oxygen depends on the presence of a nonpolypeptide unit, heme, which consists of an organic part (protoporphyrin IX) and an iron atom. Extracellular heme, derived from Hb following hemorrhage or released from dying cells, induced the expression of heme oxygenase that metabolizes heme to gaseous carbon monoxide (CO), iron, and biliverdine, which is rapidly converted to bilirubin (Stryer, 1987). The clearance of the pooled extravascular edema and blood including Hb or its breakdown products from brain parenchyma might take hours, days or even weeks and thus the surrounding neurons are exposed to Hb breakdown products for an extended period of time (Barrows *et al.*, 1955; Tortelotte *et al.* 1964; Findlay *et al.*, 1989; Yang *et al.*,1994; Zazulia *et al.*, 1999). As Hb is one of the major components of blood, it is suggested to play a major role in this neuronal damage following ICH (Reagan and Panter, 1993). In the present study, we examined the potential toxic effects of the various Hb metabolites on neuronal cell lines.

MATERIALS AND METHODS

Cell Culture

Pheochromocytoma (PC12) cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 4.5 g/l D-glucose, supplemented with 8% fetal calf serum (FCS) and 8% horse serum (HS), penicillin (100 units/ml), streptomycin (100 μg/ml), nystatin (12.5 units/ml) and 2 mmol/l L-glutamine (Biological Industries, Beit Haemek, Israel). Confluent cultures were washed with phosphate-buffered saline (PBS), pH 7.0, detached with 0.5 mmol/l EDTA (Biological Industries, Beit Haemek, Israel), centrifuged and sub-cultured (in 1% FCS and 1% HS) on 96-well microtiter plates (Corning, Corning, NY, USA), 100 μl of 3×10^5 cells/ml in each well.

Neuroblastoma SH-SY5Y (NB) maintained in DMEM, supplemented with 10% FCS, penicillin (100 units/ml), streptomycin (100 μg/ml), nystatin (12.5 units/ml) and 2 mmol/l L-glutamine. Confluent cultures were washed with trypsin–EDTA solution (0.25% trypsin and EDTA 1:2000 in puck's saline) (Biological Industries, Beit Haemek, Israel), centrifuged and sub-cultured (in 2% FCS) on 96-well microtiter plates, 100 μl of 3×10^5 cells/ml in each well.

Cell viability was evaluated by Alamar blue (Wildflower International Inc., Santa Fe, NM, USA), a fluorometric/colourimetric growth indicator of the oxidation–reduction cellular state, which is incorporated into the cells and causes fluorescence changes in response to a reduced environment. Reduction related to growth causes the indicator to change from oxidized form (non-fluorescent, blue) to reduced form (fluorescent, red). 3×10^4 cells per well were seeded in 96-well multidishes in 100 μl culture medium, and incubated for 18–24 h with Hb or Hb compounds, an antioxidant or an iron chelator. After appropriate treatment, 10 μl of Alamar blue were added to each well and plates were incubated for 4 h at 37°C. Fluorescence intensity was measured using a FLUOstar (BMG Labtechnologies, Offenburg, Germany) with excitation at 544 nm and emission at 590 nm.

Western Blot

PC12 cells (2.5×10^6) were incubated with 100 μmol/l hemin (Sigma, St. Louis, MO, USA) for 1.5, 3, 6 or 24 h in 60-mm growth surface diameter tissue culture dish (Corning). After appropriate treatment, PC12 cells (10^6 each sample) were washed with PBS, scraped and pelleted by centrifugation at 1000*g* for 10 min. The pellet was resuspended in 50 μl of cold buffer containing 105 mmol/l Tris (Sigma), 5 mmol/l EDTA (BDH Laboratory Supplies, Poole, England), 140 mmol/l NaCl (BioLab, Jerusalem, Israel), 10 mmol/l NaF (Sigma), 0.5% NP-40 (United States Biochemical Corporation, Cleveland, OH, USA), 1 μmol/l PMSF (Sigma). Cells were then incubated on ice for 30 min. During this period, cells were vortexed for 10 s at high speed every 5 min. The mixture was centrifuged at 13,000*g* for 20 min. Protein content was determined with the BCA protein assay (Pierce, Rockford, IL, USA). The protein was denatured in sample buffer (62.5 mmol/l Tris–HCl, pH 6.8, 10% Glycerol, 2% sodium dodecyl sulfate, 5% 2-β-mercaptoethanol, 0.0025% bromophenol blue) diluted 1:5 with the sample and boiled for 5 min. Each sample (100 μg protein) was loaded onto 10% SDS-polyacrylamide gel (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. After electrophoresis, the protein was transferred to polyvinylidene difluride membrane (Bio-Rad Laboratories), followed by blocking with 5% nonfat milk in Tris-buffered saline with Tween 20 (TBS-T) (Sigma) (10 mmol/l Tris, pH 7.5, 150 mmol/l NaCl, 0.05% Teen 20). Blots were probed at 4°C overnight with the rabbit anti-cleaved caspase-9 or anti-PARP

(poly ADP-ribose polymerase) polyclonal antibodies (Cell Signaling Technology, Beverly, MA, USA), diluted at 1:2000 in 0.375% nonfat milk in TBS-T. Equal loadings of proteins were probed with mouse monoclonal antibody to actin (Chemicon, Temecula, CA, USA) diluted at 1:500 in 0.375% nonfat milk in TBS-T. The next day, membranes were washed with TBS-T three times (5 min each) and then exposed to horseradish-peroxidase conjugated goat anti-rabbit IgG, or anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) diluted 1:10,000 in TBS-T, for 1 h at room temperature and washed with TBS-T three times (5 min each). Proteins of interest were detected using the enhanced Super-Signal® chemiluminescent Western blotting detection system kit (Pierce). Blots were incubated with 0.125 ml/cm^2 of blots surface in working solution for 5 min, and exposed to medical X-ray film (Fuji Photo Film, Tokyo, Japan) at room temperature in a darkroom.

Mitochondrial Transmembrane Potential

PC12 cells (2.5×10^6) were incubated with 150 μmol/l hemin in 60-mm growth surface diameter tissue culture dish (Corning). After appropriate treatment, PC12 cells (10^6 each sample) were washed with PBS, scraped and pelleted by centrifugation at 500*g* for 5 min. The pellet was resuspended in 1 ml pre-warmed incubation buffer with 7.7 μmol/l of J-aggregate-forming lipophilic cation fluorochrome 5,5′,6,6′-tetrachloro-1,1′,3,3-tetraethyl- benzimidazolcarbocyanine iodide (JC-1) (MitoCapture™ mitochondrial apoptosis detection kit, BioVision, Palo Alto, CA, USA). The mixture was incubated at 37°C in 5% CO_2 incubator for 20 min. Cells were centrifuged at 500*g* and the supernatant discarded. The pellet was resuspended in 1 ml pre-warmed incubation buffer. Cells were analyzed by flow cytometry immediately after the last step. Analysis was performed on the FACSCalibur™ flow cytometer (Becton Dickinson Immunocytometry System, San Jose, CA, USA), using an argon ion laser, adjusted to an excitation wavelength of 488 nm. CELLQuest™ version 3.0 software (Becton Dickinson Immunocytometry System) was used to acquire and analyze data from flow cytometer. Fluorescence signals were analyzed using FITC channel for green monomers (Ex/Em = 488/530 ± 30 nm) and PI channel for red aggregates (Ex/Em = 488/590 ± 42 nm) and stored in listmode data file. Each measurement contained 10^4 cells and flow rate was 60 ± 3 μl/min.

DNA Content Measurement

Neuroblastoma cells (2.5×10^6) were incubated with 150 μmol/l hemin in 60 mm growth surface diameter tissue culture dish (Corning). After appropriate treatment, neuroblastoma cells (10^6 each sample) were washed with DMEM, scraped and pelleted by centrifugation at 500*g* for 5 min. Hemin-induced nuclear changes were evaluated by flow cytometry analysis of propidum-iodide (PI) stained cell nuclei. Nuclear purification and staining were performed according to Vindelov's method (Vindelov *et al.*, 1983). Analysis was performed on FACSCalibur™ flow cytometer using an argon ion laser adjusted to an excitation wavelength of 488 nm. CELLQuest™ version 3.0 software was used to acquire data from the flow cytometer. ModFit LT™ cell-cycle analysis software (Verity Software House, Topsham, ME, USA) was used to analyze data. Each measurement contained 10^4 cells and flow rate was 12 ± 3 μl/min. PI was detected in the orange range of the spectrum using a 562–588 nm band pass filter. Evaluation of subdiploid apoptotic peak was on FL2-Area scale (reflecting emergence of nuclear particles with reduced DNA content).

Materials

Human Hb (Sigma) was solubilized in 100 mmol/l phosphate buffer at pH of about 7.2. Protoporphyrin IX or hemin (Sigma) was dissolved in 0.2 ml of 1N KOH; 1 ml of 0.2 mol/l Tris–Hcl, pH 7.8, was added and the volume was brought to 4.0 ml by the addition of 2.6 ml ddH_2O. pH was adjusted to 7.8 by adding 0.1–0.2 ml 1N HCl. Stock solution was 1 mmol/l. Human globin, purified by chromatography with carboxymethyl cellulose column according to the technique of Clegg (Clegg *et al.*,1966). Iron solutions were freshly prepared from ferrous sulfate (Sigma) and used immediately. The *N*-acetyl-L-cysteine (NAC) was obtained from Sigma, deferoxamine methasulfonic from Ciba-Geigy (Basle, Switzerland), and Proteinase-K from Promega. All solutions were sterilized by cellulose acetate (low protein binding) filter.

Statistical Analysis

All experiments of cell viability were repeated six times. Results are presented as mean ± SEM; *p* values were calculated using two-tail unpaired Student's *t*-test. In all tests, significance was assigned when $p < 0.05$.

RESULTS

Following the incubation of PC12 cells for 24 h with increasing concentrations (1–100 μmol/l) of Hb, a significant dose-dependent cell death was observed with LD_{50} of 8 μmol/l, as indicated by the Alamar blue assay (Fig. 1a).

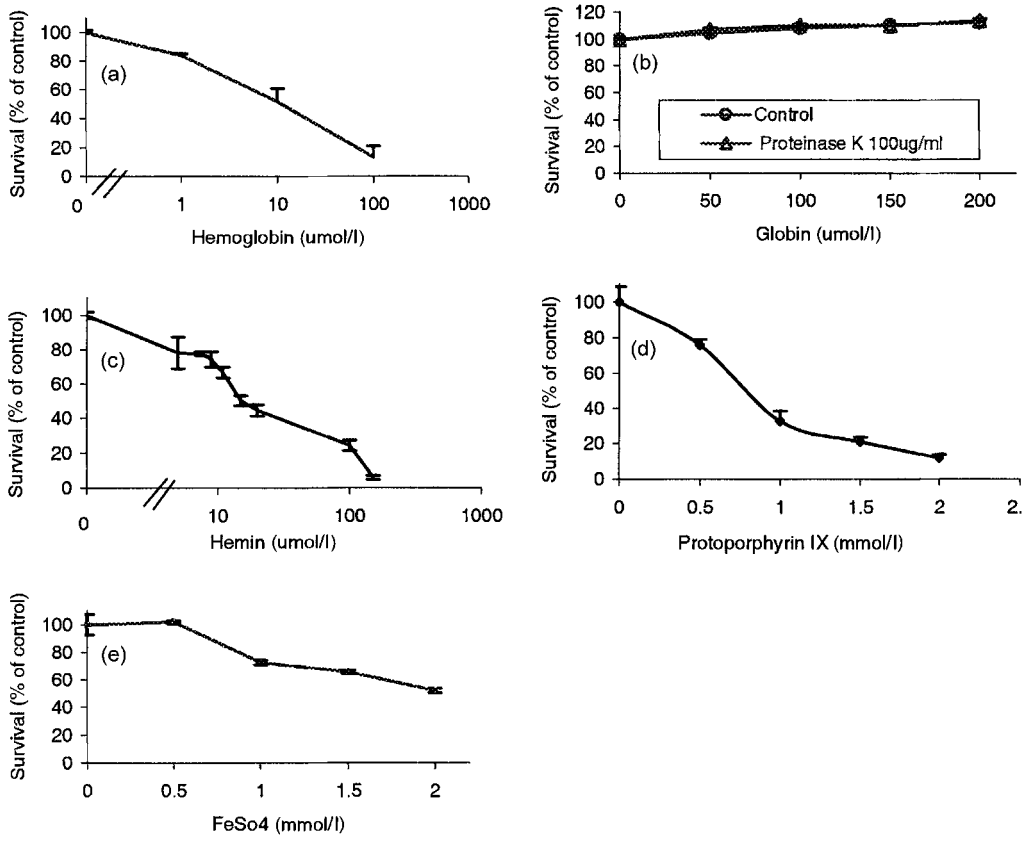

FIGURE 1 *Concentration dependence of hemoglobin compounds neurotoxicity.* PC12 cells were exposed for 24 h to various concentrations of Hb (a), globin (b), hemin (c), protoporphyrin IX (d) and ferrous sulfate (e). Cell survival was measured by Alamar blue assay (mean ± S.E.M, $n = 6$), compared to cells without treatment (control).

To examine whether hemin or globin, the two major components of Hb, are toxic, PC12 cells were incubated for 24 h with increasing concentrations of hemin or pure globin protein. We found that exposure of the cells for 24 h to 50–200 μmol/l of globin was not toxic (Fig. 1b), while hemin was highly toxic with LD_{50} of 20 μmol/l, as indicated by the Alamar blue assay (Fig. 1c).

Hemin-derived compounds, protoporphyrin IX (organic ring of heme without iron) and ferrous sulfate, were both shown to be toxic to PC12 cells, but only at relatively high concentrations with LD_{50} of 962 and 2070 μmol/l, respectively, i.e. 100 times higher than those of hemin or Hb alone (Fig. 1d, e).

As hemin was found to be the toxic compound of Hb, we studied the possible contribution of Hb protein structure to neurotoxicity. We therefore cleaved Hb with proteinase-K, a non-specific protease (100 μg/ml). Electrophoresis analysis of cleaved Hb on 12.5% SDS-polyacrylamide gel showed three bands: 16 (single chains), 32 (two alpha chains or two beta chains) and 64 kDa (complex of Hb) (Fig. 2a). Proteinase-K treatment cleaved most of Hb and revealed mostly single subunit of 16 kDa and a smear of degraded proteins (Fig. 2a). PC12 cells were then exposed for 24 h to 50 μmol/l Hb, which was preincubated with increasing amounts of proteinase-K (10–50 μg/ml). Digestion significantly increased Hb toxicity (Fig. 2b). In contrast, digested globin (with 100 μg/ml proteinase-K) or the addition of proteinase-K alone without Hb, using the same concentrations, was not toxic to cells (data not shown). Thus, our findings demonstrate that partial degradation of the protein component of Hb potentiates its toxicity.

To examine the hypothesis that hemin neurotoxicity is mediated by oxidation of cellular components, PC12 cells were exposed to hemin for 24 h in the presence of an iron chelator or an antioxidant. Treatment with deferoxamine (1.5 mmol/l), a ferric-iron chelator that also scavenges hydroxyl radicals (Halliwell and Gutteridge, 1989), attenuated hemin (100 μmol/l) neurotoxicity. Similarly, NAC, a potent thiol-containing antioxidant (at a concentration of 0.5 mmol/l), inhibited cell death induced by hemin (100–300 μmol/l) (Fig. 3).

To verify whether hemin, the most toxic compound of Hb, induces cell death by apoptosis, we followed the mitochondrial transmembrane potential and caspase activation as the intracellular events that lead to programmed cell-death. We used the JC-1 cationic dye indicator to measure the membrane potential in the mitochondria. In healthy cells, JC-1 accumulates and aggregates in the mitochondria,

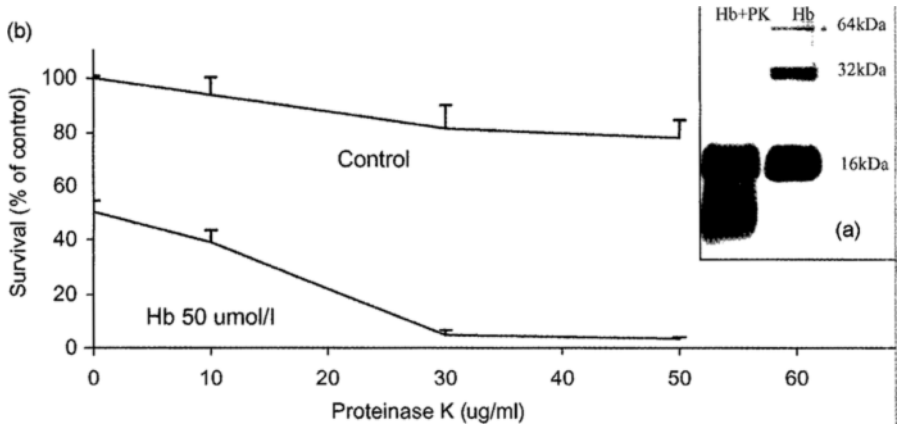

FIGURE 2 *Concentration dependence of cleaved Hb neurotoxicity.* (a) Hb cleaved with PK: SDS-polyacrylamide gel electrophoresis (15%) analysis of Hb (75 μmol/l) treated with 100 μg/ml proteinase-K. Proteins were stained with Coomassie blue. (b) PC12 cells were exposed, for 24 h, to 50 μmol/l Hb and increasing concentrations of proteinase-K. Cell survival was measured by Alamar blue assay (mean ± S.E.M, $n = 6$), relative to cells without treatment (control).

giving off a bright red fluorescence (shift from green, 525 nm, to red, 590 nm, Smiley *et al.*, 1991). In apoptotic cells, JC-1 cannot aggregate in the mitochondria due to the altered mitochondrial membrane potential, and thus it remains in the cytoplasm in its monomeric form, fluorescein green (Bedner *et al.*, 1999). We observed a drop in the mitochondrial membrane potential after PC12 cells were exposed to hemin (150 μmol/l) for 24 h, as reflected by the loss of orange fluorescence in JC-1 stained cells (Fig. 4).

Caspases are cysteine protease mediators of apoptosis, destroying normal cellular function by cleaving substrates at aspartic acid residues (Nunez *et al.*, 1998). Upon apoptotic stimulation, cytochrome C is released from the mitochondria and processes procaspase-9 into a large active subunit (either 37 kDa with prodomain or 17 kDa without) and a small nonactive subunit (10 kDa) (Li *et al.*, 1996; 1997). Using Western blot analysis with antibody against cleaved caspase-9, we followed the appearance of the active 37 kDa fragment, cleaved from the 47-kDa protein. The 37-kDa protein was first seen after 1.5 h of cell exposure to hemin (150 μmol/l) and peaked at 3 h. Comparable levels of actin were detected in each lane, indicating equal loading of sample (Fig. 5). We also used specific antibodies against poly ADP-ribose polymerase (PARP) involved in DNA repair, predominately in response to environmental stress, and cleaved by several effector caspases during apoptosis (Satoh and Lindahl, 1992; Nicholson *et al.*, 1995). PARP level compared to actin, markedly diminished after 1.5 h of hemin treatment and peaked after 24 h (Fig. 5). Moreover, DNA content of isolated nuclei was monitored by propidium iodide fluorescence staining. In the hemin-treated (150 μmol/l) neuroblastoma cells, flow cytometry analysis demonstrated a high ratio of nuclei with low content of DNA, typical for apoptotic bodies. Using flow cytometry analysis degraded DNA appeared as a peak distinguished from the normal diploid peak seen in the untreated cells. These hypoploid peaks contain 22% of the total DNA after 14 h of hemin treatment, 27% after 24 h compare with 1% in untreated cells (Fig. 6a–c).

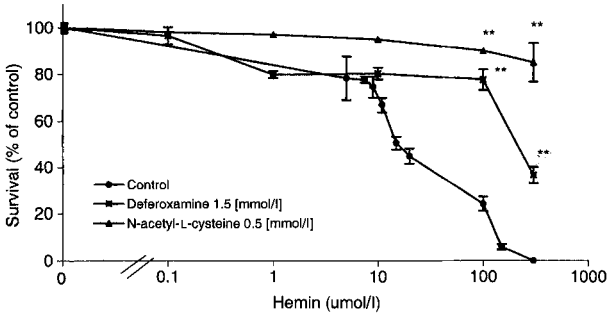

FIGURE 3 *Effect of deferoxamine or N-acety-L-cysteine on hemin toxicity in PC12 cells.* PC12 cells were exposed for 24 h to increasing concentrations of hemin alone or in the presence of deferoxamine (1.5 mmol/l) or NAC (0.5 mmol/l). Cell survival was measured by Alamar blue assay (mean ± S.E.M, $n = 6$), relative to cells without hemin (control), $^{**}P < 0.01$.

DISCUSSION

ICH or other forms of intracranial bleeding cause exposure of neurons to the whole spectrum of blood products and can induce cell death by various substances and mechanisms. In this study we investigated the toxicity of Hb and its metabolites, the major compounds of the hemolysate. We found that Hb by itself is toxic to neuron-like cells (PC12) at a relatively low concentration (10 μmol/l). Moreover, non-specific cleavage by proteinase-K potentiates its toxicity. Hemin, the intermolecular compound of Hb, is highly toxic while pure globin protein is not toxic even at a dose 20 times higher. The organic compound

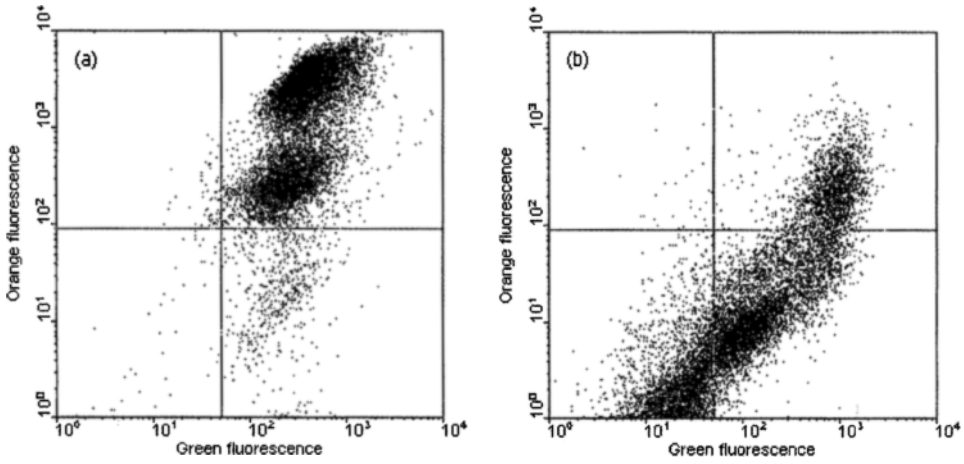

FIGURE 4 *Flowcytometry analysis of mitochondrial transmembrane potential of hemin treated cells.* JC-1, a cation fluorescence dye accumulated and aggregated in the healthy mitochondria as an orange fluorescence, while the monomer JC-1 appeared as a green fluorescence in the cytosol. Healthy mitochondria were used as controls showing a high level of orange fluorescence (a). A reduction of orange and green fluorescence in hemin-treated PC12 cells indicated for a lower mitochondrial transmembrane potential (b).

of hemin, protoporphyrin IX and iron are both toxic, although each of these compounds is less toxic than hemin as a complex. We further observed that toxicity is mediated through the induction of oxidative stress, which can be antagonized by antioxidants such as NAC and the iron-chelator, deferoxamine. Hemin-induced cell death is also associated with the destruction of the mitochondrial potential membrane, known to initiate of apoptosis. In addition, flow cytometry analysis demonstrated hemin-induced cell shrinkage and DNA fragmentation.

We chose to study products resulting from Hb breakdown as the possible inducers of neuronal damage, for several reasons. Firstly, Hb is the predominant component of blood and hemolysate. Secondly, it was shown that Hb by itself was highly toxic to cortical and hippocampal cultures and neuronal cell lines (Reagan and Panter, 1993; Wang *et al.*, 1999; Yip and Sastry, 2000). Thirdly, injection of blood hemolysate into the subarachnoid space of mice induced neuronal cell-death that was accompanied by the induction of heme oxygenase-1 expression, which is responsible for heme metabolism (Matz *et al.*, 2000; Regan *et al.*, 2000; Panahin Maines 2001). Furthermore, continued expression of heme oxygenase-1 demonstrated in humans after brain injury (Beschorner *et al.*, 2000), indicated a prolonged presence of heme in the injured area. Similarly, it was demonstrated that heme oxygenase activity was increased in PC12 and neuroblastoma (SH-SY5Y) cells following oxidative stress (Yan *et al.*, 1994; Pappolla *et al.*, 1998).

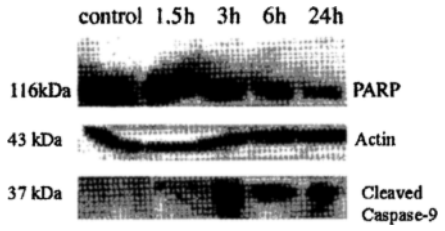

FIGURE 5 *Analysis of caspase-9 activation and PARP degradation in hemin-treated PC12 cells.* Western blot analysis of hemin-treated (100 μmol/l) PC12 cells demonstrated an elevation in the cleaved caspase-9 (37 kDa) after 3 h. PARP (116 kDa) immunoreactivity, relative to actin, was greatly diminished after 3 h in culture treated with hemin, compared with untreated cells that showed no cleaved caspase-9 and no changes in the PARP protein.

Hb was hypothesized to be associated with the pathophysiology of ICH and parenchymal ischemia that accompanies hemorrhage (Sadrzadeh *et al.*, 1987; Macdonald and Weir, 1991). Ogihara *et al.* (1999) demonstrated that OxyHb induces apoptosis in a concentration- and time-dependent manner in cultured bovine aortic artery endothelial cells. The mechanism of OxyHb-induced apoptosis and its role in the pathogenesis of cerebral vasospasm is not clear. In addition, several *in-vitro* studies showed that Hb is toxic to neuronal cells (Reagan and Panter, 1993; Wang *et al.*, 1999), while plasma alone and concentrated erythrocytes had negligible toxic effects (Xue and Del-Bigio, 2000). Although it was suggested that an apoptotic cell-death mechanism plays a role in the damaged tissue, which of the Hb components involved was not fully identified, and whether it was caused directly from Hb exposure or by the inflammatory reaction that generally follows (Stryer, 1987; Matz *et al.*, 2000). When blood or blood hemolysate was injected into the subarachnoid space or to cortical zones, apoptotic indications were accompanied with an increase in infiltration of lymphocytes (Xue and Del-Bigio, 2000) and hemoxygenase-1 (Matz *et al.*, 2000). Furthermore, induction of ICH in the rat striatum demonstrated apoptotic cell-death as indicated by various parameters (Matsushita *et al.*, 2000). To our knowledge, there have been no reports on the detailed contribution of

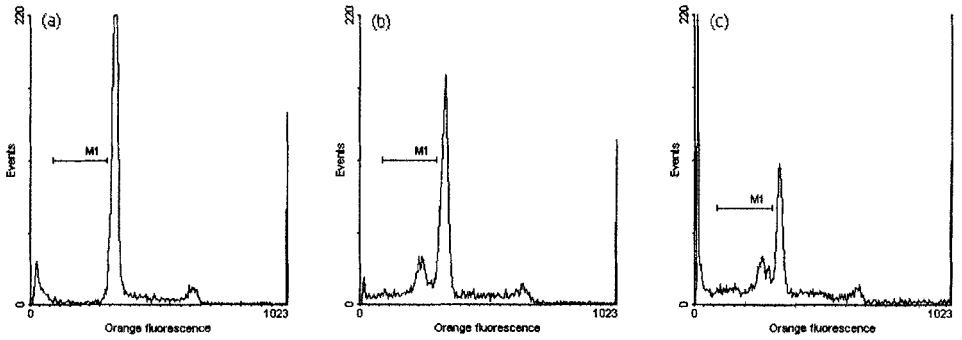

FIGURE 6 *Flow cytometry analysis of nuclei from hemin-treated neuroblastoma cells.* Nuclei of neuroblastoma cells stained with propidium-iodide, demonstrated a normal diploid peak of DNA (a). Cells treated with 150 μmol/l hemin for 14 h, generated an hipoploid apoptotic peak (b) that increased after 24 h (c).

each of the Hb components to the neuronal cell-death. Our results demonstrated that hemin-induced apoptosis, as indicated by reduced mitochondrial membrane potential, increased caspase-9 and PARP cleavage, DNA fragmentation and cell shrinkage. The undifferentiated PC12 cells, which we used as a model for neurons, have also been employed in a wide range of studies as a model for apoptosis in neurons (Offen *et al.*, 1996; Nakagawa *et al.*, 2000; Kim *et al.*, 2001).

The current study indicates that the most toxic compound is hemin, which consists of an organic molecule and iron: each on their own is much less toxic. Our observations suggest that although iron itself generates free radicals, when it is part of hemin it demonstrates greater toxicity. It seems that globin protein fraction unmasks the hemin moiety. We speculate that proteinase-K digestion changes Hb structure and enhances the production of iron-induced free radicals. Normally, cells have a number of mechanisms acting to combat against the damage induced by free radicals. Cellular damages occur when production of reactive oxygen species (ROS) such as $OH^{\bullet}$, $O_2^{-\bullet}$, NO, H_2O_2, or $ONOO^-$ exceeds their elimination by the antioxidant protective systems or when the latter is damaged (Evans, 1993; Simonian and Coyle, 1996).

Hemorrhage is an extremely complex process involving pathological permeability of blood–brain barrier, energy failure, loss of cell ion homeostasis, acidosis, increased intracellular calcium, excitotoxicity, and free radical-mediated toxicity. However, the presence of free Hb in brain tissue was suggested to exacerbate potential ROS-effected damage (Sadrzadeh *et al.*, 1987). Indeed, one of the best agents that showed benefit in an animal model of SAH was ebselen (2-phenyl-1,2-benzisoselenazol-3(2H)-one), which increased the activity of the anti-oxidant enzyme glutathione peroxidase (Watanabe *et al.*, 1997). Moreover, a controlled clinical study in 286 patients with subarachnoid hemorrhage treated with ebselen demonstrated ameliorated delayed neurological deficits (Saito *et al.*, 1998).

Free metal ions are associated with the pathology of various neurodegenerative diseases (e.g. copper in Wilson's disease, iron in the substantia nigra in Parkinson's disease). Therefore, agents like transferrin (which bind iron) ceruloplasmin (binds copper) and hemopexin (binds heme) might be used in SAH. Indeed, deferoxamine, a potent chelator of iron and redox-active metals, improved survival and physiological functions in an experimental model of SAH (Vollmer *et al.*, 1991) and in rats model of closed head injury (Zhang *et al.*, 1998).

Stroke is a leading cause of death and chronic disability in the Western world. So far, however, effective treatment is not available. If Hb degradation products play a key role in the pathogenesis of neuronal injury by way of oxidative stress, as we have shown, then a therapeutic option may be at hand. Antioxidant therapy has been suggested as a treatment for ischemic/reperfusion injury (Cuzzocrea *et al.*, 2001). Based on our data, we recommend that it should also be tried for ICH.

In conclusion, our findings suggest that Hb, and especially its compound, hemin, are highly toxic to neurons and causes neuronal apoptosis. Therapeutic strategies based on antioxidants, iron chelator or targeted anti-apoptotic agents might be effective in slowing down or arresting the progression in neuronal death.

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