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Iron Accumulation and Neurotoxicity in Cortical Cultures Treated with Holotransferrin

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Abstract

Nonheme iron accumulates in CNS tissue after ischemic and hemorrhagic insults, and may contribute to cell loss. The source of this iron has not been precisely defined. After blood-brain barrier disruption, CNS cells may be exposed to plasma concentrations of transferrin-bound iron (TBI), which exceed that in CSF by over 50-fold. In this study, the hypothesis that these concentrations of TBI produce cell iron accumulation and neurotoxicity was tested in primary cortical cultures. Treatment with 0.5-3 mg/ml holotransferrin for 24 hours resulted in loss of 20-40% of neurons, associated with increases in malondialdehyde, ferritin, heme oxygenase-1 and iron; transferrin receptor-1 expression was reduced by about 50%. Deferoxamine, 2,2'-bipyridyl, Trolox, and ascorbate prevented all injury, but apotransferrin was ineffective. Cell TBI accumulation was significantly reduced by deferoxamine, 2,2'-bipyridyl, and apotransferrin, but not by ascorbate or Trolox. After treatment with ⁵⁵Fe-transferrin, approximately 40% of cell iron was exported within 16 hours. Net export was increased by deferoxamine and 2,2'-bipyridyl, but not by apotransferrin. These results suggest that downregulation of transferrin receptor-1 expression is insufficient to prevent iron-mediated death when neurons are exposed to plasma concentrations of TBI. Chelator therapy may be beneficial for acute CNS injuries associated with loss of blood-brain barrier integrity.

Keywords

intracerebral hemorrhage; oxidative; stroke; traumatic brain injury

Introduction

Increased nonheme iron has been observed in injured tissue after experimental ischemic stroke and intracerebral hemorrhage (ICH) [1, 2], and is associated with lipid, protein, and nucleic acid oxidation. Although the protective effect of iron chelators in some models suggests that this iron may contribute to cell loss, [2-4], its source has not been precisely defined. Millimolar concentrations of iron are incorporated into the hemoglobin of an intracerebral hematoma [5], while brain tissue contains micromolar concentrations of cytochromes and other endogenous hemoproteins [6]. However, heme bound to hemoproteins is a relatively poor substrate for the HOs (HO's) [7], which catalyze the rate-limiting step in heme degradation resulting in iron release. If hemoprotein degradation is

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deleterious, then reducing HO activity would be predicted to be protective. This phenomenon has been observed in rodent [8, 9], rabbit [10], and pig ICH models [11], but both HO gene knockout and HO inhibitors consistently worsen outcome after ischemic stroke [12-14], indicating that the net effect of heme breakdown in the latter disease process is protective.

An alternate source of nonheme iron is bound to transferrin, which is present in CSF at a concentration of ~50 $\mu\text{g/ml}$ [15], and 50 to 70-fold higher in plasma. We have recently reported that CSF concentrations of diferric “holotransferrin” are not toxic to cultured neurons [16]. After stroke or trauma, disruption of the blood-brain barrier may expose CNS cells to the higher plasma concentrations of transferrin-bound iron (TBI) [2]. Uptake of this iron is limited to cells expressing transferrin receptors, and in the CNS is mediated predominantly by transferrin receptor-1 (TfR1), which is present on neurons [17] and to a lesser extent on glial cells [18]. TfR1 expression is an inverse function of iron levels in most cell populations, due to post-transcriptional regulation mediated by iron regulatory proteins (IRP's)[19]. These bind to iron regulatory elements in the 3' region of TfR1 mRNA, stabilizing it and increasing synthesis. In iron-replete cells, IRP degradation and/or reduced binding affinity downregulate TfR1 expression and TBI uptake. Recent studies in vitro and in vivo suggest that this negative feedback inhibition may be suboptimal in the CNS, with either no decrease or a paradoxical increase in TfR1 expression after iron loading of sufficient magnitude to generate oxidative stress and delayed neuronal death [1, 2, 16]. Despite its potential relevance to acute CNS injury associated with loss of blood-brain barrier integrity, the consequences of prolonged exposure of neural cells to plasma concentrations of holotransferrin has never been quantitatively investigated. In the present study, we tested the effect of purified holotransferrin on cell iron, lipid oxidation, expression of TfR1, ferritin, HO-1, and ferroportin, and cell viability in murine cortical cultures.

Methods

Cell cultures

All procedures on animals were previously approved by the Thomas Jefferson University Institutional Animal Care and Use Committee. Cortical cell cultures containing both neurons and glia were prepared from fetal mice (B6129 strain) at gestational age 14-16 days using a method that has been described in detail [20]. Two-thirds of the culture medium was replaced on day 5 and day 8 or 9 in vitro with minimal essential medium (Invitrogen, Carlsbad, CA, USA) containing 10% equine serum (Hyclone, Logan, UT, USA), 2 mM glutamine, and 23 mM glucose. After ten days in vitro, medium exchange was performed daily. Cultures were used for experiments at 12-16 days in vitro. At this time point, neurons are easily distinguished from glial cells in this culture system by their characteristic phase-bright cell bodies resting on top of the glial monolayer, and extensive processes [16, 21].

Holotransferrin exposure

Cell culture grade lyophilized human diferric transferrin (holotransferrin, Calbiochem/EMD, Gibbstown, NJ) was dissolved in distilled water, and then was desalted using Amicon Ultra centrifugal filters (nominal molecular weight limit 10 kDa, Millipore, Billerica, MA). A stock solution of the retained protein was prepared in serum-free MEM containing 10 mM glucose (MEM10). Prior experiments have demonstrated that this medium is preferable to the commonly-used Neurobasal Medium with B27 supplement for studying iron-mediated injury mechanisms, since the latter contains several antioxidants (particularly iron-poor transferrin and α -tocopherol) that attenuate iron neurotoxicity. However, the use of MEM-based media requires the presence of glial cells for neuronal survival [22], and therefore all experiments were conducted on mixed cultures. On the day prior to the experiment, the

serum concentration of the cultures was reduced to 3.3% by replacing two-thirds of the medium with MEM10. Remaining serum was washed out the following day, and holotransferrin alone or with other reagents was immediately added in MEM10. Cultures were then incubated at 37°C in a 5% CO₂ atmosphere.

Cell injury assessment

At the end of the exposure interval, cultures were examined with phase contrast microscopy. Cell death was quantified by measurement of lactate dehydrogenase (LDH) activity in the medium, as previously described [23]. The low LDH activity in sister cultures subjected to medium exchange only was subtracted from all values to yield the signal specific for the neurotoxic insult, following the protocol of Koh and Choi [24]. To facilitate comparison of experiments conducted on cultures from different platings, LDH values were scaled to the mean value in sister cultures exposed to NMDA 300 μ M for the duration of the experiment. This treatment releases all neuronal LDH in these cultures without injuring glial cells. Since holotransferrin did not injure glia, their contribution to the total signal was negligible.

LDH release is a specific marker of cell death in this culture system that correlates well with cell counts after vital staining [25, 26]; however, since it requires disruption of cell membranes, it may be insensitive to sublethal oxidative stress or to lethal injury that has not yet progressed to membrane rupture. Oxidative cell injury was therefore also assessed via malondialdehyde (MDA) assay. After precipitation of culture proteins with 4.5% trichloroacetic acid, MDA was quantified using a method that has previously been described in detail [27]. Protein content was assayed by the BCA method (Pierce, Biotechnology, Rockford, IL, USA); MDA values were expressed as nanomoles/milligram protein.

Enhanced Perls' Staining

Cultures were washed with PBS and were fixed with 4% paraformaldehyde for one hour. After another PBS wash, they were exposed to Perls' solution (1:1, 2% HCl and 2% potassium ferrocyanide) for 30 minutes at room temperature, followed by 0.05% 3,3'-diaminobenzidine (DAB, Sigma-Aldrich) and then 0.033% H₂O₂ in 0.05% DAB in PBS. Staining was quantified by measuring density of randomly-selected grayscale images, using Kodak Molecular Imaging software, Version 4.0.

Cell iron assay

Cell iron content was quantified with a ferrozine-based assay, as described by Riemer et al. [28] and modified by Dang et al. [29]. Cells were lysed in 100 μ l/well of a solution containing equal volumes of 142.5 mM potassium permanganate in 700 mM HCl (freshly prepared), 50 mM NaOH, and 10 mM HCl. After addition to each well of 50 μ l of an iron detection solution (6.5 mM ferrozine, 6.5 mM neocuproine, 2.5 M ammonium acetate and 1 M ascorbic acid in H₂O), the lysates were collected and centrifuged (15,682 $\times$ g, 1 min). The absorbance of the supernatant was then measured at 562 nm, and compared with that of a FeCl₃ standard curve. After BCA protein assay, iron content was expressed as nanomoles/milligram protein.

Quantification of iron accumulation and export

Human serum apotransferrin (Calbiochem/EMD) in MEM10 was loaded with ⁵⁵Fe³⁺ (Perkin Elmer, Waltham, MA) by incubation for at least one hour prior to use. Cultures were washed with MEM10 containing 1 mg/ml bovine serum albumin (MEM10/BSA) and were incubated in this medium for 1 hour. After medium exchange with fresh MEM10/BSA, labeled transferrin (3.3 μ g/ml) was added to cultures, alone or with study drugs. After incubation for 2 hours at 37°C, cultures were washed with fresh MEM10/BSA, and cells

were lysed in MEM10 containing 0.3% Triton X-100. Lysate radioactivity was quantified by liquid scintillation counting. TBI accumulation, defined as iron uptake minus export during the incubation period [30], was calculated from the known specific activity of the radioisotope. In order to distinguish TBI nonspecifically associated with the cell membrane from that available for internalization, control experiments were conducted in the same manner but on ice rather than at 37°C, according to the protocol of Riedel et al [31]. The latter values, which were 3.1-8.5% of the total signal at 37°C, were subtracted from the 37°C value to calculate specific iron accumulation. To quantify net iron export, defined as iron export minus reuptake during the incubation period, additional cultures were treated with labeled transferrin for 2 hours as described above. After washing with fresh MEM10/BSA, cultures were incubated in medium containing 100 µg/ml unlabeled transferrin, which is nontoxic [16], and 1 mg/ml BSA, alone or with study drugs. Excess unlabeled transferrin was present in the culture medium to minimize any isotope reuptake. Medium was sampled at defined time points and radioactivity was counted as above.

Immunoblotting

Cultures were washed with MEM10, and then were lysed in an ice-cold buffer containing 210 mM mannitol, 70 mM sucrose, 5 mM HEPES, 1 mM EDTA, 0.1 % sodium dodecyl sulfate, and 0.1 % Triton X-100. After sonication, centrifugation, and BCA protein assay, samples (30 µg in 30 µl) were diluted with 10 µl 4× loading buffer (Tris-Cl 240 mmol/L, β-mercaptoethanol 20%, sodium dodecyl sulfate (SDS) 8%, glycerol 40%, and bromophenol blue 0.2%). Samples for ferritin, HO-1, ferroportin or actin immunoblots were then heated to 95°C for 3 minutes; transferrin receptor-1 (TfR1) samples were incubated for 10 minutes at 37°C. Proteins were separated on 4-15% gradient gels and were transferred to a polyvinylidene difluoride (PVDF) transfer membrane filter (Millipore). Membranes to be probed with anti-TfR1 were then treated with 100 mM β-mercaptoethanol, 2% SDS in 62.5 mM Tris buffer (pH 6.7) following the protocol of Kaur and Bachhawat to enhance the signal of membrane-bound proteins [32]. After washing, nonspecific sites of all membranes were blocked with 5% non-fat dry milk in a buffer containing 20 mM Tris, 500 mM NaCl, and 0.1% Tween 20 (pH 7.5) for 1 h at room temperature. Membranes were then incubated at 4°C overnight with primary antibodies (rabbit anti-horse spleen ferritin 1:250 dilution, Sigma-Aldrich Cat. No. F5762, St Louis MO, USA; monoclonal anti-TfR1, 1:500, Invitrogen Cat. No. 13-6800; rabbit anti-HO-1, 1:3000, Enzo Life Sciences, Plymouth Meeting, PA, rabbit anti-MTP1/Ferroportin, Alpha Diagnostics, San Antonio, TX, 1:1000; rabbit anti-actin, 1:1000, Sigma-Aldrich Cat. No. A2066). After washing, membranes were treated with horseradish peroxidase-conjugated secondary antibody (Pierce, 1:3000). Immunoreactive proteins were visualized using Super Signal West Femto Reagent (Pierce) and Kodak Gel Logic 2200.

Statistical analysis

Data were analyzed with one-way analysis of variance, followed by the Bonferroni multiple comparisons test, with a P value < 0.05 considered statistically significant.

Results

Neurotoxicity of holotransferrin

In a prior study, we reported that neurons in this culture system express TfR1, and that CSF concentrations of apo- and holotransferrin (50-100 µg/ml) were nontoxic [16]. Treatment with apotransferrin 0.5-3 mg/ml for 24 hours similarly resulted in no change in cellular morphology or significant LDH leakage (Figs. 1, 2). Treatment with holotransferrin in this concentration range, which approximates that in serum, resulted in significant LDH release. This was accompanied by degeneration of the phase-bright cells resting on the glial

monolayer that we have previously identified by NeuN immunostaining as neurons [16, 21]. Neuronal injury was associated with a significant and proportionate increase in culture malondialdehyde (MDA, Fig. 1B). Time course experiments demonstrated little LDH leakage before 16 hours (Fig. 1C). When compared with a concentration of hemoglobin containing equimolar iron, holotransferrin was less toxic. Glial cells remained morphologically normal; no significant increase in LDH leakage or MDA was detected in cultures containing glial cells only (>90% GFAP+, 2-3% microglia [21], Table 1).

Iron chelators and antioxidants prevent neuronal injury

In order to test the hypothesis that neuronal injury was iron-dependent, cultures were treated with holotransferrin plus the cell membrane-permeable iron chelator 2,2'-bipyridyl or the less permeable ferric chelator deferoxamine (Fig. 3A,B). Both prevented all neurotoxicity. Complete protection was also provided by the amphipathic chain-breaking antioxidant Trolox and the hydrophilic antioxidant ascorbate. A protective effect was still observed when these agents were added to cultures 6 hours after holotransferrin (Fig. 3C,D).

Iron chelators and ascorbate attenuate iron staining after holotransferrin treatment

Increased Perl's staining intensity was apparent in cultures treated with 3 mg/ml holotransferrin for 24 hours (Fig. 4A,B). Although TfR1 is detected by immunostaining only in neurons in this culture system [33], much of the iron staining was localized to the glial monolayer. Iron staining density was significantly reduced by concomitant treatment with 2,2'-bipyridyl, deferoxamine, and ascorbate (density values 67.6 ± 5.9 , 73.5 ± 7.0 , and 67.0 ± 6.8 , respectively, v. 100.0 ± 5.5 for holotransferrin alone, $n = 11-14$ /condition, $P < 0.01$). However, when quantified by a ferrozine-based assay, the increase in culture iron produced by holotransferrin treatment was significantly reduced only by 2,2'-bipyridyl and deferoxamine (Fig. 4G).

Accumulation and export of transferrin-bound iron

The observation that iron chelators and ascorbate reduced cell iron staining suggested that these agents may reduce accumulation of TBI or enhance iron export. Additional experiments were conducted to assess the contribution of these mechanisms. Incubating mixed neuron/glia cultures with $3.3 \mu\text{g/ml}$ ^{55}Fe -transferrin for 2 hours resulted in accumulation of 8.60 ± 0.61 pmoles iron/mg protein (Fig. 5A); this was significantly reduced by either deferoxamine or 2,2'-bipyridyl, but not by ascorbate. When cultures were treated with the same concentration of nontransferrin-bound ^{55}Fe , accumulation of 12.22 ± 1.44 pmoles iron/mg protein was observed, which was markedly reduced by either deferoxamine or 2,2'-bipyridyl (Fig. 5B), and was enhanced by ascorbate. At two hours after ^{55}Fe -transferrin washout, approximately 30% of transferrin-bound ^{55}Fe accumulated by cells was detected in the culture medium (Fig. 6A); net iron export was significantly increased by deferoxamine and 2,2'-bipyridyl, but was not altered by ascorbate (Fig. 6B).

Effect of apotransferrin on holotransferrin neurotoxicity and iron transport

With the exception of iron overload states such as hemochromatosis, plasma transferrin is usually less than 55% iron-saturated. The effect of apotransferrin on holotransferrin neurotoxicity was therefore investigated. In contrast to the effects of deferoxamine and 2,2'-bipyridyl, apotransferrin provided no cytoprotection (Fig 7A). Equimolar apotransferrin reduced accumulation of TBI by 39%, but in contrast to the effect of low molecular weight chelators it failed to enhance iron export.

Effect of holotransferrin alone or with protective compounds on transferrin receptor-1, ferritin, HO-1 and ferroportin expression

We have recently reported that neuronal expression of TfR1 in this culture system is not attenuated by treatment with hemoglobin, which produces an iron-dependent neuronal injury [16]. In contrast, holotransferrin reduced TfR1 expression (Fig. 8A); concomitant treatment with deferoxamine prevented this effect, and 2,2'-bipyridyl increased expression over baseline. Consistent with cellular iron loading, holotransferrin increased culture ferritin (Fig. 8B), and ascorbate enhanced this induction. HO-1 expression was also increased by holotransferrin, and was further increased by addition of 2,2'-bipyridyl (Fig. 8C). Ferroportin expression was not altered by holotransferrin alone or with chelators or antioxidants (Fig. 8D).

Discussion

The present results suggest the following: 1) sustained exposure to plasma concentrations of holotransferrin are neurotoxic; 2) this toxicity is iron-dependent and oxidative, since it is blocked by iron chelators and antioxidants, and since the same concentrations of iron-poor transferrin are nontoxic; 3) holotransferrin downregulates TfR1 expression, but this is insufficient to prevent iron accumulation in cultures treated with plasma concentrations of holotransferrin; 4) about 40% of TBI taken up by cells is exported within 16 hours, with most of this occurring within 2 hours; 5) iron chelators attenuate uptake of TBI and enhance export.

In prior studies using this culture system, treatment with inorganic iron diluted in transferrin-free medium produced widespread neuronal death, with an EC₅₀ near 10 μ M for a 24 hour exposure [34]. In the present study, cultures were treated with up to 3 mg/ml holotransferrin (37.5 μ M, iron content 75 μ M) for the same interval, and yet sustained loss of only ~40% of neurons. The weaker neurotoxicity of holotransferrin may be due in part to a slower rate of iron uptake, since cultures treated with ⁵⁵Fe-holotransferrin accumulated less iron after a 2 hour treatment than sister cultures treated with ⁵⁵FeCl₃. Other mechanisms related to as yet undefined differences in cellular iron trafficking cannot be excluded. When directly compared with the neurotoxicity of hemoglobin-bound iron, TBI was likewise less toxic, consistent with prior observations that the EC₅₀ for hemoglobin in this culture system is 2-3 μ M (8-12 μ M iron)[34].

Like hemoglobin, holotransferrin is a slowly acting neurotoxin, producing minimal neuronal injury when exposure times are less than 16 hours. In vivo, it diffuses rapidly through brain tissue, with high affinity but low capacity receptor binding [35]. These characteristics may account for the observation by Nakamura et al. [36] that a single striatal injection of 0.4 mg holotransferrin produced no increase in tissue water or iron content in rats. When combined with thrombin, which increases TfR1 expression, both edema and iron deposition were detected, suggesting synergistic toxicity. However, thrombin per se disrupts the blood-brain barrier, and increases transferrin levels around the injection site [37, 38]. Leakage of plasma holotransferrin into brain interstitial fluid, resulting in maintenance of higher tissue concentrations than after holotransferrin injection alone, should be considered as another mechanism by which thrombin enhances the toxicity of injected holotransferrin.

Both deferoxamine and 2,2'-bipyridyl reduced accumulation of TBI and enhanced iron export, consistent with the observation that they decreased Perls' staining intensity in holotransferrin-treated cultures. Deferoxamine has an even greater affinity for ferric iron than transferrin (stability constant 10³¹ v. 10²⁸ [39]); its ability to remove iron directly from transferrin has been demonstrated empirically [40]. It also tends to concentrate in endosomes, where it may bind iron released from transferrin and prevent its reduction and

subsequent transfer into the cytosol [41]. 2,2'-bipyridyl has considerably weaker affinity for iron (stability constants 10^{17} for Fe^{2+} and 10^{16} for Fe^{3+} [42]), and in the present study increased TfR1 expression, likely due to its ability to cross cell membranes and enhance the binding activity of iron regulatory proteins [43]. Its effect on iron accumulation and export may be mediated at least in part by binding to iron after its endosomal removal from transferrin, as previously reported in reticulocytes [44]. The present results also indicate that it would largely prevent reuptake of any nontransferrin-bound iron exported from cells. Neither chelator altered expression of the iron exporter ferroportin in holotransferrin-treated cultures.

In contrast to its protective effect against hemoglobin neurotoxicity [16], apotransferrin did not mitigate holotransferrin neurotoxicity. These results suggest that the iron binding capacity present in normal plasma is unlikely to protect neurons from the toxicity of TBI. Apotransferrin moderately reduced TBI uptake, consistent with competition for TfR1 binding sites [45]. However, unlike deferoxamine and 2,2'-bipyridyl, it had no effect whatsoever on net iron export. The latter discrepancy may account at least in part for its failure to attenuate neuronal death in this model. Other mechanisms related to the greater availability of low molecular weight chelators at cellular sites that are vulnerable to iron-catalyzed oxidation reactions may also contribute.

Although transferrin receptor-1 expression is detected by immunostaining only in neurons in this culture system [33], much of the culture iron detected by enhanced Perls' staining after 24h holotransferrin treatment was localized to cells with a glial morphology. This observation is consistent with the hypothesis of Moos and Morgan [46] that neurons take up TBI but then tend to export it, following which it may be taken up by glial cells as nontransferrin-bound iron and stored as ferric iron. Relatively little iron staining in neurons may indicate less storage, rather than less uptake. The paucity of ferritin in primary cultured neurons compared with that in glia after iron loading is consistent with iron storage primarily in the latter [47].

The attenuated Perls' staining intensity produced by ascorbate is inconsistent with ferrozine assay results. It also conflicts with observations that ascorbate had no effect on TBI uptake and export, but facilitated nontransferrin-bound iron uptake. The latter phenomenon would be predicted to enhance reuptake of exported iron, and may be mediated by reduction of Fe^{3+} by ascorbate [48], which then increases cellular uptake by divalent metal transporter-1 [30]. These observations suggest that in the presence of ascorbate, Perls' staining is an unreliable marker of cell iron stores. Since this method preferentially stains Fe^{3+} [49], ferric iron reduction may also account for this limitation.

Plasma concentrations of holotransferrin produced iron-dependent neurotoxicity in this cell culture model despite downregulation of TfR1 expression. Two reported mechanisms may contribute to this phenomenon. First, the ~50% reduction in TfR1 expression in holotransferrin-treated cultures may simply be insufficient to prevent lethal iron overload. Considerable evidence suggests that the classic model of TfR1 regulation, i.e. loss of IRP stabilization of TfR1 mRNA, may not be sufficient to account for experimental observations in an oxidative cellular milieu. Specifically, both Andriopoulos et al. [50] and Kaur et al. [51] have observed that continuous exposure to low concentrations of H_2O_2 increased TfR1 synthesis via a mechanism that is independent of both iron regulatory proteins and hypoxia inducible factor (HIF)-1 α . The relatively high rate of H_2O_2 production by neural cell mitochondria may be sufficient to maintain some neuronal TfR1 expression despite excessive cell iron [52]. Second, it is possible that TBI may also enter cells via a route that is independent of receptor binding. The latter phenomenon, attributed to adsorptive pinocytosis or fluid phase endocytosis, has been reported in human melanoma cells [53] and cultured

hepatocytes [54]. It is saturated at higher transferrin concentrations than receptor-mediated uptake, and may be relevant when cells are exposed to plasma concentrations of TBI. Nonspecific TBI uptake has not been intensively investigated, and has not been reported in neural cells to date.

HO-1 expression was increased by holotransferrin treatment, consistent with cellular oxidative stress; however, the magnitude of this effect was less than that produced by hemoglobin in this culture system [33]. HO-1 was further increased by 2,2'-bipyridyl but not by deferoxamine. 2,2'-bipyridyl and other cell-permeable iron chelators activate HIF-1 α , which transcriptionally regulates HO-1 expression [55]. Since HO activity attenuates nonheme iron toxicity in this culture system [34], the increase in HO-1 may have mitigated the neurotoxicity of holotransferrin, and may have contributed to the beneficial effect of 2,2'-bipyridyl.

Brain transferrin levels are increased 1-3 days after experimental ICH and subarachnoid hemorrhage [1, 56], and are associated with increased expression of TfR1. The latter phenomenon has also been reported after experimental ischemic stroke [2], but tissue transferrin has not yet been quantified in the penumbra. After clinical ischemic and hemorrhagic stroke, a rapid increase in CSF transferrin has been observed that is sustained for at least 5 days [57]. The present results suggest that TBI may contribute to the perilesional iron accumulation observed in these disease processes. Further investigation of holotransferrin neurotoxicity in vivo seems warranted.

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References

1. Wu J, Hua Y, Keep RF, Nakemura T, Hoff JT, Xi G. Iron and iron- handling proteins in the brain after intracerebral hemorrhage. *Stroke*. 2003; 34:2964–2969. [PubMed: 14615611]
2. Park UJ, Lee YA, Won SM, Lee JH, Kang SH, Springer JE, Lee YB, Gwag BJ. Blood-derived iron mediates free radical production and neuronal death in the hippocampal CA1 area following transient forebrain ischemia in rat. *Acta Neuropathol*. 2011; 121:459–73. [PubMed: 21120509]
3. 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; 100:672–678. [PubMed: 15070122]
4. Hanson LR, Roeytenberg A, Martinez PM, Coppes VG, Sweet DC, Rao RJ, Marti DL, Hoekman JD, Matthews RB, Frey WH 2nd, Panter SS. Intranasal deferoxamine provides increased brain exposure and significant protection in rat ischemic stroke. *J Pharmacol Exp Ther*. 2009; 330:679–686. [PubMed: 19509317]
5. Letarte PB, Lieberman K, Nagatani K, Haworth RA, Odell GB, Duff TA. Hemin: levels in experimental subarachnoid hematoma and effects on dissociated vascular smooth muscle cells. *J Neurosurg*. 1993; 79:252–255. [PubMed: 8331409]
6. Valenzuela C, Lopez de Romana D, Olivares M, Morales MS, Pizarro F. Total Iron and Heme Iron Content and their Distribution in Beef Meat and Viscera. *Biol Trace Elem Res*. 2009
7. Abraham NG, Drummond GS, Lutton JD, Kappas A. The biological significance and physiological role of heme oxygenase. *Cell Physiol Biochem*. 1996; 6:129–168.
8. Qu Y, Chen J, Benvenisti-Zarom L, Ma X, Regan RF. Effect of targeted deletion of the heme oxygenase-2 gene on hemoglobin toxicity in the striatum. *J Cereb Blood Flow Metab*. 2005; 25:1466–1475. [PubMed: 15902196]
9. Wang J, Doré S. Heme oxygenase-1 exacerbates early brain injury after intracerebral haemorrhage. *Brain*. 2007; 130:1643–1652. [PubMed: 17525142]

10. Koeppe AH, Dickson AC, Smith J. Heme oxygenase in experimental intracerebral hemorrhage: the benefit of tin-mesoporphyrin. *J Neuropathol Exp Neurol*. 2004; 63:587–597. [PubMed: 15217087]
11. Wagner KR, Hua Y, de Courten-Myers GM, Broderick JP, Nishimura RN, Lu SY, Dwyer BE. Tin-mesoporphyrin, a potent heme oxygenase inhibitor, for treatment of intracerebral hemorrhage: in vivo and in vitro studies. *Cell Mol Biol*. 2000; 46:597–608. [PubMed: 10872746]
12. Kadoya C, Domino EF, Yang GY, Stern JD, Betz AL. Preischemic but not postischemic zinc protoporphyrin treatment reduces infarct size and edema accumulation after temporary focal cerebral ischemia in rats. *Stroke*. 1995; 26:1035–1038. [PubMed: 7762020]
13. Doré S, Sampei K, Goto S, Alkayed NJ, Guastella D, Blackshaw S, Gallagher M, Traystman RJ, Hurn PD, Koehler RC, Snyder SH. Heme oxygenase-2 is neuroprotective in cerebral ischemia. *Mol Med*. 1999; 5:656–663. [PubMed: 10602774]
14. Dore S, Goto S, Sampei K, Blackshaw S, Hester LD, Ingi T, Sawa A, Traystman RJ, Koehler RC, Snyder SH. Heme oxygenase-2 acts to prevent neuronal death in brain cultures and following transient cerebral ischemia. *Neuroscience*. 2000; 99:587–592. [PubMed: 10974422]
15. Moos T, Oates PS, Morgan EH. Iron-independent neuronal expression of transferrin receptor mRNA in the rat. *Brain Res Mol Brain Res*. 1999; 72:231–234. [PubMed: 10529482]
16. Chen-Roetling J, Chen L, Regan RF. Apotransferrin protects cortical neurons from hemoglobin toxicity. *Neuropharmacology*. 2011; 60:423–31. [PubMed: 21034753]
17. Moos T, Rosengren Nielsen T, Skjorring T, Morgan EH. Iron trafficking inside the brain. *J Neurochem*. 2007; 103:1730–1740. [PubMed: 17953660]
18. Connor JR, Menzies SL. Cellular management of iron in the brain. *J Neurol Sci*. 1995; 134(Suppl): 33–44. [PubMed: 8847543]
19. Recalcati S, Minotti G, Cairo G. Iron regulatory proteins: from molecular mechanisms to drug development. *Antioxid Redox Signal*. 2010; 13:1593–1616. [PubMed: 20214491]
20. Regan RF, Choi DW. The effect of NMDA, AMPA/kainate, and calcium channel antagonists on traumatic cortical neuronal injury in culture. *Brain Res*. 1994; 633:236–242. [PubMed: 7907934]
21. Jaremko KM, Chen-Roetling J, Chen L, Regan RF. Accelerated Hemolysis and Neurotoxicity in Neuron-Glia-Blood Clot Co-cultures. *J Neurochem*. 2010; 114:1063–1073. [PubMed: 20497302]
22. Wang XF, Cynader MS. Effects of astrocytes on neuronal attachment and survival shown in a serum-free co-culture system. *Brain Res Brain Res Protoc*. 1999; 4:209–216. [PubMed: 10446416]
23. Regan RF, Rogers B. Delayed treatment of hemoglobin neurotoxicity. *J Neurotrauma*. 2003; 20:111–120. [PubMed: 12614593]
24. Koh JY, Choi DW. Vulnerability of cultured cortical neurons to damage by excitotoxins: Differential susceptibility of neurons containing NADPH-diaphorase. *J Neurosci*. 1988; 8:2153–2163. [PubMed: 3385492]
25. Koh JY, Choi DW. Quantitative determination of glutamate mediated cortical neuronal injury in cell culture by lactate dehydrogenase efflux assay. *J Neurosci Meth*. 1987; 20:83–90.
26. Lobner D. Comparison of the LDH and MTT assays for quantifying cell death: validity for neuronal apoptosis. *J Neurosci Meth*. 2000; 96:147–152.
27. Regan RF, Jasper E, Guo YP, Panter SS. The effect of magnesium on oxidative neuronal injury in vitro. *J Neurochem*. 1998; 70:77–85. [PubMed: 9422349]
28. Riemer J, Hoepken HH, Czerwinska H, Robinson SR, Dringen R. Colorimetric ferrozine-based assay for the quantitation of iron in cultured cells. *Anal Biochem*. 2004; 331:370–375. [PubMed: 15265744]
29. Dang TN, Bishop GM, Dringen R, Robinson SR. The putative heme transporter HCP1 is expressed in cultured astrocytes and contributes to the uptake of hemin. *Glia*. 2010; 58:55–65. [PubMed: 19533605]
30. Lane DJ, Robinson SR, Czerwinska H, Bishop GM, Lawen A. Two routes of iron accumulation in astrocytes: ascorbate-dependent ferrous iron uptake via the divalent metal transporter (DMT1) plus an independent route for ferric iron. *Biochem J*. 2010; 432:123–132. [PubMed: 20819077]
31. Riedel HD, Muckenthaler MU, Gehrke SG, Mohr I, Brennan K, Herrmann T, Fitcher BA, Hentze MW, Stremmel W. HFE downregulates iron uptake from transferrin and induces iron-regulatory protein activity in stably transfected cells. *Blood*. 1999; 94:3915–3921. [PubMed: 10572108]

32. Kaur J, Bachhawat AK. A modified Western blot protocol for enhanced sensitivity in the detection of a membrane protein. *Anal Biochem.* 2009; 384:348–349. [PubMed: 18952039]
33. Chen-Roetling J, Chen L, Regan RF. Apotransferrin protects cortical neurons from hemoglobin toxicity. *Neuropharmacology.* 2011; 60:423–431. [PubMed: 21034753]
34. Rogers B, Yakopson V, Teng ZP, Guo Y, Regan RF. Heme oxygenase-2 knockout neurons are less vulnerable to hemoglobin toxicity. *Free Rad Biol Med.* 2003; 35:872–881. [PubMed: 14556851]
35. Thorne RG, Lakkaraju A, Rodriguez-Boulan E, Nicholson C. In vivo diffusion of lactoferrin in brain extracellular space is regulated by interactions with heparan sulfate. *Proc Natl Acad Sci U S A.* 2008; 105:8416–8421. [PubMed: 18541909]
36. Nakamura T, Xi G, Park JW, Hua Y, Hoff JT, Keep RF. Holo- Transferrin and Thrombin Can Interact to Cause Brain Damage. *Stroke.* 2005; 36:348–352. [PubMed: 15637325]
37. Hua Y, Keep RF, Hoff JT, Xi G. Thrombin preconditioning attenuates brain edema induced by erythrocytes and iron. *J Cereb Blood Flow Metab.* 2003; 23:1448–1454. [PubMed: 14663340]
38. Liu DZ, Ander BP, Xu H, Shen Y, Kaur P, Deng W, Sharp FR. Blood-brain barrier breakdown and repair by Src after thrombin-induced injury. *Ann Neurol.* 2010; 67:526–533. [PubMed: 20437588]
39. Halliwell, B.; Gutteridge, JMC. *Free Radicals in Biology and Medicine.* Oxford: Oxford University Press; 1999.
40. Kim CM, Park YJ, Shin SH. A widespread deferoxamine-mediated iron- uptake system in *Vibrio vulnificus*. *J Infect Dis.* 2007; 196:1537–1545. [PubMed: 18008234]
41. Persson HL, Richardson DR. Iron-binding drugs targeted to lysosomes: a potential strategy to treat inflammatory lung disorders. *Expert Opin Investig Drugs.* 2005; 14:997–1008.
42. Liu ZD, Hider RC. Design of clinically useful iron(III)-selective chelators. *Med Res Rev.* 2002; 22:26–64. [PubMed: 11746175]
43. Romeo AM, Christen L, Niles EG, Kosman DJ. Intracellular chelation of iron by bipyridyl inhibits DNA virus replication: ribonucleotide reductase maturation as a probe of intracellular iron pools. *J Biol Chem.* 2001; 276:24301–24308. [PubMed: 11301321]
44. Morgan EH. Chelator-mediated iron efflux from reticulocytes. *Biochim Biophys Acta.* 1983; 733:39–50. [PubMed: 6882755]
45. Klausner RD, Ashwell G, van Renswoude J, Harford JB, Bridges KR. Binding of apotransferrin to K562 cells: explanation of the transferrin cycle. *Proc Natl Acad Sci U S A.* 1983; 80:2263–2266. [PubMed: 6300904]
46. Moos T, Morgan EH. The metabolism of neuronal iron and its pathogenic role in neurological disease: review. *Ann N Y Acad Sci.* 2004; 1012:14–26. [PubMed: 15105252]
47. Regan RF, Chen M, Li Z, Zhang X, Benvenisti-Zarom L, Chen-Roetling J. Neurons lacking iron regulatory protein-2 are highly resistant to the toxicity of hemoglobin. *Neurobiol Dis.* 2008; 31:242–249. [PubMed: 18571425]
48. May JM, Qu ZC, Mendiratta S. Role of ascorbic acid in transferrin- independent reduction and uptake of iron by U-937 cells. *Biochem Pharmacol.* 1999; 57:1275–1282. [PubMed: 10230771]
49. Meguro R, Asano Y, Odagiri S, Li C, Iwatsuki H, Shoumura K. Nonheme-iron histochemistry for light and electron microscopy: a historical, theoretical and technical review. *Arch Histol Cytol.* 2007; 70:1–19. [PubMed: 17558140]
50. Andriopoulos B, Hegedusch S, Mangin J, Riedel HD, Hebling U, Wang J, Pantopoulos K, Mueller S. Sustained hydrogen peroxide induces iron uptake by transferrin receptor-1 independent of the iron regulatory protein/iron-responsive element network. *J Biol Chem.* 2007; 282:20301–20308. [PubMed: 17517884]
51. Kaur D, Lee D, Ragapalan S, Andersen JK. Glutathione depletion in immortalized midbrain-derived dopaminergic neurons results in increases in the labile iron pool: implications for Parkinson's disease. *Free Radic Biol Med.* 2009; 46:593–598. [PubMed: 19118623]
52. Bao L, Avshalumov MV, Patel JC, Lee CR, Miller EW, Chang CJ, Rice ME. Mitochondria are the source of hydrogen peroxide for dynamic brain-cell signaling. *J Neurosci.* 2009; 29:9002–9010. [PubMed: 19605638]
53. Richardson DR, Baker E. Two saturable mechanisms of iron uptake from transferrin in human melanoma cells: the effect of transferrin concentration, chelators, and metabolic probes on transferrin and iron uptake. *J Cell Physiol.* 1994; 161:160–168. [PubMed: 7929602]

54. Sibille JC, Octave JN, Schneider YJ, Trouet A, Crichton RR. Transferrin protein and iron uptake by cultured hepatocytes. *FEBS Lett.* 1982; 150:365–369. [PubMed: 7160481]
55. Lee DW, Andersen JK. Role of HIF-1 in iron regulation: potential therapeutic strategy for neurodegenerative disorders. *Curr Mol Med.* 2006; 6:883–893. [PubMed: 17168739]
56. Lee JY, Keep RF, He Y, Sagher O, Hua Y, Xi G. Hemoglobin and iron handling in brain after subarachnoid hemorrhage and the effect of deferoxamine on early brain injury. *J Cereb Blood Flow Metab.* 2010; 30:1793–1803. [PubMed: 20736956]
57. Strand T, Alling C, Karlsson B, Karlsson I, Winblad B. Brain and plasma proteins in spinal fluid as markers for brain damage and severity of stroke. *Stroke.* 1984; 15:138–144. [PubMed: 6198785]

List of Abbreviations

DAB	diaminobenzidine
HIF	hypoxia inducible factor
HO	heme oxygenase
ICH	Intracerebral hemorrhage
IRP	Iron regulatory protein
LDH	lactate dehydrogenase
MDA	malondialdehyde
MEM10	Minimal essential medium containing 10 mM glucose
MEM10/BSA	MEM10 with 1 mg/ml bovine serum albumin
TBI	Transferrin-bound iron
TfR1	Transferrin receptor-1

Highlights

- > Plasma concentrations of holotransferrin produce iron accumulation and neurotoxicity.
- > Cell death is prevented by iron chelators and antioxidants. > Chelators reduce transferrin-bound iron accumulation and enhance export. > Apotransferrin attenuates only accumulation, and is not protective.> Chelator therapy may be beneficial after blood-brain barrier breakdown.

Fig. 1. Diferric transferrin (holotransferrin) is neurotoxic. A) Culture medium LDH activity ($\pm$ S.E.M, $n = 8-9$ /condition) after 24 hour treatment with indicated concentrations of apotransferrin (Apo) or holotransferrin (Holo). Medium LDH values are scaled to those in sister cultures treated with 300 μ M NMDA (=100), which releases all neuronal LDH without injuring glial cells. The weak signal in sister cultures subjected to medium exchange only (sham) was subtracted from all values to yield the LDH activity associated with neurotoxicity. B) Cultures (6/condition) were treated as in A, and were assayed for malondialdehyde (MDA) at the end of the exposure period. C) Cultures (6-9/condition) were treated for indicated intervals with 3 mg/ml holotransferrin or 18.75 μ M purified

hemoglobin (Hb), which have the same iron concentration (75 μ M) *P < 0.05, *P < 0.01, ***P < 0.001 v. corresponding apotransferrin or Hb-treated conditions, Bonferroni multiple comparisons test.

Fig. 2. Morphologic appearance of cultures treated with transferrin alone or with deferoxamine. Phase contrast photomicrographs of cultures after 24 hour treatment with: A) sham medium exchange only; neurons (arrows) are easily distinguished from the background glial monolayer by their prominent phase-bright cell bodies, which aggregate; B) apotransferrin 3 mg/ml; C) holotransferrin 3 mg/ml; many neurons have degenerated, resulting in loss of definition of somata; D) holotransferrin 3 mg/ml plus 100 μ M deferoxamine; neuronal morphology is preserved. Scale bar = 100 μ m.

Fig. 3.

Holotransferrin neurotoxicity is prevented by iron chelators and antioxidants. A) Culture medium LDH activity ($\pm$ S.E.M, $n = 19-20$ /condition) after 24 hour treatment with 3 mg/ml holotransferrin alone (Holo) or with 100 μ M Trolox (Tro), ascorbate (Asc), deferoxamine (DFO), or 2,2'-bipyridyl (BP); chelators and antioxidants were added at the same time as holotransferrin. Medium LDH values are scaled to those in sister cultures treated with NMDA 300 μ M (=100), which releases all neuronal LDH without injuring glial cells. The weak signal in sister cultures subjected to medium exchange only (sham) was subtracted from all values to yield the LDH activity associated with neurotoxicity. B) Cultures (8/condition) were treated as in A, and were assayed for malondialdehyde (MDA) at the end of the exposure period. C, D) as in A, B, except that chelators and antioxidants were added 6 hours after holotransferrin ($n = 6-8$ /condition in C, 4-5/condition in D). ** $P < 0.01$, *** $P < 0.001$ v. mean value in cultures treated with holotransferrin alone, Bonferroni multiple comparisons test.

Fig. 4.

Effect of holotransferrin alone or with chelators/antioxidants on culture iron. Bright field photomicrographs of Perls' stained cultures, fixed after 24-hour treatment with: A) sham medium exchange only; B) holotransferrin 3 mg/ml alone; C-F) holotransferrin 3 mg/ml plus Trolox 100 μ M (C), ascorbate 100 μ M (D), deferoxamine 100 μ M (E), or 2,2'-bipyridyl 100 μ M (F). Bars represent mean iron cell iron ($\pm$ S.E.M., 5/condition) detected by a ferrozine-based assay after 24-hour treatment with holotransferrin alone or with protective drugs, normalized to culture protein. * $P < 0.05$, *** $P < 0.001$ v. mean signal in holotransferrin alone group, ### $P < 0.001$ v. value in sham group, Bonferroni multiple comparisons test. Scale bar = 100 μ m.

Fig. 5. Effect of chelators and ascorbate on culture iron accumulation. Mean cell ^{55}Fe ($\pm$ S.E.M.), normalized to cell protein content, in cultures treated for 2 hours with: A) 75 nM transferrin-bound ^{55}Fe (Trf-Fe, 0.1 $\mu\text{Ci/ml}$) alone or with 100 μM ascorbate (ASC), deferoxamine (DFO), or 2,2'-bipyridyl (BP); B) 75 nM nontransferrin-bound $^{55}\text{FeCl}_3$ (Fe), alone or with study drugs. ** $P < 0.01$, *** $P < 0.001$ v. mean signal in Trf-Fe or Fe groups, Bonferroni multiple comparisons test, $n = 4-6$ cultures/condition.

Fig. 6.

Net export of iron after transferrin-bound iron treatment is increased by chelators. A) Mean percentage of ^{55}Fe in medium and cells ($\pm$ S.E.M., $n = 5-6$ /condition) in cultures treated with 75 nM transferrin-bound ^{55}Fe ($0.1 \mu\text{Ci/ml}$) for 2 hours, followed by isotope washout and incubation at 37°C in MEM10 containing 1 mg/ml BSA and $100 \mu\text{g/ml}$ unlabeled transferrin (50% saturated) for indicated interval. B) Medium ^{55}Fe , normalized to cell protein content, in cultures treated with transferrin-bound iron (Trf-Fe) as in A, then incubated in MEM10 containing $100 \mu\text{g/ml}$ unlabeled transferrin alone or with $100 \mu\text{M}$ ascorbate (ASC), deferoxamine (DFO), or 2,2'-bipyridyl for 2 hours. ** $P < 0.01$, *** $P < 0.001$ v. mean signal in Trf-Fe group, $n = 5$ /condition.

Fig. 7.

Effect of apotransferrin on holotransferrin toxicity and iron transport. A) Culture medium LDH activity ($\pm$ S.E.M, $n = 14$ /condition) after 24 hour treatment with 1.5 mg/ml holotransferrin (Holo) alone or with 1.5 mg/ml apotransferrin (Apo). Medium LDH values are scaled to those in sister cultures treated with 300 μ M NMDA (=100), which releases all neuronal LDH without injuring glial cells. The weak signal in sister cultures subjected to medium exchange only (sham) was subtracted from all values to yield the LDH activity associated with neurotoxicity. B) Mean cell ^{55}Fe ($\pm$ S.E.M., 6/condition) in cultures treated for 2 hours with transferrin-bound ^{55}Fe as in Fig. 5 (Trf-Fe, 0.1 $\mu\text{Ci/ml}$), alone or with equimolar apotransferrin. C) Medium ^{55}Fe , normalized to cell protein content, in cultures

treated with transferrin-bound iron as in B, followed by washout and 2 hour incubation in MEM10 containing 100 $\mu\text{g/ml}$ holotransferrin (Holo), 100 $\mu\text{g/ml}$ apotransferrin (Apo), or both. *** $P < 0.001$ v. signal in Trf-Fe condition.

Fig. 8. Effect of holotransferrin alone or with chelators/antioxidants on transferrin receptor-1 (TfR1), ferritin, heme oxygenase-1 (HO-1) and ferroportin expression. Bar graphs represent mean TfR1 (A, $\pm$ S.E.M, 6/condition), ferritin (B, 10/condition), HO-1 (C, 6/condition) and ferroportin (D, 5-9/condition) immunoblot band densities from cultures treated for 24h with 3 mg/ml holotransferrin (Holo) alone or with 100 μ M Trolox (Tro), ascorbate (Asc), deferoxamine (DFO) or 2,2'-bipyridyl. Lane order of representative immunoblots stained with anti-TfR1, anti-ferritin, anti-HO-1, anti-ferroportin, or anti-actin (gel loading control) is the same as bar order. *** $P < 0.001$ v. mean signal in holotransferrin group, # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ v. mean value in sham group, multiple comparisons test.

Table 1

Holotransferrin is not toxic to glial cells. Glial cultures were treated with 3 mg/ml holotransferrin (Holo) for 24 hours or subjected to medium change only (Sham). LDH values are scaled to the mean in sister cultures treated with 0.1% Triton X-100, which lyses all cells. Malondialdehyde (MDA) values are normalized to culture protein content. $P > 0.05$, holotransferrin v. sham.

Glia	% LDH Release	MDA (nmol/mg)
Sham	0 ± 1.24 ; n=16	0.47 ± 0.13 ; n=8
Holo	2.91 ± 1.23 ; n=18	0.70 ± 0.28 ; n=8