Full Paper

Human Umbilical Cord Blood–Derived CD34+ Cells Can Be Used as a Prophylactic Agent for Experimental Heatstroke

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Abstract. We attempted to assess the prophylactic effect of human umbilical cord blood–derived CD34+ cells in experimental heatstroke. Anesthetized rats, 1 day before heat stress, were divided into 2 major groups and given CD34− cells (defined by 1 × 10^6 human cord blood lymphocytes and monocytes that contained <0.2% CD34+ cells) or CD34+ cells (defined by 1 × 10^6 human cord blood lymphocytes and monocytes that contained >95% CD34+ cells). They were exposed to ambient temperature of 43°C for 70 min to induce heatstroke. When the CD34− cells–treated or untreated rats underwent heat stress, their survival time values were found to be 20 – 24 min. Pretreatment with CD34+ cells significantly increased survival time (123 – 351 min). As compared with normothermic controls, all CD34− cells–treated heatstroke animals displayed hypotension, hepatic and renal failure, hypercoagulable state, activated inflammation, and cerebral ischemia and injury. However, these heatstroke reactions all were significantly suppressed by CD34+ cells pretreatment. In addition, the levels of interlukin-10 in plasma and glial cell line–derived neurotrophic factors in brain were all significantly increased after CD34+ cell administration during heatstroke. Our data indicate that human umbilical cord–derived CD34+ cells can be used as a prophylactic agent for experimental heatstroke.

Keywords: heatstroke, CD34+ cell, cytokine, multiorgan dysfunction, coagulation

Introduction

Human umbilical cord blood cells (HUCBC) transplantation is a promising therapeutic technique against central nervous system disorders such as conventional stroke (1, 2), traumatic brain injury (3), and spinal cord injury (4). Recently we have also effectively demonstrated that an early in exposure to HUCBC treatment is able to attenuate the cerebral ischemia and neuronal damage that occurred during heatstroke (5). It has been estimated that CD34− cells defined by the lymphocytes and monocytes derived from HUCBC contain less than 0.2% of CD34−-expressing cells (6, 7). It is not known whether CD34− cells (defined by HUCBC lymphocytes and monocytes that contained over 95% CD34−-expressing cells) can be used as a prophylactic agent for experimental heatstroke.

To deal with the question, the current experiments were performed to assess the temporal profiles of cerebrovascular function [e.g., arterial blood pressure, intracranial pressure, and cerebral blood flow (CBF)]; hepatic and renal function [e.g., serum urea nitrogen (SUN), creatinine, aspartate aminotransferase (SGOT), alanine aminotransferase (SGPT), and alkaline phosphatase (ALP) levels in plasma]; coagulable state (e.g., prothrombin time, activated partial thromboplastin time, D-dimer, platelet count, and protein C levels in plasma); and inflammation [e.g., tumor necrosis factor-α (TNF-α) and interleukin-10 (IL-10) levels in serum] during heatstroke in rats (8) with prior administration of CD34− or CD34+ cells 1 day before the initiation of heatstroke.
stress.

In this study, we show that prior administration of CD34+, but not CD34−, cells 1 day before initiation of heat stress do protect against the above-mentioned heatstroke reactions in a rat model of heatstroke.

Materials and Methods

Animals

Adult, male Sprague-Dawley rats (weight: 255 ± 14 g) were obtained from the Animal Resource Center of the National Science Council of the Republic of China (Taipei, Taiwan). The animals were housed 4 in a group at an ambient temperature of 24 ± 1°C, with a 12-h light/dark cycle. Pellet rat chow and tap water were available ad libitum. All protocols were approved by the Animal Ethics Committee of the Chi Mei Medical Center (Tainan, Taiwan) in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, as well as the guide lines of the Animal Welfare Act. Adequate anesthesia was maintained to abolish the corneal reflex and pain reflexes induced by tail pinching throughout all experiments (approximately 8 h) by a single intraperitoneal dose of urethane (1.4 g/kg wt). At the end of the experiments, control rats and any rats that had survived heatstroke were killed with an overdose of urethane.

Surgery and physiologic parameter monitoring

The right femoral artery and vein of rats were cannulated with polyethylene tubing (PE 50), under urethane anesthesia, for blood pressure monitoring and drug administration. Core temperature (Tco) was monitored continuously by a thermocouple inserted into the rectum, while both mean arterial pressure (MAP) and heart rate (HR) were continuously monitored with a pressure transducer. For measurement of intracranial pressure (ICP), the animals were positioned in a stereotaxic apparatus (Kopf 1406; Grass Instrument, Quincy, MA, USA) to insert probes for a Statham P23AC transducer via 20-gauge stainless-steel–needled probe (diameter, 0.90 mm; length, 38 cm), which was introduced into the right cerebral ventricle according to the stereotaxic coordinates of Paxinos and Watson (9).

Induction of heatstroke

The Tco of the anesthetized animals were maintained at about 36°C with an infrared light lamp except in the heat stress experiments. Heatstroke was induced by putting the animals in a folded heating pad of 43°C controlled by circulating hot water. The instant at which the MAP dropped to 25 mmHg from the peak level was taken as the onset of heatstroke (8, 9). After the onset of heatstroke, the heating pad was removed and the animals were allowed to recover at room temperature (24°C). Our pilot results showed that the latency for onset of heatstroke in vehicle-treated rats was found to be 70 ± 2 min (n = 8). Therefore, in the following heatstroke groups of rats, all animals were exposed to 43°C for exactly 70 min and then allowed to recover at room temperature (24°C).

Experimental groups

Two major groups of animals were designated for the experiments. In the normothermic control group, the animals were treated or untreated with an i.v. dose of CD34− or CD34+ (1 × 10^6) in 0.3 ml of phosphate-buffered saline (PBS) per rat and their core temperatures were maintained at about 36°C with an infrared light lamp throughout the entire experiments. In the heatstroke groups, the animals were untreated or treated with an i.v. dose of CD34− cells (1 × 10^6/0.3 ml PBS) or CD34+ cells (1 × 10^5 – 1 × 10^6/0.3 ml PBS), exposed to 43°C for exactly 70 min, and then allowed to recover at room temperature (24°C). Both physiologic parameters and survival time (interval between the initiation of heatstroke and animal death) were observed for up to 480 min (or at the end of experiment).

Human CD34+ cell preparation

Human umbilical cord derived CD34+ cells were obtained from healthy subjects without any diseases using a Direct CD34 Progenitor Cell Isolation kit (Miltenyi Biotec, Bergiseh Gladbach, Germany) and CD34 Multisort kit (Miltenyi Biotec), according to the manufacturer’s protocol. Twenty-four hours before the start of heat stroke, human CD34+ cells (defined by 1 × 10^6 – 1 × 10^6 human cord blood lymphocytes and monocytes that contained >95% CD34+ cells) isolated from human cord blood or CD34+ cells (defined by 1 × 10^6 human cord blood lymphocytes and monocytes that contained less than 0.2% CD34+ cells) were administered via the femoral vein.

Measurements of CBF, brain O2, and brain temperature

A 100-µm-diameter thermocouple and two 230-µm fibers were attached to the oxygen probe. This combined probe measures oxygen, temperature and microvascular blood flow. The measurement requires OxyLite™ and OxyFlo™ instruments. OxyLite 2000 (Oxford Optronix, Ltd., Oxford, UK) is a 2-channel device (measuring PO2 and temperature at two sites simultaneously), whereas OxyFlo 2000 is a 2-channel Laser Doppler perfusion monitoring instrument. The OxyLite has been designed to operate in conjunction with OxyFlo. The combination of these 2 instruments provides simultaneous tissue
blood flow, oxygenation and temperature data. Under urethane anesthesia, the animal was placed in a stereotaxic apparatus, and the combined probe was implanted into the striatum using the atlas and coordinates of Paxinos and Watson (10). The detailed procedures for measurement of brain temperature, PO$_2$, and CBF were described previously (11, 12).

**Biochemical measurements**

Blood samples at 0, 70, and 85 min after initiation of heat stress were drawn by arterial femoral cannulation. Properly calibrated and controlled automated devices were used to determine complete blood counts (Cell Dyn 400; Abbott Diagnostics, Santa Clara, CA, USA), liver profiles (Hitachi 912; Mannheim-Boehringer, Mannheim, Germany), and coagulation profiles (BCS; Dade Behring, Miami, FL, USA). For determination of plasma protein C, recombinant mouse and rat protein C were prepared essentially as described previously (13), and mouse protein C was needed to obtain rabbit anti-mouse protein C polyclonal antibody that was cross-reactive to rat for use in a standard enzyme-linked immunoabsorbant assay. For determination of circulating protein C concentrations, plasma samples for each animal were diluted and assayed in triplicate using this enzyme-linked immunoabsorbant assay; recombinant rat protein C was used as the reference standard.

**Measurement for serum TNF-α, nitric oxide metabolite (NO$_3^-$), and IL-10**

Blood samples were taken at 0, 70, or 85 min after the start of heat exposure for determination of TNF-α, NO$_3^-$, and IL-10 levels. For measurement of serum cytokines, 5 ml of blood was withdrawn from the femoral vein of each rat. The amounts of the cytokines including TNF-α and IL-10 in serum were determined by using a double-antibody sandwich enzyme-linked immunoabsorbant assay (R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s instructions. Optical densities were read on a plate reader set at 450 nm for TNF-α and IL-10. The concentration of TNF-α or IL-10 in the serum samples was calculated from the standard curve multiplied by the dilution factor and was expressed as pg/ml. The NO$_3^-$ concentrations in the dialysates were measured with the Eicom ENO-20 NO$_3^-$ analysis system (Eicom, Kyoto). In the Eicom ENO-20 NO$_3^-$ analysis system, after the NO$_3^-$ and NO$_2^-$ in the sample have been separated by the column, the NO$_2^-$ reacts in the acidic solution with the primary aromatic amine to produce an azo compound. Following this, the addition of aromatic amines to the azo compound results in a coupling that produces a diazo compound and the absorbance rate of the red color in this compound is then measured.

**Neuronal damage score**

At the end of the experiments, animals were killed by an overdose of urethane and their brains were fixed in situ and left in the skull in 10% neutral-buffered formalin for at least 24 h prior to removal from the skull. The brain was removed and embedded in paraffin blocks. Serial (10 μm) sections through the striatum were stained with hematoxylin and eosin for microscopic evaluation. The extent of cerebral neuronal damage structures was scored on a scale of 0 – 3, modified from the grading system of Pulsinelli et al. (14), in which 0 is normal, 1 indicates approximately 30% of the neurons are damaged, 2 indicates that approximately 60% of the neurons are damaged, and 3 indicates that 100% of the neurons are damaged. Each hemisphere was evaluated independently without the examiner knowing the experimental conditions.

**ELISA**

Supernatants were collected from the tissue homogenates of the striatum and ELISA measurements were done with the Promega GDNF Immunoassay System following the recommendations of the manufacturer (Promega, Madison, WI, USA). A standard curve of pure GDNF protein provided in the kit was used to quantify the production of the neurotrophic factors by CD34$^+$ cell therapy.

**Statistical analyses**

Data are presented as means ± S.D. Repeated-measures ANOVA was conducted to test the treatment by time interactions and the effect of treatment over time on each score. The Duncan’s multiple-range test was used for post hoc multiple comparison among means. The Wilcoxon tests were used for evaluation of neuronal damage scores. The Wilcoxon test converts the scores or values of a variable to ranks, require calculation of a sum of the ranks, and provide critical values for the sum necessary to test the null hypothesis at a given significant level. These data were presented as “median”, followed by first (Q1) and third (Q3) quartile. A $P$ value less than 0.05 was calculated as statistical significance.

**Results**

**CD34$^+$ cells improve survival during heatstroke**

Table 1 summarizes survival time values for untreated, CD34$^+$ cells–treated, and CD34$^+$ cells–treated heatstroke rats. It can be seen from the table that the survival time values were found to be 20 – 24 min for untreated or CD34$^+$ cells–treated heatstroke rats. The
values of survival time for CD34+ cells–treated heatstroke rats were not distinguishable from those of the untreated heatstroke rats. However, as compared with those of CD34+ cells–treated heatstroke rats, the survival time values were significantly and dose-dependently greater in those of CD34+ cells–treated heatstroke rats (123 – 351 min over a dose range of 1 x 10^6 – 1 x 10^8 /0.3 ml).

**CD34+ cells protect against cerebrovascular dysfunction and neuronal damage during heatstroke**

Figure 1 depicts the effect of heat exposure (43°C for 70 min) on Tco, MAP, ICP, CPP (MAP-ICP), and CBF in rats pretreated with CD34+ cells (1 x 10^6 cells/0.3 ml PBS) and in rats pretreated with CD34+ cells (1 x 10^6 cells/0.3 ml PBS) one day before the start of heat exposure. A shown in this figure, heat exposure induced significant increases in CPP, MAP, and CBF; and the increases were more pronounced in the CD34+ cell–treated group. However, 15 min after the termination of heat exposure in the CD34+ cells–treated group, all the MAP, CPP, CBF, and PO2 values were significantly lower than those of the normothermic controls (P<0.05). On the other hand, the values of Tco and ICP in the CD34+ cells–treated group were significantly higher 15 min after the termination of 70-min heat exposure than in those of the normothermic controls. Heatstroke-induced arterial hypotension, cerebral ischemia, and hypoxia, but not hyperthermia, were significantly attenuated by treatment with CD34+ cells one day before the start of heat exposure.

In separate experiments, 15 min after the onset of heatstroke, animals were killed for determination of neuronal damage score in the striatum. The data are summarized in Table 2. No neuronal changes were observed in CD34+ cells–treated or CD34+ cells–treated normothermic rats. However, after the onset of heatstroke, animals treated with CD34+ cells displayed higher values of striatal neuronal damage score compared with those of normothermic controls. With the CD34+ cells treatment, neuroprotection ensured.

Figure 2 depicts the plasma levels of activated partial thromboplastin time (aPTT), prothrombin time (PT), platelet count, protein C, and D-dimer for normothermic controls, CD34+ cells–treated heatstroke rats, and CD34+ cells (1 x 10^6/0.3 ml)–treated heatstroke rats. The figure reveals that aPTT, PT, and D-dimer values for rats pretreated with CD34+ cells (1 x 10^6/0.3 ml) 24 h before start of heat stress were all significantly higher at 70 – 85 min after the start of heat exposure than they were for normothermic controls. In contrast, the values for plasma protein C and platelet count were all significantly lower than those of normothermic controls. Pretreatment with CD34+ cells 24 h before initiation of heat exposure significantly attenuated the heat stress-induced increased plasma levels of aPTT, PT, and D-dimers, as well as the decreased plasma levels of protein C and platelet count.

**CD34+ cells attenuate renal and hepatic dysfunction during heatstroke**

Figure 3 depicts the plasma levels of creatinine, SUN, SGOT, SGPT, and ALP for normothermic controls, CD34+ cells–treated heatstroke rats, and CD34+ cells–treated heatstroke rats. It can be seen form the table that the plasma levels of creatinine, SUN, SGOT, SGPT, and ALP for CD34+ cells–treated rats were all significantly higher at 70 – 85 min after the start of heat exposure than they were for normothermic controls. Pretreatment with CD34+ cells 24 h before start of heat exposure.
exposure significantly attenuated the heat stress-induced increased plasma levels of creatinine, SUN, SGOT, SGPT, and ALP.

**CD34**^+ cells attenuate both TNF-α and NO$_x$⁻ over-production but stimulate IL-10 production during heatstroke

Figure 4 depicts the values of serum TNF-α, NO$_x$⁻, and IL-10 in different groups of rats. The values of serum TNF-α and NO$_x$⁻ of CD34^- treated heatstroke rats obtained at 70 or 85 min after initiation of heat exposure were significantly higher than those of normothermic controls. In rats, CD34^+ cells injected intravenously 24 h before heat stress significantly suppressed the increased levels of serum TNF-α and NO$_x$⁻ obtained at 70 or 85 min after initiation of heatstroke. In CD34^- treated heatstroke rats or normothermic controls, their serum levels of IL-10 were maintained at a negligible level. However, 70 or 85 min following an intravenous dose of CD34^+ cells (1 x 10^6/0.3 ml), the serum levels of IL-10 were greatly elevated in heatstroke rats.
Table 2. Effects of heat exposure (43°C for 70 min) plus 15 min of room temperature (24°C) exposure on neuronal damage score values of the striatum from normothermic controls, CD34− cells–treated heatstroke rats, and CD34+ cells–treated heatstroke rats

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Neuronal damage score (0 – 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Normothermic controls</td>
<td>(0, 0)</td>
</tr>
<tr>
<td>2. CD34− cells–treated heatstroke rats (1 × 10^6 in 0.3 ml per rat)</td>
<td>2 (2, 2)*</td>
</tr>
<tr>
<td>3. CD34+ cells–treated heatstroke rats (1 × 10^6 in 0.3 ml per rat)</td>
<td>1 (0.25, 0.75)†</td>
</tr>
</tbody>
</table>

Values represent the median with the first and third quartile in parentheses of 8 rats per group. For determination of neuronal damage score, animals were killed after 70 min of heat exposure plus 15 min of room temperature exposure after termination of heat exposure. No neuronal damages were observed in CD34− cells–treated and CD34+ cells–treated normothermic rats. The data were evaluated by a Wilcoxon signed rank test followed by the Duncan test when appropriate. *P<0.05, compared with group 1; †P<0.05, compared with group 2 (ANOVA followed by the Duncan test).

Fig. 2. Values of activated partial thromboplastin time (aPTT), prothrombin time (PT), platelet counts, protein C, and D-dimer for normothermic control (NC), CD34− cells–treated heatstroke rats (CD34− cells + HS), and CD34+ cells–treated heatstroke rats (CD34+ cells + HS). The values were obtained 0 (empty bar), 70 (diagonally shaded bar), or 85 (cross-hatched bar) min after the initiation of heat exposure in heatstroke rats or the equivalent times in normothermic controls. *P<0.05, in comparison with NC group; †P<0.05, in comparison with CD34− cells + HS group. All heatstroke groups had heat exposure (43°C) withdrawn exactly at 70 min and were then allowed to recover at room temperature (24°C). Bars are each the mean ± S.D. of 8 rats for each group.
**CD34+ cells secrete GDNF in brain**

To elucidate the nature of the soluble factor secreted by the CD34+ cells, analysis of homogenates’ supernatants by specific ELISA for GDNF was done. These revealed that CD34+ cells secreted GDNF (116 ± 18 pg/g) in the striatum of rat brain obtained 85 min after the start of heat stress (Table 3).

**Discussion**

Our previous (15) and present results have shown that hypercoagulable state (e.g., an increase in PT, aPTT, and D-dimer and a decrease in both platelet count and protein C); systemic inflammation (e.g., an increase in plasma levels of TNF-α); and tissue ischemia and injury (e.g., increased plasma levels of SUN, creatinine, SGOT, SGPT, and ALP, and increased cerebral levels of glutamate, glycerol, and lactate/pyruvate ratio) occur during heatstroke. In addition, arterial hypotension, intracranial hypertension, and cerebral hypoperfusion and hypoxia are observed during heatstroke. The present results further show that an early CD34+ cells treatment causes attenuation of the coagulatory, inflammatory, and cerebrovascular dysfunction of a rodent model to heat...
stress. Accordingly, CD34+ cells delivered systemically improves survival during heatstroke in a dose-dependent manner. These findings indicate that prior administration of CD34+ can be a good choice for preventing heatstroke occurrence.

It has been shown that peripherally transplanted adult human bone marrow cells in patients with hematologic malignancies entered the central nervous system and generated neurons (16, 17). Migration and differentiation of bone marrow-derived neural cells within the central nervous system has also been reported (18). After the onset of heatstroke (9) or 24 h prior to the initiation of heat exposure (present results), intravenously delivered CD34+ cells were found in different brain structures. However, it is known that central nervous system availability of grafted cells is not a prerequisite for acute neuroprotection provided that therapeutic molecules secreted by these cells could cross the blood-brain-barrier (19).

Indeed, in the present study, we further demonstrated that intravenously delivered CD34+ cells caused secretion of GDNF in brain during heatstroke. GDNF, a
member of the transforming growth factor-β superfamily, is a potent neurotrophic factor that promotes the survival and morphological differentiation of dopaminergic neurons (20). Intrastriatal grafting of encapsulated GDNF-producing cells in a rat model of Parkinson’s disease displays neuroprotective and restorative effects (21). Adenovirus-mediated GDNF gene transfer protects against ischemic brain damage after transient middle cerebral artery occlusion in the rat (22–24). A more recent report has also demonstrated that mesenchymal stem cells that produce neurotrophic factor reduce ischemic damage in the rat middle cerebral artery occlusion model (25). In the present study, CD34+ cells therapy may have attenuated cerebral ischemia and injury by inducing overproduction of endogenous GDNF during heatstroke.

Intestinal mucosal permeability to endotoxin increases during heatstroke in the rat (26); this alteration enhances production of cytokines that induce nitric oxide (NO) overproduction in the rat (27). Indeed, plasma or cerebral levels of NO are elevated in heatstroke rats (27, 28; present results). Aminoguanidine, an inducible nitric oxide synthase (iNOS) inhibitor, improves heat tolerance in the rat by preserving the splanchnic blood flow (29) and reducing intracranial hypertension and cerebral ischemia and damage (30). An early exposure to HUCBC (5), in addition to ameliorating iNOS-dependent NO overproduction in brain, causes attenuation of cerebrovascular dysfunction or injury that occurred during heatstroke in the rat. In fact, iNOS-dependent NO overproduction may trigger formation of peroxynitrite and subsequently damage lipids, proteins, and DNA, and lead to cell death (31). Indeed, the NO overproduction could be observed (ref. 5 and the present results) and iNOS inhibitors (30) or CD34+ cell (present results) could exert the prophylactic effects in the heatstroke model used in this study.

It has also been shown that acute heart failure (32) and septic shock (33) are associated with overproduction of TNF-α. On the other hand, IL-10 has been shown to possess important anti-inflammatory and immunosuppressive properties through attenuation of TNF-α and other proinflammatory cytokines (34). In the present results, like brain cooling therapy (28), CD34+ cells may ameliorate circulatory shock and cerebral ischemia and injury during heatstroke by suppressing TNF-α overproduction but increasing IL-10 production.

It should be mentioned that the heatstroke-induced hyperpyrexia is not affected by prior administration of CD34+ cells. This indicates that hyperpyrexia may not be the sole cause of heatstroke. CD34+ cells may exert their prophylactic effects through attenuation of multiple organ dysfunction or failure during heatstroke. This hypothesis is supported by several reports. For example, it was found that despite adequate body cooling, heatstroke was often fatal in humans (35, 36). Tissue injury continued to develop after cooling to normal body temperature in 25% of heatstroke patients (36).

The empirical triad used for the diagnosis of classic human heatstroke includes hyperpyrexia, central nervous system disorders, and a history of heat stress (37). Based on this triad, the anesthetized rat, unanesthetized rat, unanesthetized rabbit, unanesthetized mouse, and anesthetized baboon all displayed a uniform response and reacted similarly to humans with heatstroke. It can be derived from this review (37) that the anesthetized rat model used in the current study can nearly mirror the full spectrum of signs and symptoms occurring during heatstroke in humans.

In summary, our results demonstrate that human umbilical cord blood-derived CD34+ cells can be used as a prophylactic agent for heatstroke occurrence. Prior administration of CD34+ cells, in addition to stimulating production of both IL-10 and GDNF as well as reducing NO production, significantly protects against heatstroke reactions. Future studies are required to assess the benefits of using CD34+ cells instead of iNOS inhibitors, IL-10, or GDNF for heatstroke.

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