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Human umbilical cord blood effect on sod mice (amyotrophic lateral sclerosis)☆

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Abstract

In previous studies we observed that human umbilical cord blood (HUCB) could have a protective effect on the onset of disease and time of death in MRL Lpr/Lpr mice which have an autoimmune disease that may be considered similar to human lupus. We believed a temporary xenograph may have occurred in these animals with the disease process delayed and the life span markedly increased. When HUCB is stored at 4°C in gas permeable bags, there is a decrease of the cell reaction in mixed lymphocyte cultures. The blood, however, maintains a significant number of cells capable of producing replatable colonies. This study attempted to determine the effect of HUCB on SOD1 mice (transgenic B6SJL-TgN(SOD1-G93A)1GUR), which have a mutation of the human transgene, (CuZn superoxide dismutase gene SOD1) that has been associated with amyotrophic lateral sclerosis. We previously developed evidence that the survival of lethally irradiated mice was related to the number of human mononuclear cells administered. In the present study, we decided to investigate the effect of a relatively large dose of human mononuclear cord blood cells on SOD1 mice subjected to a sublethal dose of irradiation preceded by antikiller sera (rabbit anti-asialo). The SOD1 mice show evidence of paralysis at 4 to 5 months. The average expected lifetime of these mice is reported to be 130 days (Jackson Laboratory). In this experiment, there were 23 mice. Two mice died before the onset of paralysis. The remainder were divided into three groups: group I: control group of 4 untreated mice; group II: an experimental group of 6 mice treated with antikiller sera, 800 cGy irradiation plus 5×10^6 cogenic bone marrow mononuclear cells; group III: another experimental group of 11 mice treated with antikiller sera, 800 cGy irradiation plus $34.2–35.6 \times 10^6$ HUCB mononuclear cells, previously stored for 17–20 days at 4°C in gas permeable bags. The results were as follows: the average age at death was: (I) 127 days for the untreated control group, (II) 138 days for the group that received 800 cGy of irradiation and cogenic bone marrow (BM) and (III) 148 days for the group that received irradiation and HUCB. ($P < 0.001$ HUCB vs control, $p < 0.01$ HUCB vs BM). The longest surviving mouse in each group was 131, 153, and 182 days old respectively. In summary, large doses of HUCB mononuclear cells produced considerable delay in the onset of symptoms and death of SOD1 mice. These preliminary results may not only indicate that amyotrophic lateral sclerosis is an autoimmune disease, but may also

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indicate a possible treatment for a devastating disease and possibly others.

Keywords: SOD mice; Umbilical cord blood; Amyotrophic lateral sclerosis

Introduction

Experimentally, human umbilical cord blood (1) and clinically bone marrow transplantation, can modify and improve the course of autoimmune disease (2). In xenograph experiments we have found evidence that human umbilical cord blood cells given to MRL Lpr/Lpr mice could significantly increase the length of life of the animal and delay the onset of pathological changes (1). These animals have an autoimmune disease which is somewhat similar to Lupus in humans. Although controversial, there have been arguments presented that amyotrophic lateral sclerosis is an autoimmune disease (3).

One of the primary limitations of the use of human umbilical cord blood (HUCB) for clinical marrow transplantation has been the limitation of the number of stem cells available from a given donor. This is particularly true if marrow transplantation is attempted in an adult and a close HLA match is desirable. From recent publications (4,5), however, there is strong indications that an exact HLA match is unnecessary and it may be possible to combine cord blood samples without regard to HLA typing (6). If pooling of specimens is feasible, large numbers of umbilical cord stem cells can be made available for use in a single clinical case.

Having previously demonstrated that in xenograph experiments that survival of mice receiving lethal irradiation was dependent on the number of cells transfused (7), we undertook this study to explore the possibility that a large number of HUCB could modify the disease process or extend the life of the SOD1 mice. Human umbilical cord blood from multiple donors was combined and relatively large doses of human cord blood mononuclear cells 34.2–35.0 $\times$ 10^6 were given to SOD1 mice which were exposed to sublethal irradiation. These mice have a mutation of the human transgene CuZn superoxide dismutase (8) and this mutation has been related to amyotrophic lateral sclerosis (8).

Materials and Methods

Cord Blood Collection and Storage

The study was approved by the Institutional Review Board of the New Jersey Medical School, Newark, NJ, under federal regulations, Title 45, part 46, paragraph 46, 110 no. 2. Eleven human umbilical cord blood samples were obtained from the placentas of healthy full-term neonates. Each cord blood sample was collected into a 50 ml sterile polypropylene test tube containing 5 ml of citrate phosphate dextrose as an anticoagulant. The volume of the HUCB collected varied from 20 ml to 40 ml. Samples were kept at room temperature until they were sent to the blood bank for storage. The HUCB sample was then transferred into a special polyolefin blood collection bag (Cryocyte Freezing Container, Baxter Healthcare,

Deerfield, IL) that allows gaseous transfer and stored in a 4°C blood bank refrigerator for 17 to 21 days.

Preparing Cord Blood for Injection

Eleven donor specimens were combined according to their blood type (ABO). After storage for 17 and 21 days HUCB units were placed in a 15 ml disposable centrifuge tube and mononuclear cells (MNC) were separated from the whole cord blood by centrifugation for 30 minutes at 1700 RPMs with ficol histopaque (Sigma, St. Louis, MO). Portions of each stored bag were removed to provide the desired number of mononuclear cells ($34.0–35.0 \times 10^6$) per mouse. The MNC cells were then washed twice with phosphate buffered saline (PBS) and centrifuged for 10 minutes at 1000 RPMs. One ml of PBS was added to the pellet for counting. After the viability and counting was determined the MNC were centrifuged for 10 minutes at 1000 RPMs. 0.2 ml of PBS solution was added for final dilution and injection into the mouse (retro-ocular). This process was repeated the next day to bring the total number of HUCB mononuclear cells up to $34.0–35.0 \times 10^6$.

Preparing Bone Marrow for Injection

Bone marrow was obtained from a wild type mouse B6SJL-TgN(SOD1) 2 Gur. These donor mice have the human transgene without the mutation and do not develop paralysis. After euthanization of the mouse, the bone marrow was extracted from the femur and tibia by lavage with PBS. The bone marrow cells were prepared and injected in the same manner as the cord blood cells.

Animals

The test animals were 8 weeks old, transgenice mice B6SJL-TgN(SOD1-G93A)1Gur which have a mutation of the human transgene CuZn superoxide dismutase gene(SOD1). These mice develop paralysis at 4–5 months and the average expected lifetime of these mice is 130 days (Jackson Laboratory, Bar Harbor, ME).

There were 23 mice in the study, 2 of which died prior to onset of paralysis. The remaining 21 animals were divided into 3 groups:

Group I
: A control group of four animals 8 weeks old, randomly selected, with no evidence of paralysis and no treatment.

Group II
: Six animals that received 0.1 ml of Anti-Asialo GM1 antikiller sera 24 hours before irradiation. On the day of injection, each mouse received 800 cGy of irradiation from a Mark-1 irradiator (Cs 137). Irradiation was followed by a transfusion of 5×10^6 nucleated cells (retro-ocular) obtained from the marrow of a wild type congenic female mouse approximately 8 weeks old B6SJL-TgN(SOD1)2Gur.

Group III
: Eleven test animals received 1 cc of anti-killer sera 24 hours prior to 800 cGy of irradiation. Following irradiation they received $34.2–35.0 \times 10^6$ human mononuclear cells

(retro-ocular) that had been separated by Ficol Histopaque after 17–20 days of storage at 4°C in gas permeable bags.

Statistics: The P values are based on 2-tailed student's t-test

Reverse transcriptase-Polymerase chain reaction for detecting human DNA

All organs were taken out immediately after the mice were euthanized. RNA was extracted (using Rneasy, QIAGEN Inc., Valencia, CA 91355) from the whole spleen, liver, thymus, brain, thoracic lymph nodes and bone marrow from both hind femurs. The RNA extracted from human umbilical cord blood was used as positive control (detecting human DNA). An additional negative control to rule out false positive results was RNA extracted from tissue of mice that did not receive any human umbilical cord blood. The RNA was reversed transcribed and cDNA amplified, using kits obtained from Perkin Elmer (Foster City, CA). Primers human growth hormone (HGH) specific for human 5′-TGC CTT CCC AAC CAT TCC CTT A-3′ and 5′-CCA CTC ACG GAT TTC TGT TGT GTT TC-3′ (Product size 434 base pairs), and the housekeeping gene HPRT were employed. The PCR reaction consisted of one cycle of 95°C for 105 seconds, 40 cycles of 95°C for 15 seconds, 60°C for 30 seconds and 72°C for 5 minutes in a GeneAmp PCR System 9600 (applied Biosystems, Weiterstadt, Germany). PCR products were run on an agarose gel (1.5%) (Biozym, Oldndorf, Germany) on a horizontal electrophoresis apparatus (Gibio BRL, Eggenstein, Germany) and visualized with ethidium bromide on a fluorimager (Molecular dynamics, Sunnyvale, CA). Band densities were analyzed using Image Quant software.

End point of experiment

The animals were checked daily for evidence of paralysis. The end point of the animals' life span was death or when the animals became unable to feed themselves. The determination to euthanize, without knowledge of therapy, was made by the animal facility laboratory technicians. The animal facility is an AAA LAC-1 approved laboratory.

Results

The results (Fig. 1) revealed that, as expected (from data received from Jackson laboratory), when the four control mice (Group I, no treatment) were 115 days old they began to show evidence of paralysis and were all dead by 130 days. All but one of the mice receiving bone marrow from the wild type mouse (Group II) congenic, were dead by day 140. In the animals that received HUCB (Group III), only 5 out of the 11 were dead on day 140. By day 165, thirty-five days after the death of the last control mouse (Group I) two mice that had received HUCB were still alive, one showing partial paralysis. At 52 days after the death of the last control animal, the last mouse that had received human cord blood developed evidence of paralysis and was sacrificed 4 days later.

Human DNA was found in four (4) of the eleven (11) SOD1 mice (Group III) Fig. 1. All four (4) of these mice lived 149 days or more, while the last control without treatment (Group I) died at 131 days. The four (4) animals in which human DNA was found in various organs is as follows: In two (2) animals human DNA was found only in the lungs. In one an-

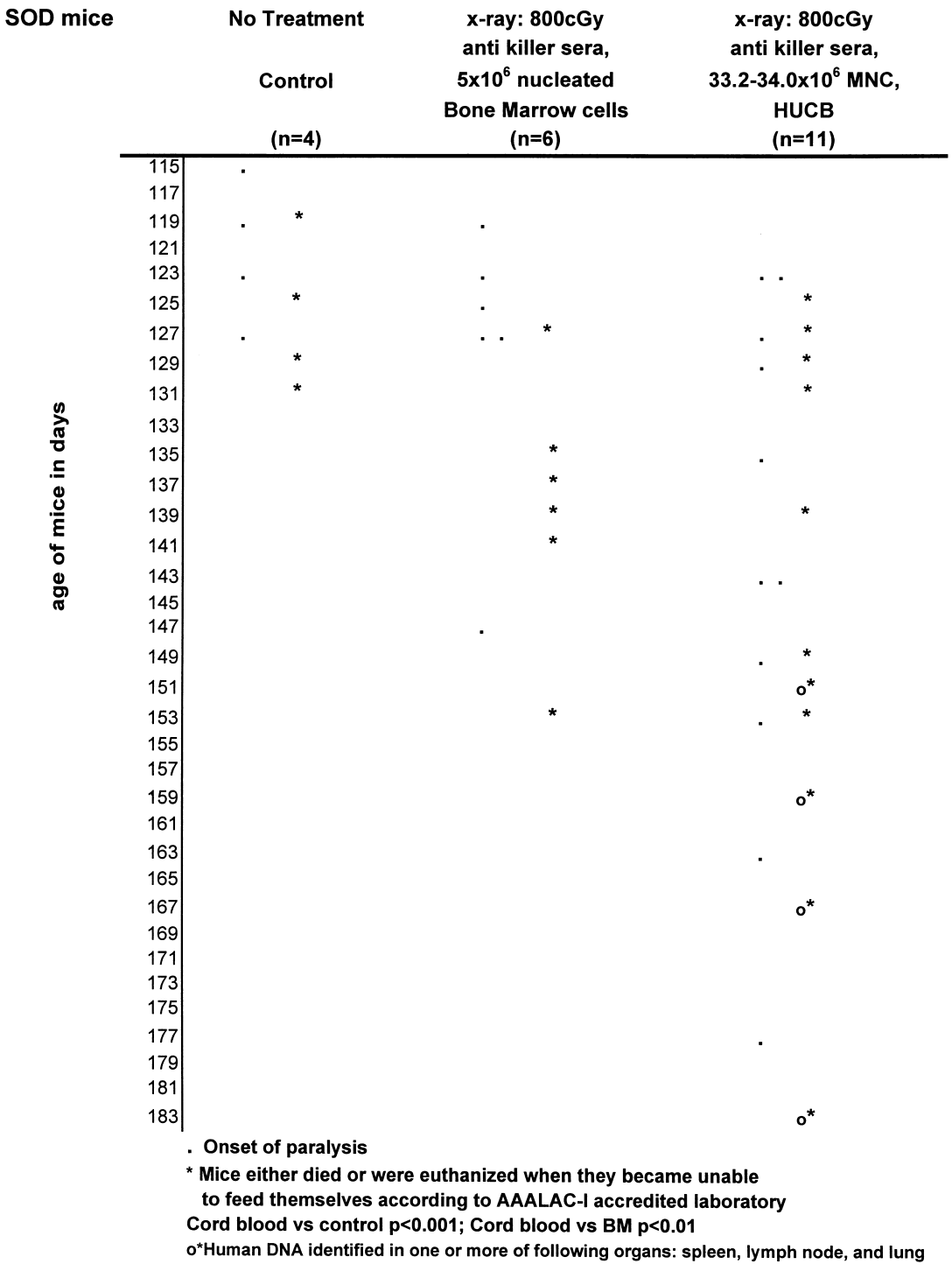

Fig. 1. The cord human blood was stored for 17 to 21 days at 4°C in permeable bags. The bone marrow was obtained from B65JL-Tg (SOD1)2Gur that have the human transgene but do not come down with the disease.

imal that lived 159 days, human DNA was found only in the spleen and one animal that lived 167 days it was found in two organs, the spleen and lymph nodes. Human DNA was not found in the organs examined of the SOD1 mice (Group III) that lived 147 days or less, and was not found in one animal that lived 152 days (Fig. 1). The entire brain, but without the spinal cord was sampled for DNA analysis, but human DNA was not found.

The human blood groups given the mice were A,B&O. No definite pattern as to blood type and survival was detected but the number of donors were only eleven and represented too small a group for accurate analysis.

This study has been repeated and confirms the preliminary study. By increasing the doses of human umbilical cord blood mononuclear cells there was an increased length of survival of SODI mice. Report in progress.

Discussion

Although amyotrophic lateral sclerosis has been considered by some authors as an autoimmune disease, evidence of successful marrow transplants in this condition have not been forthcoming. A search of the literature on this matter was unsuccessful. If, however, this type of therapy (cord blood for marrow transplantation) was to be tried in adult humans, a large number of cells from a close HLA match would have been necessary. In adults the use of HUBC has been limited by the number of stem cells, closely matched or otherwise, from a single donor. In our recent work we found evidence that if multiple cord blood specimens were pooled together they could potentially provide enough stem cells for a successful marrow transplant in an adult (6). This concept of providing an adequate number of cord blood stem cells for transplantation was aided by evidence that by storage at 4°C for 10 to 14 days the reaction of mixed lymphocyte cultures was markedly reduced and stem cells capable of producing replatable colonies remained (6). Furthermore, mixing of cord blood samples from different donors, not HLA matched, did not appear to produce a harmful effect on the colony counts of cell cultures.

Our earlier finding that survival of lethally irradiated mice (7) was related to the number of mononuclear cells given to the animals, led us to consider a megadose approach in treating the SOD1 mice.

In this present experiment the animals that received human cord blood showed a wide variability of the time of the onset of paralysis and death. This can be readily explained by the wide variability of the primitive stem cells found in individual samples of cord blood stored at 4°C (6,9). Based on replating efficiency, the number of replatable colonies varied greatly with different donors (6,9). In the study reported here, the human stem cells given to the mice came from 11 different donors, which could account for the observed wide difference of animal survival. Nevertheless, the results demonstrate the superior quality of this approach as compared with bone marrow transplantation (Group II).

Our previous research has shown that for 30 days human DNA can be demonstrated in various organs of lethally irradiated mice receiving human cord blood transfusion. After this, trace amounts could be detected in the animals for up to 1 year (10). The increased survival of the SOD1 mice receiving cord blood can be explained by the temporary human graft directly or indirectly providing adequate (non mutant) superoxide dismutase and thereby de-

laying the onset of symptoms and death. The other possibility is that the cord blood cells provide enhanced hematopoietic reconsititution of the irradiated hosts own stem cells (11), which in turn provide adequate normal superoxide dismutase to delay the onset of symptoms and death. If an increase in the delay of symptoms and death can be further improved by giving even larger doses of HUCB mononuclear cells, it could have great clinical significance and possibly be relevant to other late onset neurological diseases.

It is well established that the immune system ages. Perhaps vital factors that normally protect the organism are diminished or cease to exist and thereby allow the abnormal process (the mutant SOD1) to be activated and destroy the nerve cells. It is quite possible that a similar process occurs in other diseases, not currently closely aligned with autoimmune diseases, particularly those diseases that have a late onset in life.

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