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THE POTENTIAL FOR THE USE OF MONONUCLEAR CELLS FROM HUMAN UMBILICAL CORD BLOOD IN THE TREATMENT OF AMYOTROPHIC LATERAL SCLEROSIS IN SOD1 MICE

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Abbreviations: ALS = amyotrophic lateral sclerosis, HGH = human growth hormone, MNC = monomuclear cells, PBS = phosphate buffered saline.

Abstract

The SOD1 mice (transgenic B6SJL-TgN(SOD1-G93A)1GUR) have a mutation of the human transgene (CuZn superoxide dismutase gene SOD1) that has been associated with amyotrophic lateral sclerosis (ALS). In a preliminary study, we demonstrated that a megadose of human umbilical cord blood mononuclear cells given intravenously after 800cGy of irradiation could substantially increase the life span of SOD1 mice. This report is an attempt to confirm and expand the preliminary findings. By repeating the study and raising the number of human cord

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blood cells from 33.2-34.0 x 10^6 to 70.2-73.3 x 10^6 there was a further significant increase in the life span of the SOD1 mice. The average life of the controls was 123.5 days while that of mice receiving the larger megadose of cells was 162 days. While all the controls were dead by 130 days, the treated group receiving 70.2-73.3 x 10^6 cells had one animal living up to 187 days and one 210 days. In order to obtain a megadose of cells, pooled blood from different donors was used and did not appear to have a negative effect, but indicated a beneficial effect on survival.

The clinical significance of these findings may extend beyond the potential treatment for amyotrophic lateral sclerosis. This study confirms and extends the preliminary study whereby increasing the dose of human umbilical cord blood cells we were able to substantially further increase the survival of SOD1 mice.

Introduction

The SOD1 mice (transgenic B6SJL-TgN(SOD1-G93A)1GUR) have a mutation of the human transgene (CuZn superoxide dismutase gene SOD1) that has been associated with amyotrophic lateral sclerosis (ALS) (Gurney, Pu, *et al.*, 1994). In a preliminary study, we demonstrated that a megadose of human umbilical cord blood mononuclear cells given intravenously after 800cGy of irradiation could substantially increase the life span of SOD1 mice (Ende, Chen, *et al.*, 2000).

This study was undertaken to attempt to confirm and expand the preliminary findings by repeating the experiment with a larger dose of human umbilical cord monocular cells and the same and lower doses of preparatory irradiation. It was frequently necessary to pool cord blood specimens to provide a megadose of cord blood cells (70.2-73.3 x 10^6).

Materials and Methods

The study was approved by the Institutional Review Board of the New Jersey Medical School, Newark, NJ. The mice were housed in an AAALAC-1 approved animal laboratory. The project was approved by the institutional Animal Review Committee.

Collection and Preparation of Cord Blood

Human umbilical cord blood samples were obtained from the placentae of healthy full-term neonates. Each cord blood sample was collected into a 50mL sterile polypropylene test tube containing 5mL of citrate phosphate dextrose as an anticoagulant. The volume collected

varied from 20mL to 40mL. Samples were kept at room temperature until they were sent to the blood bank for storage. The samples were then transferred into a polyolefin blood collection bag (Cryocyte Freezing Container, Baxter Healthcare, Deerfield, IL) that allows gaseous transfer and then were stored in a 4°C blood bank refrigerator. Donor specimens were combined according to their blood type (ABO) (Ende, Lu, *et al.*, 1999; Lu and Ende, 1997). However, since both the availability of cord blood and the volume required to obtain the desired number of cells varied widely, many of the mice received all the cells from a single donor. After storage for 10-13 days, units were placed in a 15mL disposable centrifuge tube and mononuclear cells (MNC) were separated from the whole cord blood by centrifugation for 30 minutes at 1700 RPM with ficol histopaque (Sigma, St. Louis, MO). Portions or all of each stored bag were removed to provide the desired number of mononuclear cells (70.2-73.3 x 10^6) per mouse. The cells were then washed twice with phosphate buffered saline (PBS) and centrifuged for 10 minutes at 1000 RPM. One mL of PBS was added to the pellet for counting. After the viability and counting were determined, the MNC were centrifuged for 10 minutes at 1000 RPM. 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 mononuclear cells given the animals up to 70.2-73.3 x 10^6.

Preparing Bone Marrow for Injection

Bone marrow was obtained from a wild type mouse B6SJL-TgN(SOD1)2Gur. These donor mice have the human transgene without the mutation and do not develop paralysis. After euthanization, 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 seven weeks old (when received), transgenic mice B6SJL-TgN(SOD1-G93A)1Gur which have a mutation of the human transgene CuZn superoxide dismutase gene (SOD1) (Gurney, Pu *et al.*, 1994). 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 30 mice in the study of which two died prior to the onset of paralysis. The remaining 28 animals were divided into three groups:

Group I: A control group of five animals that received no treatment.

Group II: A control group that received congenic bone marrow consisted of nine animals that received 0.1 mL of Anti-Asialo GM1 antikiller sera 24 hours before irradiation. On the day of injection, five of these mice received 800cGy of irradiation and four mice 400cGy from a Mark-1 irradiator (Cs137). Irradiation was followed by a transfusion of 5×10^6 nucleated cells (retro-ocular) obtained form the marrow of a wild type congenic female mouse approximately eight weeks old B6SJL-TgN(SOD1)2Gur).

Group III: This group consisted of sixteen animals (B6S)JL-TgN(SOD1-G93A)1Gur. Eight test animals received 1 mL of anti-killer serum 24 hours prior to 800cGy of irradiation and another group of eight mice (two died prior to the onset of paralysis) received 400cGy of irradiation. Following irradiation, they received $70.2\text{-}73.3 \times 10^6$ human mononuclear cells (retro-ocular) that had been separated by Ficol Histopaque after 10-13 days of storage at 4°C in gas permeable bags. Each animal received mononuclear cells from pooled cells of one blood type. However, because of the limitation of the availability of donor cells, some animals received all of their mononuclear cells from a single donor (not pooled).

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 removed 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 obtained from both the hind femurs. RNA extracted from human umbilical cord blood was used as a 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 reverse transcribed and cDNA amplified, using kits obtained from Perkin Elmer (Foster City, CA). Primers of human growth hormone (HGH) specific for

human 5'TGC CTT CCC ACC AT TCC 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 at 95 °C for 105 seconds, 40 cycles at 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, Eggensein, Germany) and visualized with ethidium bromide on a flourimager (Molecular Dynamics, Sunnyvale, CA). Band densities were analyzed using Image Quant software.

End Point of the 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. This determination (without knowledge of the treatment) was made by the animal facility laboratory technicians and the animals were euthanized by them.

Results

The present study produced results similar to our preliminary findings (Ende, Chen, *et al.*, 2000). In addition, the increase in the life span of the SOD1 mice that received 800cGy of irradiation appeared to be related to the number of MNC given to the animals. In our preliminary study, where the average life span of the mice was 147 days for the animals receiving 33.2-34.0 x 10^6 MNC and for the untreated controls it was 123.5 days; the average life span of the animals was 162 days for those receiving 70.2-73.3 x 10^6 human umbilical cord blood MNC (Table I).

In the preliminary study where one out or eleven mice lived 183 days, when the numbers of human MNC given to the animals was increased to 70.2-73.3 x 10^6 MNC, three out of eight mice lived for 183 days; one mouse had the onset of paralysis at 204 days and was sacrificed at 210 days. At the time of death of the last control animal in the preliminary experiment, four out of 11 SOD1 mice were dead when 33.2-34.0x10^6 MNC were given (Ende, Chen, *et al.*, 2000). When 70.2-73.3 x 10^6 MNC were given, only one out of eight animals was dead while all the controls were dead.

In the animals receiving only 400cGy of irradiation (1/2 the sublethal dose given the other animals) and 70.2-73.3 x 10^6 MNC, three out of six of the animals lived beyond 130 days, but the death of two mice before the onset of paralysis, made this group difficult to evaluate.

The data indicate that there is also evidence that pooled samples of cord blood may be more dose-effective than individual samples from a single donor (Figure 1).

It may be noted that both the first two animals to die of those receiving 70.2-73.3 x 10^6 MNC and 800cGy of irradiation, received MNC that came from a single donor. A similar finding was noted in animals receiving 400cGy where the first animals to die received MNC from a single donor.

In the preliminary finding, human DNA was found in the longer surviving animals (Ende, Chen, *et al.*, 2000). A similar finding occurred in mice receiving 70.2-73.3 x 10^6 MNC in the present study. Details of these results are presented in Figure 1.

Discussion

This study provides a confirmation of previously reported preliminary findings, wherein the use of human umbilical cord blood mononuclear cells significantly increased the life span of SOD1 mice (Ende, Chen, *et al.*, 2000). In addition, by doubling the dose of human umbilical cord blood cells given the SOD1 mice in this current study, there was a significant further improvement in the length of the life span of these animals.

From our previous studies, using the number of replatable colonies obtained per mL of cord blood as a measure of the most primitive circulating human cord blood stem cells, the cells that produce replatable colonies appear to be relatively rare (Ende, Lu, *et al.*, 1999; Lu and Ende, 1997). For lack of a name and until better identification can be obtained, we have identified these cells as "Berashis" cells (meaning "in the beginning") (Ende, 1995). After 14 days of storage at 4°C in gas permeable bags, the number of replatable colonies obtained from one mL of human cord blood averaged 8 ± 2.9 colonies (or 8 cells) per mL, and some samples of cord blood have none or up to 18 replatable colonies per mL. The variability of replatable colonies could readily explain the variability of animal survival, particularly those receiving all the mononuclear cells from a single donor. If a given donor had very few of the most primitive cells a shorter life span could be expected for the

TABLE I

Effect of Treatment on the Survival of SOD1 Mice

			Average Days of Survival		
CONTROL	No Treatment	n' + n = 9	123.5		
ANTI-KILLER SERA, BONE MARROW					
5 x 10^6 nucleated cells	800 cGy	n' + n = 11	132.5	BM vs Control: $p < 0.05$	
	400 cGy	n = 4	121		
ANTI-KILLER SERA, HUCB					
33.2-34.0x10^6 MNC	800 cGy	n' = 11	147	CB vs Control: $p < 0.01$ CB vs BM: $p < 0.05$	(n vs n' $p<0.05$)
70.2-73.3x10^6 MNC	800 cGy	n = 8	162	CB vs Control: $p < 0.001$ CB vs BM: $p < 0.01$	
70x10^6 MNC	400 cGy	n = 6	131		

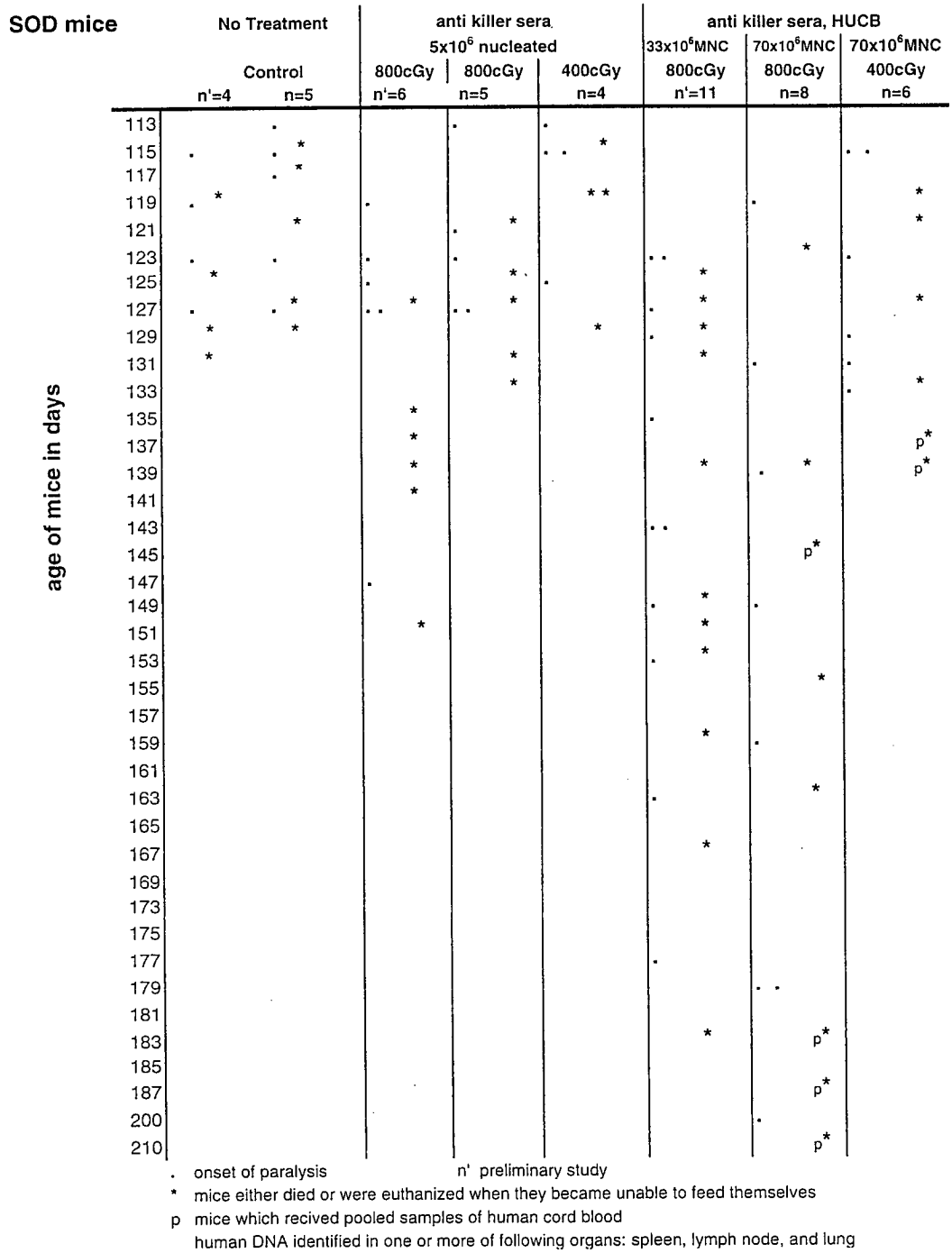

Figure 1.: SOD mice

recipient and a high number of primitive cells could provide a significant increase to the animal's life span.

In a retrospective analysis of the data, it should be noted that all mice that received human cord blood and died within the same time-period as the control mice receiving no treatment, the cells came from a single human donor. In addition, we were unable to find human DNA in the organs examined from the animals that died in the same time-period as the controls (Figure 1).

In a previous study we found a direct relationship between the number of human umbilical cord MNC given and the mice and survival of the animals following lethal irradiation (Ende, Lu, *et al.*, 1996). Because of the factors given above, we postulated it would be necessary to use a large number of cord blood MNC (megadose) if a xenograph, temporary or permanent, was to be produced in SOD1 mice.

One of the primary limitations of the use of human umbilical cord blood is the volume and number of primitive cells recovered from a single donor. The ability to combine and store multiple human umbilical cord blood samples unmatched for HLA and provide enough cells capable of establishing a marrow transplant, producing a partial or complete chimera, has enormous potential for clinical use (Ende, 1995; Ende, Lu, *et al.*, 1999). In addition, the significance of a fetal immune system, as a chimera superimposed on an adult immune system, likewise may have broad clinical significance.

The basic rationale for undertaking this experiment was that the animal's immune system held the mutant transgene in check for a certain time period, after which the mutant gene either increased in strength or the immune system could no longer retard the mutant gene expression. This concept is being further explored since it is quite possible that a similar process applies to other chronic diseases, clinically unrelated, which occur later in life such as multiple sclerosis, Huntington's disease, Alzheimer disease, ulcerative colitis and others.

In summary, the improvement of the life span of SOD1 mice (amyotrophic lateral sclerosis) with the use of megadoses of human umbilical cord blood mononuclear cells, not only suggests that amyotrophic lateral sclerosis may be an autoimmune disease, but also indicates a potential therapy for this debilitating and lethal disease.

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