

Cryobiophysical Characteristics of Genetically Modified Hematopoietic Progenitor Cells

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The freezing responses of hematopoietic progenitor cells isolated from normal donors and from donors with mucopolysaccharidosis type I (MPS I) were determined using cryomicroscopy and analyzed using theoretical models for water transport and intracellular ice formation. The cells from donors with MPS I used in this investigation were cultured and transduced with a retroviral vector for the α -L-iduronidase (IDUA) enzyme in preclinical studies for human gene therapy. The water transport and intracellular ice formation (IIF) characteristics were determined at different time points in the culture and transduction process for hematopoietic progenitor cells expressing CD34 antigen from donors with MPS I and from normal donors. There were statistically significant changes in water transport, osmotically inactive cell volume fraction, and permeability between cells from different sources (normal donors vs donors with MPSI) and different culture conditions (freshly isolated vs cultured and transduced). Specifically, L_{pg} and E_n increased after *ex vivo* culture of the cells and the changes in permeability parameters were observed after as little as 3 days in culture. Similarly, the IIF characteristics of hematopoietic progenitor cells can also be influenced by the culture and transduction process. The IIF characteristics of freshly isolated cells from donors with MPS I were statistically distinct from those of cultured and transduced cells from the same donor. The ability to cryopreserve cells which are cultured *ex vivo* for therapeutic purposes will require an understanding of the biophysical changes resulting from the culture conditions and the manner in which these changes influence viability. © 1999 Academic Press

Key Words: water transport; hematopoietic stem cells; gene therapy.

There has been a significant interest in gene therapy involving hematopoietic progenitor cells. One reason for the interest in this specific cell type is the accessibility of these cells, which are found principally in bone marrow. Additionally, successful gene transfer into primitive progenitor cells can permit repopulation of the donor with modified cells capable of self renewal and therefore resulting in the potential alleviation of the symptoms of the genetic defect for the lifetime of the donor. Finally, advances in our understanding of hematopoiesis and methods of *ex vivo* manipulation of progenitor cells have provided a strong foundation for the development of clinical protocols for human gene therapy.

Mucopolysaccharidosis type I (MPS I) is a disorder resulting from deficiency of α -L-iduronidase enzyme (IDUA), an enzyme partially

responsible for the catabolism of glycosaminoglycans (GAGs). Without IDUA, GAGs accumulate in the lysosome of cells. Clinical manifestation of MPS I can include hepatosplenomegaly, mental retardation, and cardiac and pulmonary failure (14, 24). Human studies using allogeneic bone marrow transplantation have shown alleviation of the symptoms of MPS I (25). Genetically modified progenitor cells may permit a similar therapeutic effect without the additional risks associated with allogeneic bone marrow transplantation (i.e., graft versus host disease).

The ability to cryopreserve cells that have been genetically modified is important for the clinical application of human gene therapy. Specifically, cryopreservation permits transportation of cells to a clinical location, pooling of cells to reach a therapeutic dose, and time for the completion of safety and quality control testing. Cryopreservation also permits coordination of the infusion of cell-based gene therapy products with donor care regimes.

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Bone marrow has been successfully cryopreserved since 1955 (1). At present, cryopreservation of bone marrow for autologous transplantation, in particular, has become standard clinical practice (19). Emerging progenitor cell therapies (both somatic cell and gene therapies) involve the use of a series of processing procedures. Typically, progenitor cells are positively selected from a mixed population of cells obtained from bone marrow, apheresis products, or umbilical cord blood. The cells are subsequently cultured/expanded in a stem cell growth medium. In the case of gene therapy, the cells are then transduced with the desired gene.

It is not clear whether conventional bone marrow cryopreservation protocols would be appropriate for use in the preservation of progenitor cells that have been manipulated for use in gene therapy. Studies of other cell types have shown that culturing freshly isolated cells produces a change in the freezing characteristics of the cells as a function of time (7, 29). The additional concern with the culturing of hematopoietic progenitor cells is that culturing will promote differentiation of the progenitor cells (25). The potential exists that conventional freezing protocols developed for minimally processed bone marrow may not be appropriate for use on progenitor cells that have been extensively manipulated. In addition, it will be important to establish the similarities and differences between the freezing behavior of cells from normal donors and that from donors with a genetic defect, which in this investigation was MPS I.

Multiple processes have been found to affect the post freeze-thaw survival of cells. During freezing, solute is rejected from the solid phase, producing an abrupt change in concentration in the unfrozen portion of the solution. A cell responds to this perturbation by expressing water to reach a new equilibrium state between intracellular and extracellular solutions. At high cooling rates, equilibrium cannot be maintained because the rate at which the chemical potential in the extracellular solution is being lowered is much greater than the rate at which water can diffuse out of the cell. The end result of this

imbalance is that intracellular ice formation (IIF), which is lethal to the cell (cf. 21 for review), is observed. At low cooling rates, cells are exposed for long periods of time at high subzero temperatures to high extracellular concentrations, resulting in potentially damaging high intracellular concentrations (10, 18). Mechanical stresses during slow freezing are also considered to contribute to damage (12).

Water content and the transport of water are common elements in the previous description of the response of a biological system to freezing. Similarly, the correlation between IIF and lethal damage necessitates the determination of IIF characteristics. The purpose of this investigation was to quantify the water transport and IIF characteristics of progenitor cells which were modified for use in gene therapy to treat a donor with MPS I. A comparison was also made between the freezing characteristics of cells from a normal donor and those from a donor with MPS I. Determination of IIF and water transport characteristics are important in quantifying the effect of the manipulation protocols on the physical properties of the cells and in developing appropriate cryopreservation protocols for gene therapy products.

MATERIALS AND METHODS

Cell Isolation

Mononuclear cells (MNCs) were obtained from normal donors and donors with MPS I. Informed consent and permission from the local Institutional Board were obtained. The donors were given 7.5 $\mu\text{g}/\text{kg}$ of granulocyte colony stimulating factor (G-CSF) (Amgen, Thousand Oaks, CA, U.S.A.) for 4 days prior to apheresis in order to increase the number of hematopoietic progenitor cells in circulation.

Upon completion of the apheresis, using positive selection, the cell population was enriched in cells expressing the CD34⁺ antigen. In the case of apheresis collections from donors with MPS I, enrichment of cells expressing the CD34⁺ antigen was performed using the Ceparate SC system (CellPro, Inc., Bothell, WA, U.S.A.). The cells obtained from the apheresis

product were treated with a monoclonal antibody to the CD34 antigen. The cells were then passed over a column of avidin-coated beads, which allowed the CD⁺ cells to bind to the beads in the column. Unlabeled cells were not retained in the column and passed through. Finally, the CD34⁺ cells that were bound to the beads in the column were removed using gentle agitation.

For cells obtained from normal donors, positive selection for cells expressing the CD34⁺ antigen was performed using the Isolex 50 (Baxter, Deerfield, IL, U.S.A.). Anti-CD34 murine monoclonal antibody (primary antibody) was mixed with the cell suspension to permit binding of the antibody to the target cell. The suspension was washed to remove unbound antibody, and target cells (CD34⁺ cells) were rosetted with Dynal paramagnetic microspheres coated with sheep anti-murine antibody. The rosetted target cells were then separated from the rest of the cell suspension using the Isolex 50 magnet cell separator. After washing, the target cells were released from the beads by the use of PR34⁺ stem cell releasing agent. The remaining Dynabeads were then separated from the freed target cells using the magnetic separator.

The final purity of the cells (using either method of separation) was determined using flow cytometry. Cells were labeled with phycoerythrin-conjugated antibody to CD34 and the fraction of cells expressing the antigen was determined. The percentage of cells expressing the CD34 antigen after purification with the column ranged from 60 to 80%.

Cell Culture

For cells obtained from donors with MPS I, approximately $15\text{--}20 \times 10^6$ cells were inoculated into a cellulose acetate hollow-fiber bioreactor (HFBR) cartridge (Cellco, Germantown, MD, U.S.A.). The cells were cultured using a serum-free medium containing X-Vivo 10 (BioWhittaker, Walkersville, MD, U.S.A.) supplemented with 1% human serum albumin (Baxter, Round Lake, IL, U.S.A.), 2 mM L-glutamine, 20 ng/ml rIL-3, 75 ng/ml rIL-6, and 25 ng/ml stem

cell factor (R & D Systems, Minneapolis, MN, U.S.A.). The culture medium was circulated using a media pump (Quad Pump, Cellco) according to manufacturer instructions.

Cell Transduction

After approximately 72 h in culture, the cells from donors with MPS I were transduced with the retroviral vector LP1CD (obtained from Dr. C. Whitley). The construction and validation of the vector have been described previously (17). The cells were transduced twice per day for 2 days, resulting in a total of four transductions. A frozen bag of supernatant containing the retroviral vector was thawed in a 30°C water bath and then supplemented with protamine sulfate to a final concentration of 5 µg/ml. The average viral titer of LP1CD was 5×10^5 colony forming units/ml. The multiplicity of infection was 2. The transduced cells were harvested on the morning of day 5. For experiments using cells from normal donors, the cells were transduced with retroviral vector LNGFR described previously (20), using the same culture media and transduction protocols used with cells from donors with MPS I.

Cryomicroscopy System

The controlled freezing experiments were performed using a cryomicroscope that consisted of a Zeiss general research microscope (Carl Zeiss, FRG) fitted with a specially designed cryostage described in detail elsewhere (3). The response of the cells during freezing was detected by a camera (Optronics LX450A) and transmitted to a video cassette recorder (JVC HR-S6900U) and a color monitor (Sony PVM 1344Q) for subsequent image analysis. A computer-based temperature controller and video interface (Thermascope and Datavideo, Interface Techniques, Cambridge, MA, U.S.A.) were used to specify and control the stage temperature. A 40× bright-field objective (Carl Zeiss) was used with a 2× optivar (Carl Zeiss) for magnification of the experiments. The freezing studies were performed on a thin convection stage. The experimental sample was placed on a composite window consisting of an optically

transparent heating layer made of indium tin oxide, a thermocouple for sensing temperature, and a sapphire substrate designed to isolate the thermocouple from the freezing solution and reduce temperature gradients. Gaseous nitrogen, chilled in a bath of liquid nitrogen, provided refrigeration for the cryostage. Stage temperature during experiments was controlled by adjusting the amount of power to the heating layer using a Tempsoft software package (Interface Techniques). For a freezing experiment, a 3- to 5- μL sample of the cell suspension was placed between the cryostage window and a glass coverslip. Silicone grease was used to seal the edges of this system to prevent evaporation of the solution and to act as an anchor for the coverslip. The sample was cooled from room temperature to a holding temperature of approximately -2°C . At that time, a chilled probe was used to seed ice formation in the extracellular solution. Immediately after seeding, the sample was cooled at a constant rate to a final temperature of -60°C . The stage was then warmed to room temperature and the sample removed. No viability tests were performed on the samples because of the difficulty of recovering cells from the stage after a freezing protocol.

The total water content of the cell includes both water available for transport and osmotically inactive water. In a series of independent experiments, the osmotically inactive cell volumes were determined. A series of solutions of increasing osmolarity (0.3 to 1.0 osm) were made by adding sucrose to Iscove's modified Dulbecco's medium (IMDM, GIBCO, Grand Island, NY, U.S.A.). The cells were added to these solutions. The static cell volumes for the cells resuspended in these hypertonic solutions were determined using image analysis.

Water Transport Model

The flow of water across the cell membrane at subzero temperature has been modeled using a nonequilibrium model developed by Mazur (11). Assuming that equilibria of temperature and pressure prevail between the intra- and the

extracellular media (11), the following equation can be written,

$$\frac{dV}{dT} = \frac{L_p A R T}{\nu_w B} \left[\ln \left(\frac{V - V_b}{(V - V_b) + \nu_w (\nu_s n_s)} \right) - \frac{\Delta H_f}{R} \left(\frac{1}{T_r} - \frac{1}{T} \right) \right], \quad [1]$$

where V is the cell volume, T is the temperature (absolute), L_{pg} is the hydraulic permeability, A is the surface area of the cell, B is the cooling rate, ν_w is the partial molar volume of water, ν_s is the partial molar volume of salt, V_b is the osmotically inactive cell volume fraction, n_s is the dissociation constant for NaCl, T_r is the equilibrium freezing temperature for pure water (273.15 K), and ΔH_f is the latent heat of fusion of water.

The hydraulic permeability of the membrane, L_p , is a function of the temperature. Assuming an Arrhenius relationship (8, 23), the permeability is expressed as a function of temperature

$$L_p = L_{pg} \exp \left(- \frac{E_a}{R} \left(\frac{1}{T} - \frac{1}{T_r} \right) \right), \quad [2]$$

where L_{pg} is the permeability of the cell membrane to water at the reference temperature, T_r , and E_a is the apparent activation energy for the water transport process.

Implicit within the development of these equations is the assumption that thermal equilibrium exists within the immediate region around the cell and the temperature difference across the cell membrane is less than 0.01°C . The intracellular solution is assumed to be ideal and dilute. As with most mammalian cells, it is assumed that the hydrostatic or turgor pressure across the cell membrane is negligible. A more complete discussion of the assumptions behind this model and the errors associated with these assumptions can be found in Levin *et al.* (9) and more recently in Toner *et al.* (23).

Intracellular Ice-Nucleation Model

Experimental evidence suggests that there is a correlation between IIF and fatal cell injury for many cell types (21). As such, cryobiolo-

gists have attempted to develop models to represent the process of IIF and to characterize the mechanism by which IIF damages the cell. Recently, a model for ice formation in biological cells has been developed (22) based on heterogeneous nucleation theory for condensed systems (24). Subsequently, the model has been expanded to include the effects of different factors, such as the presence of cryoprotective agents (CPAs) and diffusion-limited crystal growth (5, 6).

If the nucleation of ice crystals within cells results from a series of bimolecular reactions and if IIF results from the heterogeneous nucleation seeded by a structure within the cell, the nucleation rate can be written as

$$J_{\text{het}} = \Omega_o \frac{N^s}{N^o} \frac{\eta_o}{\eta} \left[\frac{T}{T_{fo}} \right]^{1/2} \exp \left[\frac{-\kappa_o}{\Delta T^2 T^3} \left(\frac{T_f}{T_{fo}} \right)^4 \right], \quad [3]$$

where $\Delta T = (T_m - T)$ is the undercooling, T_m is the equilibrium melting point, and Ω_o and κ_o are the kinetic and thermodynamic coefficients of the nucleation rate. A more complete description of Eq. [3] can be found in Ref. (22). The prefactor Ω_o is a function of the number of water monomers present, the viscosity of the solution, and a heterogeneous nucleation factor, which accounts for the interaction between the catalytic surface and a cluster of water molecules (6). The exponential factor, κ_o , is a function of the interfacial free energy between the ice and the solution and a heterogeneous nucleation factor, which accounts for the interaction between the catalytic surface and a cluster of water molecules. At present, theoretical models to predict values of Ω_o and κ_o for specific cell types do not exist. As with the water permeability parameters, values for Ω_o and κ_o must be obtained empirically from experimental data.

Toner *et al.* have hypothesized that IIF is catalyzed by the plasma membrane. Assuming sporadic nucleation of identical cells, the probability of IIF assuming surface-catalyzed nucleation, PIF^{SCN} can be expressed as

$$\text{PIF}^{\text{SCN}} = 1 - \exp \left[- \int_{T_{\text{seed}}}^T A J_{\text{het}} dt \right], \quad [4]$$

where A is the surface area of the plasma membrane and t is time. The nucleation rate not only reflects the influence of the nucleation parameters but also reflects the solution undercooling. An estimate for the undercooling of the cytosol can be obtained using the water transport equations (Eqs. [1] and [2]). The fraction of cells with ice observed during a freezing protocol reflects a coupling between IIF and water transport.

This model for IIF has been used to analyze a variety of biological systems, including mouse oocytes, islet B-cells, *Drosophila melanogaster* embryos, rat hepatocytes, and isolated protoplasts from *secale cereale* (21). This analysis has involved the correlation of experimentally measured kinetics of IIF with the theory and the determination of multistep protocols based on model predictions.

Data Analysis

The volumetric response of the cells during a freezing experiment was analyzed to provide an estimate of the water transport from each cell. The corresponding image analysis was done on a Gateway PS90 computer (Gateway Corp., North Sioux City, SD, U.S.A.) using MetaMorph image processing software (Universal Imaging Corp., West Chester, PA, U.S.A.). Images obtained at specific time points were digitized from the videotape and stored on optical discs. A cursor was used to trace the outline of the cell boundary and the enclosed area was calculated and entered into a spreadsheet that included other information, such as the temperature. The cross-sectional area of the cell was related to the cell volume, assuming spherical geometry. For each cooling rate, cell volumes were obtained at approximately 10 different temperatures, and for the same experimental conditions, between 6 and 12 cells were analyzed.

As indicated previously, the hydraulic permeability, L_{pg} , and its temperature dependence, E_a ,

can be estimated from the experimental measurements of cell area as a function of time/temperature using the previously described model. The inverse curve-fitting method used to relate the experimental measurements to the models uses nonlinear regression analysis to produce the permeability values that would yield the best fit between experimental volume measurements and theoretical volumes with the criteria of minimizing the χ^2 statistic (9). The values of L_{pg} and E_a can be used to predict water transport and water content for an arbitrary freezing protocol. The predictions of water content as a function of temperature used in this investigation were calculated using Cryosim software (26).

Cells with internal ice exhibit an increase in opacity during the freezing process, which can be observed and recorded as a function of cooling rate, time, and temperature. Estimates for the values of Ω_o and κ_o can be obtained from the fraction of cells which form internal ice as a function of temperature for a specific cooling rate using equations and an inverse fitting technique very similar to those described for the water transport analysis.

Statistical analysis of the water transport and IIF data obtained from different donors and culture conditions was performed using Stat-View software (Berkeley, CA, U.S.A.). Cell size (cross-sectional area) was analyzed using an unpaired t test. Water transport and dehydration characteristics were compared using analysis of variance (ANOVA). The statistical analysis of the fraction of IIF as a function of temperature and the cumulative fraction of cells with IIF as a function of cooling rate was performed using the Wilcoxon signed-rank test.

RESULTS

Water Permeability Parameters

The cell size and corresponding initial cell volume are of interest in freezing studies because of the influence of total water content on cell freezing response. It was observed that there was a change in cell size with time as the cells were cultured and transduced. In order to

quantify those differences, the cross-sectional area was determined using the image analysis software previously described. The cell size, represented by the cross-sectional area of the cell, increases during the culture and transduction protocol. Freshly isolated cells from a donor with MPS I had an average cross-sectional area of approximately $50.1 \pm 11.1 \mu\text{m}^2$ (mean \pm SD). After 3 days in culture but before transduction with the retroviral vector, the average area increased to $111 \pm 43 \mu\text{m}^2$. Upon completion of the culture and transduction process (day 5 in culture), the average area of cells from patients with MPS I was $108 \pm 29 \mu\text{m}^2$, which was similar to that measured on day 3. Control experiments were performed in which the cells from the same donor were cultured for 5 days using the stem cell culture media previously described. On days 3 and 4 of the protocol, the cells were sham transduced with culture medium that did not contain viral particles. As such, the cells in the control experiments were cultured under the same conditions but not exposed to the viral supernatant and therefore not genetically modified. The average area for the cells in these control cultures was $126 \pm 33 \mu\text{m}^2$. The cell cross-sectional area for cells at day 0 (freshly isolated cells) was statistically different from that measured at either day 3 or day 5 ($P < 0.0001$). The cross-sectional area of the cultured and transduced cells from donors with MPS I after culture and transduction was less than that of cells from the same donor that had been cultured but sham transduced ($P < 0.0002$).

The average cross-sectional area of a freshly isolated CD34⁺ cell from a normal donor was $57 \pm 13 \mu\text{m}^2$, and after culture and transduction the average cross-sectional area increased to $116 \pm 33 \mu\text{m}^2$. As with the cells from donors with MPS I, the differences in cross-sectional area measurements were statistically distinct when freshly isolated cells were compared with cells after 5 days in culture ($P < 0.0001$). The differences in cross-sectional area of the cells from normal donors after culture and transduction (day 5) and CD34⁺ cells cultured for 5 days with sham transduction were not statistically

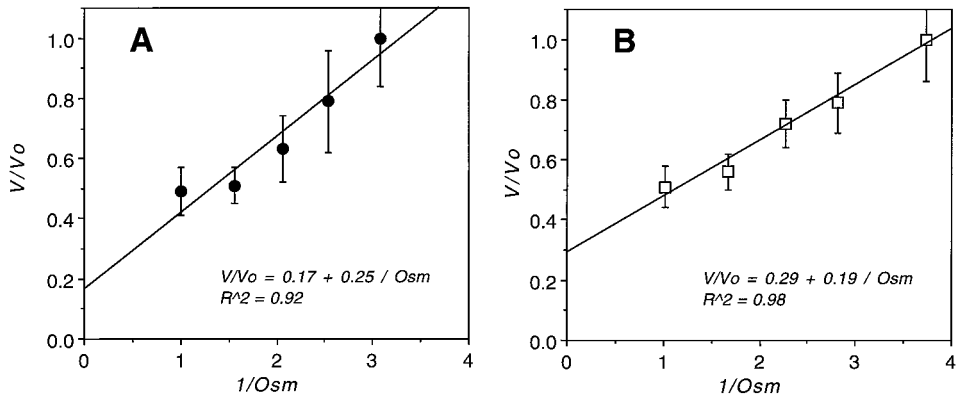


FIG. 1. Normalized cell volume fraction, V/V_o , as a function of the inverse solution osmolarity for freshly isolated cells from (A) donors with MPS I and (B) normal donors. Error bars indicate standard deviations of the mean. Linear regression was used to determine the Boyle–van’t Hoff relationship for the data represented. The number of cells used in these experiments ranged between 41 and 100.

significant, in contrast to that observed in cells from donors with MPS I.

The total volume of the cell consists of the volume of water available for water transport plus the osmotically inactive volume fraction of the cell. The osmotically inactive cell volume fraction was determined based on the equilibrium volume of cells in hypertonic solutions of increasing concentration. Assuming that cells behave as perfect osmometers, the decrease in cell volume observed at increasing solution concentrations can be extrapolated linearly to obtain an estimate for the cell volume fraction which is osmotically inactive.

In Fig. 1, the normalized cell volume (volume at a given concentration divided by the volume under isotonic conditions) as a function of the inverse of solution osmolarity is given for donors with MPS I and for normal donors (Boyle–van’t Hoff plot). Extrapolating to infinite concentration, the osmotically inactive cell volume fraction for freshly isolated cells was 0.17 for donors with MPS I and 0.29 for normal donors. After culture and transduction, the osmotically inactive cell volume fraction was 0.21 for donors with MPS I and 0.20 for cells from normal donors. Osmotically inactive cell volume fractions were determined for cells after 3 days in culture (pretransduction) and these values were not statistically different from those

obtained after culture and transduction (data not shown).

The increase in concentration of the extracellular solution observed during freezing provides a driving force of the exoosmosis of water. The decrease in cell volume as a function of temperature for a given cooling rate was determined from the video recordings of the experiments. Based on these measurements, estimates for the hydraulic permeability, L_{pg} , and activation energy, E_a , can be obtained. The normalized volume, V/V_o (volume at a given temperature, T , divided by the initial volume), as a function of temperature for cultured and transduced cells obtained from donors with MPS I frozen at a rate of $10^\circ\text{C}/\text{min}$ is given in Fig. 2. Based on the normalized volume as a function of temperature, L_{pg} was calculated to be $1.35 \times 10^{-14} \text{ m}^3/\text{N s}$ and E_a was 103 kJ/mol .² The dehydration behavior of the cells was also determined as a function of cooling rate. The cells from a donor with MSP I which were cultured and transduced were frozen at cooling rates ranging from 10 to $40^\circ\text{C}/\text{min}$. For the cells frozen at $10^\circ\text{C}/\text{min}$, there is extensive dehydration. As the cooling rate increases, the time for transport out of the cell decreases and this is reflected in the nor-

² To convert L_{pg} to $\mu\text{m}/\text{atm}/\text{s}$, multiply by 6.078×10^{12} . To convert E_a to kcal/mol , multiply by 0.239 .

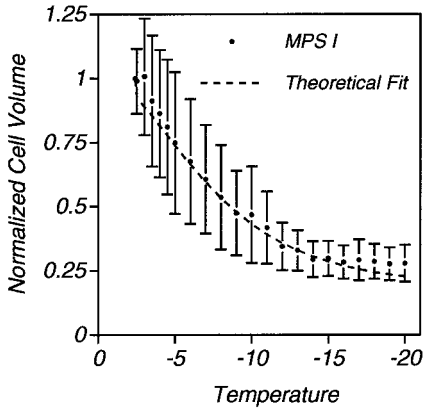


FIG. 2. Normalized cell volume fraction, V/V_0 , as a function of temperature for cultured and transduced cells from donors with MPS I frozen in IMDM at a cooling rate of $10^\circ\text{C}/\text{min}$. The dashed line indicates the best fit for data for the water transport parameters L_{pg} and E_a . (cf. Eqs. [1] and [2]). Five cells were used in these experiments.

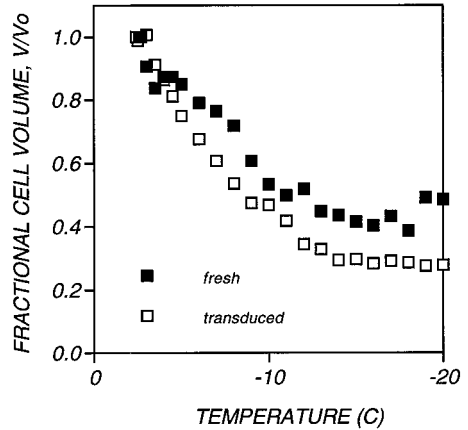


FIG. 3. Normalized cell volume fraction, V/V_0 , as a function of temperature for freshly isolated cells and for cultured and transduced cells from donors with MPS I. The cells were frozen in IMDM at a cooling rate of $10^\circ\text{C}/\text{min}$. Error bars were omitted to improve clarity. The experiments used 5 cultured and transduced cells and 11 freshly isolated cells.

malized volumes measured. Estimates for L_{pg} and E_a were obtained from the normalized volumes as a function of temperature measured at each cooling rate (cf. Table 1).

Changes in the membrane permeability with time resulting from the culture and transduction process should be reflected in differences in the observed dehydration under specific freezing conditions and in the values for L_{pg} and E_a . In

Fig. 3, the normalized volume as a function of temperature for freshly isolated $\text{CD}34^+$ cells and cultured and transduced cells from donors with MPS I is given for a cooling rate of $10^\circ\text{C}/\text{min}$. The differences in dehydration between fresh and cultured and transduced cells from donors with MPS I as a function of temperature for a constant cooling rate were statistically significant ($P < 0.001$) using ANOVA. Water transport characteristics were determined for cells from donors with MPS I during specific points in the culture process. The average values of L_{pg} and E_a for freshly isolated cells, cells at day 3 in culture (pretransduction), cultured and transduced cells (day 5), and control cultures (day 5, nontransduced) are given in Table 2. These results indicate that the water permeability does not change after an additional 2 days in culture.

TABLE 1
Water Transport Characteristics of $\text{CD}34^+$ Cells Obtained from Donors with MPS I and Cultured and Transduced at Various Cooling Rates

B^a ($^\circ\text{C}/\text{min}$)	L_{pg}^b ($10^{14} \text{ m}^3/\text{N s}$)	E_a^c (kJ/mol)	n^d	κ^e (10^4)
10	1.35	130	17	6.7
20	0.78	41	21	3.3
30	1.35	71	30	3.2
40	0.58	24	24	1.8
Avg \pm std	1.0 ± 0.4	60 ± 35		

^a Cooling rate.

^b Hydraulic permeability at reference temperature, $T_r = 273 \text{ K}$.

^c Apparent activation energy.

^d Number of cells.

^e χ^2 statistic as a measure of goodness-of-fit.

In order to compare the osmotic response of a less purified population of cells, cryomicroscopy experiments were performed using freshly isolated MNCs from donors with MPS I. Freshly isolated MNCs from donors with MPS I were frozen at three different cooling rates (10, 20, and $40^\circ\text{C}/\text{min}$). The average value of

TABLE 2
Average Water Transport Characteristics of CD34⁺ Cells
Obtained from Donors with MPS I at Different Time Points
in Culture

Culture condition	L_{pg} ($10^{14} \text{ m}^3/\text{N s}$)	E_a (kJ/mol)	V_b (%) ^a
Freshly isolated Pretransduction (day 3)	0.25	33	40
Transduced (day 5)	1.9	70	— ^b
Control (day 5, nontransduced)	1.0	60	17
	1.2	70	— ^b

^a Percentage of volume which is osmotically inactive.

^b Not determined.

L_{pg} was $1.7 \pm 1.0 \times 10^{-14} \text{ m}^3/\text{N s}$ and that of E_a was $153 \pm 18 \text{ kJ/mol}$. ANOVA of the dehydration of MNCs and CD34⁺ from donors with MPS I indicated that the dehydration characteristics of the two different cell populations were different ($P < 0.001$).

The dehydration characteristics of freshly isolated and of cultured and transduced CD34⁺ cells from normal donors were also analyzed (cf. Table 3). The values of L_{pg} and E_a measured for cultured and transduced cells from normal donors were quite similar to those obtained from a donor with MPS I. There were more distinct differences between the values of L_{pg} and E_a determined using fresh cells. These differences result principally from differences in dehydration at fairly high subzero temperatures which result in the higher estimate for E_a for freshly isolated cells from a normal donor. As with cells from donors with MPS I, there was a

TABLE 3
Average Water Transport Characteristics of CD34⁺ Cells
Obtained from Normal Donors at Different Time Points in
Culture

Culture condition	L_{pg} ($10^{14} \text{ m}^3/\text{N s}$)	E_a (kJ/mol)	V_b (%)
Freshly isolated	6.4 ± 3.7	69 ± 45	29
Transduced (day 5)	0.41 ± 0.19	29 ± 33	20

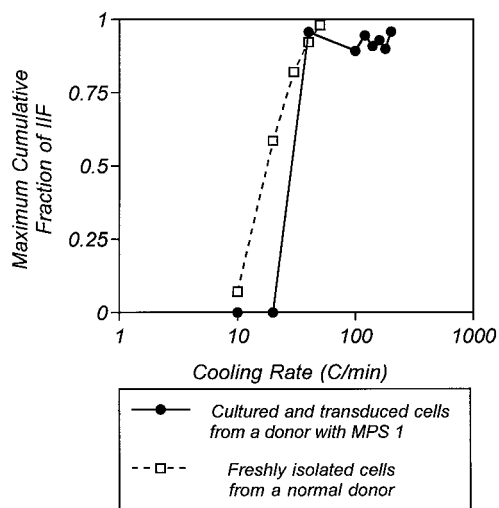


FIG. 4. Maximum cumulative fraction of cells with IIF as a function of cooling rate for CD34⁺ cells from donors with MPS I that were cultured and transduced and for freshly isolated cells from normal donors. The cells were frozen in IMDM. The number of cells per data point ranged between 32 and 134.

distinct decrease in the estimated value of E_a based on dehydration characteristics of cultured and transduced cells. The value of L_{pg} for cells from normal donors that had been cultured and transduced was less than that observed for freshly isolated cells.

Intracellular Ice Formation Parameters

The maximum cumulative fraction of cells with IIF is strongly influenced by cooling rate. The cumulative fraction of cells with IIF as a function of cooling rate is given in Fig. 4 for cultured and transduced cells from a donor with MPS I and freshly isolated cells from a normal donor. The fraction of cells with IIF increased from 0% at approximately 10°C/min to 100% at 40°C/min for cells from a donor with MPS I. For freshly isolated cells from a normal donor, the cumulative fraction of cells with IIF increased from 0 at 5°C/min to 100% at 50°C/min. The cumulative fraction of cells with IIF as a function of cooling rate for cultured and transduced cells from a normal donor was also determined and exhibited little difference from

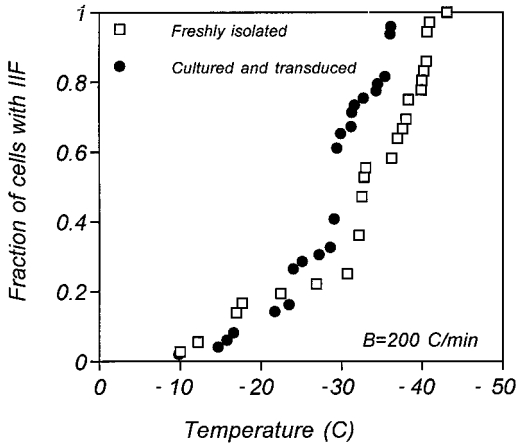


FIG. 5. Cumulative fraction of cells with IIF as a function of temperature for freshly isolated cells and for cultured and transduced cells from donors with MPS I. The cells were frozen in IMDM at a cooling rate of 200°C/min. The number of cells used in these experiments was 48 and 49, respectively.

that observed in freshly isolated cells from a normal donor (data for donors with MSP I not shown).

The ability to predict the probability of IIF requires the experimental determination of the ice nucleation parameters, Ω_0 and κ_0 . If the cooling rate used is sufficiently high to suppress water transport out of the cell, the cumulative fraction of cells as a function of temperature for a specific cooling rate (200°C/min for this investigation) can be used to estimate the IIF parameters. In Fig. 5, the cumulative fraction of cells with IIF as a function of temperature for a cooling rate of 200°C/min cells obtained from donors with MPS I is given. For both freshly isolated and cultured and transduced cells, the fraction of cells with IIF increases with decreasing temperature.

Using a Wilcoxon signed-rank test, the fraction of cells with IIF as a function of temperature for freshly isolated cells from donors with MPS I is statistically different from that of cultured and transduced cells from the same donor ($P = 0.001$), from that of freshly isolated cells from normal donors ($P < 0.001$), and from that of cultured and transduced cells from normal donors ($P < 0.0001$). For freshly

isolated cells and cultured and transduced cells from normal donors and for cultured and transduced cells from normal donors and from donors with MPS I, the fraction of cells with IIF as a function of temperature is *not* statistically distinct.

The kinetics of IIF obtained at 200°C/min for the cell types tested can be used in conjunction with the model for IIF described previously to obtain estimates for the values of Ω_0 and κ_0 (cf. Table 4). The results indicate that there is a decrease in the value of both Ω_0 and κ_0 after culture and transduction.

DISCUSSION

Water Permeability Parameters

The change in cell size over the duration of the culture and transduction process is one of the most noticeable changes that take place with in the cells. There is an approximately 55% increase in cross-sectional area of the cell (two- to threefold increase in cell volume) over the period of culture and transduction. It is not clear why the observed changes in volume are occurring. One potential explanation is that the *ex vivo* culture of the cells results in differentiation of the cells and the changes in size reflect such changes in differentiation. The percentage of cells expressing the CD34 antigen was determined using flow cytometry on the freshly isolated cells and on the cells which had been cultured and transduced for all the experiments. A dimming in the average fluorescence intensity of cells which had been cultured and transduced was noted but the percentage of cells expressing the CD34 antigen at the end of the

TABLE 4
IIF Characteristics of CD34⁺ Cells Obtained
from Donors with MPS I

Culture condition	Ω^a ($10^{-8} \text{ m}^{-2} \text{ s}^{-1}$)	κ_0^b (10^{-8} K^5)
Fresh	2337	439
Cultured and transduced	1064	347

^a Prefactor (cf. Eq. [3]).

^b Exponential factor (cf. Eq. [3]).

culture and transduction process was similar to that obtained from freshly isolated cells seeded into the HFBR. The similarity between the fraction of $CD34^+$ cells before and after culture makes it unlikely that changes in differentiation could account for the changes in cell size observed.

It is noteworthy that there were statistical differences in the cross-sectional areas of the cells from a donor with MPS I under different culture conditions. Specifically, the size of the cultured and transduced cells was less than the size of the cells from the control experiments that had been cultured for the same period of time but not sham transduced. The retroviral supernatant contains high levels of IDUA enzyme produced by the packaging cells line (17). In a previous study using genetically modified lymphocytes for the treatment of MPS II, reduced levels of glycosaminoglycan (CAC) accumulation were observed for cells that had been transduced with the corrective gene. Levels of GAG accumulation were also reduced when cells were simply exposed to culture media containing lysosomal enzymes but no infectious particles (2). These results suggest that the size difference observed between the cultured and transduced cells and the sham-transduced cells from donors with MPS I may have resulted from production of IDUA after genetic correction and/or from uptake of IDUA from the supernatant that permitted the completion of the catabolism of GAGs.

As indicated under Results, the osmotically inactive cell volume fraction, V_b , for fresh cells from a donor with MPS I was approximately 0.17. After culture and transduction, the osmotically inactive cell volume fraction was largely the same (0.21). In contrast, there was a change in the osmotically inactive cell volume fraction observed when cells from a normal donor were studied (0.29 for freshly isolated cells and 0.20 for cultured and transduced cells). The osmotically inactive cell volume fractions measured in this investigation are consistent with those from a recent study by Gao and colleagues (4) in which the osmotically inactive cell volume fraction for freshly isolated hematopoietic progeni-

tor cells ($CD34^+CD33^-$) from normal donors was determined to be 0.21.

The culture and transduction of the $CD34^+$ cells also influenced the water transport characteristics. Specifically, there was an increase in the values of L_{pg} and E_a after as few as 3 days in culture for cells from donors with MPS I. The observed changes in water transport remained largely constant for an additional 2 days in culture and there was little difference observed in the water permeability between the cultured and transduced and the cultured and sham-transduced cells. In contrast, the *ex vivo* culture of cells from normal donors resulted in a decrease in the values of L_{pg} and E_a with time in culture, which is consistent with a previous study of water permeability characteristics of hepatocytes cultured *ex vivo* (29). In that study, L_{pg} and E_a were determined to decrease with time in culture.

The permeability parameters determined in this investigation are consistent with those determined in a study of the subzero water transport characteristics of monocytes by McCaa and colleagues (13). In this study performed at suprazero temperatures, a value of L_{pg} of $5.8 \times 10^{-14} \text{ m}^3/\text{N s}$ and a value of E_a of 61 kJ/mol were determined for monocytes. The hydraulic permeability parameters for hematopoietic progenitor cells were determined previously in a study by McGann and colleagues (14). In this study performed at suprazero temperatures, L_{pg} was determined to be $0.46 \times 10^{-14} \text{ m}^3/\text{N s}$ and E_a was 27 kJ/mol. The value of L_{pg} determined in the study by McGann and colleagues was consistent with the measurements of freshly isolated cells from normal donors found in this investigation ($6.4 \times 10^{-14} \text{ m}^3/\text{N s}$) and slightly larger than that observed for freshly isolated cells from donors with MPS I ($0.25 \times 10^{-14} \text{ m}^3/\text{N s}$). The activation energy, E_a , for freshly isolated cells from normal donors determined in this investigation was 69 kJ/mol, which was considerably higher than that observed for freshly isolated cells from donors with MPS I (33 kJ/mol) and that observed in the study by McGann and colleagues (27 kJ/mol). The different values for E_a may reflect the difference in

experimental conditions between the two studies. The studies performed in this investigation were performed at subzero temperatures in the presence of external ice. As has been observed in other cell types, there can be a discontinuity in the value of the activation energy between suprazero and subzero temperatures (cf. 15 for review). The differences between the activation energy measured in this investigation and that of McGann and colleagues may reflect such differences in experimental conditions.

In analyzing IIF characteristics for the cells studied in this investigation, the interaction between water transport and IIF and the cell-specific IIF kinetics have been studied. The increase in the cumulative fraction of cells with IIF as a function of cooling rate from 0 to 100% is consistent with the IIF characteristics determined for other cell types (21) and reflects the interaction between water transport and IIF. The similarity in the cumulative fraction of cells with IIF as a function of cooling rate for cultured and transduced cells from donors with MPS I and for freshly isolated cells from normal donors is consistent with the fact that the dehydration and IIF kinetics for those two cell populations are not statistically different (for the same cooling rate).

The fraction of cells with IIF as a function of temperature for a high cooling rate reflects a cell-specific IIF characteristic (21). There are statistically significant differences between the fraction of cells with IIF as a function of temperature for freshly isolated cells from donors with MPS I and those fractions for: (1) cultured and transduced cells from donors with MPS I, (2) freshly isolated cells from normal donors, and (3) cultured and transduced cells from normal donors. The fraction of cells with IIF as a function of temperature for freshly isolated cells from normal donors is not statistically distinct from cultured and transduced cells from normal donors or from donors with MPS I.

The model for IIF used in this investigation assumes surface catalysis of IIF. As shown in Fig. 5, there is a slight discontinuity in the slope of the data for freshly isolated cells from donors with MPS I at approximately -30°C . It has

been hypothesized that a change in the slope of kinetics of IIF at low temperatures may correspond to a shift in the mechanism of IIF. It has been hypothesized that, at lower temperatures, catalysis results from molecular structures within the cells. This mechanism is known as volume-catalyzed nucleation (21, 22). The potential exists that lysosomal inclusions could act as nucleating sites for ice formation and the model used for the prediction of IIF should be modified to include both surface- and volume-catalyzed IIF nucleation. Further studies would be needed to verify the site of nucleation within the cell during freezing and the correlation of those sites with the observed kinetics of IIF.

The results of this investigation indicate that there can be changes in the subzero characteristics of hematopoietic progenitor cells when subjected to relatively short periods of *ex vivo* culture (5 days). Specifically, the cell cross-sectional area increases and water permeability parameters change after *ex vivo* culture. Furthermore, cells obtained from patients with MPS I exhibit cryobiophysical characteristics different from those observed in cells obtained from normal donors. For freshly isolated cells, the potential exists that the differences in cryobiophysical characteristics may reflect the influence of the method of isolation. The changes in water permeability parameters for donors with MPS I after culture and transduction are different from those observed for normal donors. The IIF kinetics for freshly isolated cells from normal donors was not statistically different from that of cultured and transduced cells from the same donor. For donors with MPS I, however, the culture and transduction process does result in statistically significant changes in IIF characteristics. The studies performed in this investigation used CD34^{+} cells, a subset of hematopoietic progenitor cells. This population is still somewhat heterogeneous, containing uncommitted and lineage-specific progenitor cells. The potential exists that the heterogeneity of the cell population may contribute to the scatter of values obtained in some of the experiments, in particular, the water transport experiments.

The results of this investigation do not pro-

vide the specific mechanisms for the change in cryobiophysical characteristics of cells after culture and transduction nor an elucidation of the role that a specific genetic defect (MPS I) may play in determining the values of the parameters measured in this investigation. Further studies are needed to determine the mechanisms responsible for the observed changes in water transport and IIF and the influences of these factors on the protocol. In experiments using genetically modified peripheral blood lymphocytes, the optimal survival of the genetically modified lymphocytes is considerably less than that of freshly isolated lymphocytes from the same donor (data not shown). This study reinforces the point that in the development of new hematopoietic therapies careful consideration should be given to understanding the influence of cell source and manipulation on the freezing response of cells.

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