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Mesenchymal Stem Cell Deformability and Implications for Microvascular Sequestration

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Abstract

Mesenchymal stem cells (MSCs) have received considerable attention in regenerative medicine, particularly in light of prospects for targeted delivery by intra-arterial injection. However, little is known about the mechanics of MSC sequestration in the microvasculature and the yield pressure (P_Y), above which MSCs will pass through microvessels of a given diameter. The objectives of the current study were to delineate the dependency of P_Y on cell size and the heterogeneity of cell mechanical properties and diameters (D_{CELL}) of cultured MSCs. To this end the transient filtration test was employed to elucidate the mean filtration pressure ($\langle P_Y \rangle$) for an ensemble of pores of a given size (D_{PORE}) similar to *in vivo* microvessels. Cultured MSCs had a log-normal distribution of cell diameters (D_{CELL}) with a mean of 15.8 ± 0.73 SD μm . MSC clearance from track-etched polycarbonate filters was studied for pore diameters of 7.3 to 15.4 μm . The pressure required to clear cells from filters with $30\text{--}85 \times 10^3$ pores rose exponentially with the ratio $\lambda = D_{CELL}/D_{PORE}$ for $1.1 < \lambda < 2.2$. The clearance of cells from each filter was characterized by a log-normal distribution in P_Y , with a mean filtration pressure of $0.02 < \langle P_Y \rangle < 6.7$ cmH₂O. For $\lambda = 1.56$, the yield pressure (P_Y) was well represented by the cortical shell model of a cell with a viscous interior encapsulated by a shell under cortical tension $\tau_0 = 0.99 \pm 0.42$ SD dyn/cm. For $\lambda > 1.56$, the $\langle P_Y \rangle$ characteristic of the cell population rose exponentially with λ . Analysis of the mean filtration pressure ($\langle P_Y \rangle$) of each sample suggested that the larger diameter cells that skewed the distribution of D_{CELL} contributed to about 20% of the mean filtration pressure. Further, if all cells had the same deformability (i.e. P_Y as a function of λ) as the average cell population, then $\langle P_Y \rangle$ would have risen an order of magnitude above the average from 5-fold at $\lambda = 1.56$ to 200-fold at $\lambda = 2.1$. Comparison of $\langle P_Y \rangle$ to published microvascular pressures suggested that $\langle P_Y \rangle$ may exceed microvessel pressure drops for λ exceeding 2.1, and rise 14-fold above capillary pressure drop at $\lambda = 3$ leading to 100% sequestration. However, due to the large variance of *in vivo* microvascular pressures entrapment of MSCs may be mitigated. Thus it is suggested that selecting fractions of the MSC population according to cell deformability may permit optimization of entrapment at sites targeted for tissue regeneration.

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Conflict of Interest:

No conflicts of interest are apparent.

Keywords

Mesenchymal stem cells; microvascular sequestration; deformability; cortical tension

Introduction

Strategies for using human embryonic stem cells, adult stem cells, progenitor cells from a variety of tissues, and induced pluripotent stem cells have formed the foundation of regenerative medicine for tissue and organ reconstruction¹. One of the most promising applications of stem cell therapy is the treatment of acute myocardial infarction and chronic ischemic heart disease^{14, 15}. One of the major stem cell candidates for cardiac repair are mesenchymal stem cells (MSCs)²⁵, which can be isolated from bone marrow, adipose tissue and umbilical cord¹³. MSCs have the capacity to differentiate into a broad range of tissues, including, bone, cartilage, adipose tissue and muscle^{13, 62}, exhibit immune allotolerance, produce regenerative paracrine effects^{8, 27} and may be readily expanded in culture^{45, 56}. Although the relative roles of MSC differentiation and their paracrine effects remain unclear there is no doubt that effective MSC therapy hinges on their efficient sequestration at a specific target site⁴⁰.

In the case of cardiac tissue regeneration, multiple routes of MSC delivery to tissue have been explored: (1) intravenous (systemic) infusion, (2) direct intraventricular cavity infusion², (3) direct injection into cardiac tissue⁶⁴ and (4) intra-arterial injection³. Although systemic delivery of MSCs is minimally invasive, first pass entrapment in the lungs severely limits delivery to target sites^{2, 23, 26, 56}. Direct left ventricular cavity infusion appears to enhance migration and preferential sequestration in the ischemic myocardium². Intra-arterial injection of stem cells has shown promise in several studies^{3-5, 56, 59}. Regardless of the route of administration, stem cell sequestration appears to be mainly dictated by cell size^{25, 58}. Furthermore, although it has been shown that MSCs exhibit adhesive interactions with vascular endothelium, as evidenced by rolling mediated by P-selectin⁴⁸ and firm adhesion mediated by VLA-4/VCAM-1 interactions^{48, 51}, sequestration appears to be mainly determined by MSC mechanical properties.

The ability of circulating cells to traverse the microvasculature has been of considerable interest in blood rheological disorders and inflammation. For example, the entrapment of stiffened red cells during deoxygenation in sickle cell disease may lead to a vicious cycle of ischemia and tissue hypoxia³⁸. In inflammation, leukocyte plugging of capillaries may exacerbate ischemia/reperfusion injury¹⁸. Several studies have performed direct observations of stem cell transit through the microvascular network and the mechanisms of entrapment. Intravital microscopy of the capillary network in the dorsal skin-fold chamber (mouse) revealed that 13–23% of circulating mouse embryonic endothelial progenitor cells (eEPCs) passively plugged blood vessels within tumors grown within the chamber⁵⁸. In contrast, 87% of infused eEPCs adhered to the tumor endothelium without affecting blood flow in this vascular segment. Real-time intravital microscopy of the parasagittal region of the calvarium (mouse) revealed that human MSCs (that normally do not express ligands for E-selectin) could be induced to infiltrate the marrow within hours of infusion and adhere to

constitutively expressed E-selectin⁴⁹. These studies also demonstrated that MSCs rolling on cultured endothelium in a flow chamber could be modified to adhere under conditions of high fluid shear stresses. Studies of the kinetics of human MSC trafficking through mouse cremaster muscle suggested that vaso-occlusion may occur in the microvasculature due to the relatively large size of MSCs²⁵. Tracking of intra-arterially delivered MSCs from arteriole to venule in rat cremaster muscle revealed that 92% of MSCs arrest and interrupt flow at the precapillary level during first pass, thus resulting in decreased flow in the feeding arteriole⁵⁶. Direct observations of coronary feeding arterioles, nutritive capillaries, and post-capillary venules in grafted syngeneic heart transplants revealed that infused bone marrow derived MSCs were exclusively sequestered in myocardial capillaries, but not in coronary arterioles or post-capillary venules⁵⁷. Homing of circulating MSCs to perivascular sites and their effect on vascular regeneration and microvessel hemodynamics in mouse skin flap revealed increased functional capillary density and flow during vascular regeneration⁵⁰. Intracerebral transplantation of bone marrow MSCs revealed increased microvessel density of the microvascular network and increased reactivity of arterioles in rat pia mater⁵⁴. Adhesion and flow of hematopoietic stem cells (HSCs) were examined by intravital microscopy in a murine model of renal ischemia-reperfusion injury, and revealed that increasing HSC deformability by treatment with the chemokine SDF-1 α prolonged their systemic circulation and enhanced their renal retention at sites of damaged tissue⁶¹. The increased adhesion shown therein, although partially attributed to increased HSC deformability, is consistent with prior studies of enhanced rolling and adhesion of leukocytes (WBCs) in post-capillary venules attendant to increased WBC deformability^{31, 39}. Adhesion of HPC-7, a murine HSC line, within microvessels of ischemia-reperfusion injured mouse intestine or cremaster muscle was found to increase, and intestinal adhesion was dependent upon CD18 whereas muscle adhesion predominantly relied on CD49d³⁵. Intestinal adhesion also increased in the presence of reactive oxygen species³⁴.

Thus, a common determinant of stem cell sequestration in these studies is the potential for and variability of alterations in stem cell (SC) deformability. SC deformability has been examined *in vitro* by a variety of techniques: micropipette aspiration^{41, 44, 47}, optical tweezers^{41, 55} and optical stretching^{36, 42}, capillary-based microfluidic systems^{36, 37}, analysis of diffraction patterns (ektacytometry) of suspensions under shear⁶⁰, cell manipulation using atomic force microscopy^{12, 33, 42}, imaging cell stretching in extensional flows within a microfluidic chamber^{9, 28}, transit time through a microfluidic chamber³², mechanical manipulation through artificial microscale barriers⁶³, and pressure driven filtration through porous filters⁵⁶. It is now well accepted that SCs are viscoelastic in nature, since the more rapid the deformation the stiffer the cell appears, and similar to leukocytes, the cell nucleus contributes overwhelmingly to its resistance to deformation during passage through capillary sized pores^{11, 47}. It is well recognized that SC differentiation can be directed by the extracellular stiffness of its surroundings, and SC differentiation is often accompanied by alteration of structural properties of the nucleus^{11, 12, 42, 43} and the cell membrane⁵⁵. Deformability has been identified as an early biomarker for pluripotent stem cell differentiation and is likely linked to nuclear structural changes²⁸. Thus, it has been hypothesized that the therapeutic efficacy of mesenchymal stem cells (MSCs) in tissue

engineering and regenerative medicine is determined by their unique biological, mechanical, and physicochemical characteristics, which remain to be fully elucidated⁵⁵.

To elucidate the potential for cell sequestration within the microvasculature, *in vitro* studies of blood cell deformability have aimed to simulate the passage of blood cells through capillary sized pores in filters. The seminal studies of Gregersen et al.²⁹ using polycarbonate sieves with uniform cylindrical pores (typically on the order of 5 μm) have been followed by numerous studies using either steady state or transient filtration methods to characterize cell deformability. It is generally recognized that the initial cell deformation of a cell entering an orifice of a given size is the primary determinant of the ease with which a blood cell may enter a capillary^{10, 46, 52}. Quantitative indices of cell deformability have been derived from measurement of the flow of a suspension of cells through a filter in terms of the resistance to flow for a specified pore density and pore diameter. Using steady-flow filtration methods, a quantitative index of cell deformability has been defined in terms of the ratio (β) of the resistance to flow ($R = P/Q$) through a filter pore in the presence and absence of cells ($\beta = R_{\text{CELLS}}/R_{\text{BUFFER}}$)⁵². In contrast, transient filtration techniques^{7, 16, 17} have demonstrated the ability to elucidate the heterogeneity of cell properties within a given sample. To explicitly describe the heterogeneous properties of a population of cells, prior studies have extended the bolus filtration technique described by Downey and Worthen¹⁷, to assess the average deformability of the circulating population of WBCs in terms of the yield pressure (P_y) required to dislodge an individual WBC trapped within the pores of a Nuclepore filter²¹.

Based on analysis of the probability of entrapment, measurement of the graded clearance of trapped WBCs with increasing pressure drop across a Nuclepore filter was used to calculate a mean filtration pressure, $\langle P_y \rangle$, and the statistical distribution of P_y within a WBC suspension²¹. These studies also suggested that the cortical shell model²² of a WBC may provide a single parameter, the cortical tension (τ_0), that is representative of cell deformability. As shown therein, this analysis was extended to describe the filterability of a population of WBCs of heterogeneous diameter through the capillary network of the cremaster muscle (hamster) with a known heterogeneous distribution of capillary diameters to determine a comparable *in vivo* measure of (P_y).

Within this framework, the present studies were undertaken to elucidate the deformability characteristics of a population of MSCs grown in culture and the relationship between MSC deformability, cell diameters and intravascular pressures encountered in the terminal microvascular network. To this end, samples of MSCs expanded in culture were analyzed by the transient filtration test to determine the relation between mean filtration pressure (P_y), cortical tension (τ_0), and the diameter of an MSC relative to a filter pore or microvessel diameter, $\lambda = D_{\text{CELL}}/D_{\text{PORE}}$.

Methods

Mesenchymal Stem Cells

Mouse (C57BL/6) mesenchymal stem cells (MSCs, GIBCO, Thermo Fisher, Inc.), cryopreserved at passage 9, were expanded in culture through about 15 passages. Cells were cultured in complete growth media consisting of minimum essential media (α -MEM)

supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 µg/ml streptomycin. MSCs were lifted from the plates with TrypLE™ and suspended in growth media to yield about 10⁶ cells per ml. Cell viability was routinely assayed by Trypan blue exclusion, and exceeded 95%. Cell concentration was adjusted to a total cell count in 0.2 ml equal to the number of pores in each filter, typically 30–85 × 10³ cells.

Transient Filtration Protocol

The transient filtration method for determination of cell deformability has been described previously²¹. The basic approach is illustrated in Fig. 1 and modifications and improvements are summarized as follows. The underlying assumption in the test is that a cell of diameter D_{CELL} cannot pass through a filter pore of diameter D_{PORE} unless a minimum pressure drop (P) is applied as schematized in Fig. 1A. The P required for a cell to pass through a pore is dictated by the size, structure and material properties of the cell. These parameters may be related with the assumption of the cortical shell model where the cell is assumed to consist of a spherical elastic shell surrounding a viscous fluid, as described by Evans and Yeung²². For the cortical shell model shown therein, a cell entering a pore will remain in stationary equilibrium when the balance of surface forces produced by P is balanced by the cortical tension (τ_0 , dyn/cm). The maximum pressure drop across a trapped cell before the cell flows through the pore (P_Y) is related to the cell to pore diameter ratio ($\lambda = D_{CELL}/D_{PORE}$) and the cortical tension in the shell (τ_0 , dyn/cm) by the relation,

$$P_Y = \frac{4\tau_0}{D_{CELL}} (\lambda - 1). \quad (1)$$

That is, as the pressure drop (P) across the cell rises above the equilibrium value P_Y , the cell will flow through the pore. The deformability of each cell may thus be represented by the constant of proportionality between yield pressure and cell/pore geometry, τ_0 . Given that the population of cells in a sample will be heterogeneous in diameter and mechanical properties, a cell sample will have statistical distributions of P_Y and λ that may be obtained from the washout of cells trapped in a filter with N pores.

To evaluate the distribution of yield pressures characteristic of a cell sample the distribution of P_Y was obtained from the washout of cells (initially trapped at low pressure drops) in track-etched polycarbonate filters during stepwise increases in P . Nuclepore track-etched polycarbonate filters (13 mm diameter and 11 µm thickness) with nominal pore diameters of 7 to 20 µm (Poretics, Inc. and Structure Probes Inc., West Chester, PA) were held in a Lucite support and perfused with either cell suspensions or buffer using a syringe pump (PhD 2000, Harvard Apparatus, Inc., Holliston, MA). An aliquot of cells (0.2 ml) was loaded in the antechamber of the filtration device, as schematized in Fig. 1B, and the filter was perfused with buffer at a very low flow rate (0.25 ml/min) until the maximum amount of pores was plugged with cells. The flow rate of buffer was then increased in stepwise increments of 30 sec duration (Fig. 1C) and the resultant pressure drop across the filter was measured (Fig. 1D). Pressure drop was measured with a differential transducer (MP-45, Validyne, Northridge, CA) with a range of 0–10 cmH₂O and resolution of 0.01 cmH₂O. The pump rate

was controlled by computer and increased in stepwise increments every 30 sec (Fig. 1C). Pressures were digitized (12 bit A/D, Metrabyte, PCMCIA interface, Keithley Instruments, Cleveland, OH) at a rate of 10^3 samples/sec. As shown in Fig. 1D, with each stepwise increase in flow the pressure drop rose and then rapidly fell slightly during the first 5–10 sec as cells were washed from plugged pores. The average pressure was acquired over the last 15 sec of each step to calculate the resistance of the filter (R_{FILTER}) and the fraction of pores plugged relative to that obtained during loading of the filter, i.e. $N_{PLUG}/N_{PLUG\ MAX}$.

The initial maximum number of plugged pores ($N_{PLUG\ MAX}$) was calculated from R_{FILTER} during loading with cells ($R_{PLUGGED\ MAX} = P_{LOAD}/Q_{LOAD}$). The number of plugged pores was taken as the total number of pores less the number of open pores, i.e. $N_{PLUGGED} = N_{TOTAL} - N_{OPEN}$, where N_{TOTAL} was obtained by microscopic examination of the filter prior to the test. The resistance of the filter (R_{FILTER}) was assumed to reflect the resistance of N parallel unplugged (open) pores, i.e. $R_{FILTER} = R_{PORE}/N_{OPEN}$. With increasing pressure drop across the filter, the resistance of the filter fell from that at maximum plugging ($R_{PLUG\ MAX}$) as trapped cells were washed out. It was assumed that R_{FILTER} results from two parallel sets of pores: (1) those initially open at maximum plugging, which contribute to $R_{PLUG\ MAX}$, and (2) those that undergo cell washout, represented by R_{OPEN} . The filter resistance was thus calculated from $1/R_{FILTER} = 1/R_{OPEN} + 1/R_{PLUG\ MAX}$. Thus, as pressure was increased with each step, the fraction of plugged pores that underwent washout (f) fell from 1 to 0 as:

$$f = \frac{N_{PLUG}}{N_{PLUG\ MAX}} = \frac{Q/P - Q_{LOAD}/P_{LOAD}}{Q_{MAX}/P_{MAX} - Q_{LOAD}/P_{LOAD}} \quad (2)$$

where Q_{MAX} and P_{MAX} are the flow and pressure at maximum flow rate (10 ml/min).

Pore diameter was calculated prior to each cell sample from measurement of pressure drop for a flow of 1 ml/min, using the theoretical formulation for resistance based on Poiseuille's equation and entry effects, $R_{PORE} = 128 \mu L / \pi D_{PORE}^4 + 24 \mu / D_{PORE}^3$ where μ is the buffer viscosity and L is the filter thickness⁵².

Calculation of Mean Filtration Pressure

Given that the distribution of pore sizes for a specific filter is fairly uniform (< 5% coefficient of variation²¹), the average yield pressures (P_Y) for a sample of trapped cells released from the filter was estimated by considering the probability that a cell will be washed out as pressure drop increases. As shown in Fig. 2A for a representative sample of MSCs loaded into a filter with 5.9×10^4 pores and 9.7 μ m mean pore diameter, flow (Q) increased non-linearly with filtration pressure (P). The fraction of plugged pores ($f = N_{PLUG}/N_{PLUG\ MAX}$) fell precipitously (Fig. 2B) as P increased.

The cumulative distribution function representative of the probability of a cell being washed out of the filter as pressure exceeds a given P was calculated as $1-f$, Fig. 2C. It has been shown that the best fit of the cumulative distribution function was found to be that of a log-normal distribution²¹.

$$CDF = \frac{1}{2} + \frac{1}{2} \operatorname{erf} \left[\frac{\ln P_Y - \mu}{\sqrt{2}\sigma} \right] \quad (3)$$

with probability density distribution (PDF) given by:

$$PDF = \frac{1}{P_Y \sigma \sqrt{2\pi}} e^{-\frac{(\ln P_Y - \mu)^2}{2\sigma^2}} \quad (4)$$

where P_Y is yield pressure and μ and σ^2 are the mean and variance, respectively, of the natural log of P_Y . These parameters are related to the mean ($\langle P_Y \rangle$) and variance (var) of yield pressures by:

$$\langle P_Y \rangle = e^{(\mu + \sigma^2/2)} \text{ and } \text{var} = (e^{\sigma^2} - 1) (e^{(2\mu + \sigma^2)}) \quad (5)$$

Measurements of I - f were fit to the CDF using the Levenberg-Marquardt algorithm, as implemented in SigmaPlot (Systat Software, Inc., San Jose, CA). The CDF for this sample was found to fit the data well with $R^2 = 0.996$, (Fig. 2C). The corresponding PDF is shown in Fig. 2D with a mean filtration pressure of 1.2 ± 1.3 SD cmH₂O.

Measurement Protocol

MSCs were lifted from their culture plates and suspended in media at a concentration of $1-2 \times 10^6$ cells/ml. Microscopic cell counts were performed using a hemocytometer with a $10\times$ objective. The cell suspension was filtered through a Nuclepore filter with 20 μ m pore diameter to remove debris. Cell diameters were measured in a hanging drop with a Zeiss $40\times/0.75$ NA water immersion objective for 100–300 cells from the sample. Measurements of cell count and mean cell diameter revealed no loss of cells during filtration through a 20 μ m filter. Cell samples were prepared for the filtration test by dilution with media to give a total number of cells in 0.2 ml equal to the number of pores in the test filter. The test filter was perfused with media for 1 min at a flow of 1 ml/min, the pressure drop recorded (typically 0.1–0.4 cmH₂O), and the filter resistance (P/Q) and mean pore diameter were calculated. The cell sample was introduced in the filter chamber, the filter loaded with cells at 0.25 ml/min for 2 min and the sequence of stepwise increases in flow begun. The filtration procedure was automated using code written in Microsoft Visual Basic.

Cell Morphometry

To aid interpretation of the trends of filtration pressure versus cell diameter, a small sampling of cells was stained with the fluorescent dye acridine orange (0.01% in PBS) to roughly delimit the cell nucleus relative to the cell diameter. An apparent nucleus diameter was calculated as the diameter of a circle with the nucleus area observed in the diametral plane of the cell.

Statistics

Statistical analyses were performed using SigmaPlot (Systat Software, Inc., San Jose, CA). Significance of regressions were performed using a t-test with significance of the null hypothesis taken at the 5% level or less, i.e. $p < 0.05$.

Results

A total of 31 filtration tests were run using 9 different cell samples and 12 different batches of filters with pore diameters ranging from 7.3 to 15.4 μm . The measured and calculated parameters are given in Table 1 for all values of mean cell diameter to pore diameter, $\lambda = \langle D_{\text{CELL}} \rangle / D_{\text{PORE}}$, and for λ less than and greater than 1.56. This cutoff was determined by analysis of the trends of $\langle P_Y \rangle$ vs λ (shown in the following).

Cell Diameters

Cell diameters were measured in 9 samples with an average mean diameter ($\langle D_{\text{CELL}} \rangle$) of 15.8 ± 0.73 SD μm and a range of the mean from 15.0 to 17.1 μm . A histogram of 2090 cell diameters pooled from all 9 samples is presented in Fig. 3A and revealed a mean value of 16.2 ± 3.0 SD μm (median = 16.0) with a range from 6.1 to 30.6 μm . The pooled diameters were found to be well represented by a log normal distribution, $R^2 = 0.989$ (solid line in Fig. 3A). For a small sample of cells ($N = 60$), the apparent diameter of the cell nucleus was found to average 10.5 ± 2.16 SD μm , with a ratio of nucleus to cell diameter of 0.63 ± 0.09 SD μm .

Outflow cells were collected in six experiments and their diameter distributions measured. The ratio of mean diameter from outflow to inflow was compared to pore diameter, D_{PORE} , Fig. 3B. No significant variation of D_{CELL} with D_{PORE} was found, as evidenced by R^2 of its linear regression equal to 0.016, $p = 0.49$. Thus it appears that the larger cells in the tail of the distribution were responsible for the permanent plugging of the filter. No significant trends in skewness of the diameter distributions from inflow to outflow with diminishing pore size were discernible, presumably due to the diminishing fraction of the total inflow population that was permanently trapped as pore diameter was increased.

Filter Pore Plugging

For all 31 filtration tests, the fraction of pores initially plugged was calculated from the filter resistance upon completion of loading and averaged 0.81 ± 0.12 SD. This fraction did not vary significantly with the ratio of mean cell diameter of the sample to pore diameter, λ ($= \langle D_{\text{CELL}} \rangle / D_{\text{PORE}}$), $R^2 = 0.007$, $p = 0.65$ (Fig. 4A). The fraction of pores permanently plugged was calculated from the filter resistance at the maximum flow (10 ml/min) and rose significantly with increasing λ from about 30% to 70% for $1.1 < \lambda < 2.2$ (Fig. 4B). It should be noted that permanently plugged pores do not contribute to computation of the fraction of plugged pores washed out since their resistance to flow (in parallel with the fractions open and washed out) is infinite. Examination of several filters following the test revealed that the permanently plugged pores resulted from debris (cell membrane fragments) and multiple cells lodged at a pore entrance.

Mean Filtration Pressure

Trends of mean filtration pressure ($\langle P_Y \rangle$) vs λ are presented in Fig. 5 and revealed a significant exponential rise (solid line) given by $\langle P_Y \rangle = Ae^{\lambda B}$, with $A = 0.019$ ($p = 0.08$), $B = 2.85$ ($p < 0.05$) and $R^2 = 0.79$. To facilitate estimation of the average cortical tension in the cell population linear regions of the curve were determined by iteratively specifying a cutoff value (λ_{cutoff}) that resulted in linear regressions above and below λ_{cutoff} that intersected at $\lambda_{\text{cutoff}} = 1.56$. It was found that for $\lambda < 1.56$, $\langle P_Y \rangle$ varied linearly with λ , $R^2 = 0.31$, $p < 0.01$, and for $\lambda > 1.56$, $R^2 = 0.48$, $p < 0.03$. Although both regions had significant linear regressions, for $\lambda < 1.56$ the standard error of estimate ($S_{y|x}$) equaled 0.40 vs 1.3 for $\lambda > 1.56$. By comparison, for $\lambda < 1.56$ the exponential fit yielded $S_{y|x}$ equal to 0.48 for the exponential fit. Thus a linear fit in this region appears as the best representation of the trends of $\langle P_Y \rangle$ vs λ . For $\lambda > 1.56$, the linear and exponential fits yielded values of $S_{y|x}$ equal to 1.28 and 1.33, respectively. The larger errors incurred as λ rose above the cutoff may have resulted from the increase in the number of pores permanently plugged by larger cells in the population (cf. Fig. 4) and the smaller number of pores cleared with increasing pressure. Based upon the log-normal distribution of pooled diameters, the fraction of cells in the sample with λ greater than its mean was approximately 47% for all pore diameters. However, with diminishing pore diameter the fraction of cells with $\lambda > \lambda_{\text{cutoff}}$ rose from 5% at $D_{\text{PORE}} = 15.4 \mu\text{m}$ (maximum studied), to 95% at $D_{\text{PORE}} = 7.34 \mu\text{m}$ (minimum studied). At $D_{\text{PORE}} = 10 \mu\text{m}$, about 50% of the cells have diameters greater than λ_{cutoff} . Thus the exponential rise in mean filtration pressure and attendant increase in $S_{y|x}$ with $\lambda > \lambda_{\text{cutoff}}$ reflects the increasing fraction of pores in the sample subject to permanent plugging.

Cortical Tension

The cortical tension (τ_0) was calculated from the ratio of $\langle P_Y \rangle$ to $\lambda - 1$ using Eq. 1 and found to average 1.33 ± 0.13 dyn/cm. Regression of τ_0 vs $\lambda - 1$ (Fig. 6A) revealed no significant correlation, $R^2 = 0.086$, $p = 0.122$. Also, τ_0 was calculated from the slope of $\langle P_Y \rangle$ vs $\lambda - 1$, obtained by linear regression of all points ($N = 31$) forced through the origin (Fig. 6B). The slope was significant and equaled 3.95 ± 0.342 SE, with $R^2 = 0.608$, $p < 0.05$. The resulting τ_0 equaled 1.55 ± 0.72 SD dyn/cm. Given the exponential variation of $\langle P_Y \rangle$ vs λ (Fig. 5), the best estimate of τ_0 consistent with the cortical shell model was calculated from the regression slope (through the origin) over its linear range $0 < \lambda < 1.56$. The resultant τ_0 was 0.99 ± 0.42 SD dyn/cm, with $N = 20$, and $R^2 = 0.159$, $p < 0.2905$. For $\lambda > 1.56$, τ_0 equaled 1.79 ± 0.72 SD dyn/cm ($N = 11$), which was significantly greater ($p < 0.05$). This range of $\langle P_Y \rangle$ vs λ also had a substantially larger error variance about the regression line, and thus reflected a poorer fit of the cortical shell model.

Discussion

The importance of cell deformability as a determinant of blood viscosity and plugging of capillaries has spawned development of multiple approaches to its assessment. Micropipette aspiration of individual cells^{22, 30} and steady flow filtration of cell suspensions through Nuclepore filters²⁹ have provided insights into the potential for plugging capillaries in the microvasculature. Steady state filtration, however, provides only an average assessment of the cell population whereas micropipette aspiration may provide an assessment of individual

cells, albeit time consuming and technically challenging. In contrast, transient filtration methods may illuminate the heterogeneous properties of a population of cells^{16, 17}. Using the cortical shell model of the cell described by Evans and Young²² the transient filtration method of Downey and Worthen¹⁷ was extended to obtain a statistical appraisal of the heterogeneous distribution of cell size and deformability²¹. Given 30,000 to 85,000 pores in a given filter, in some respects, the transient filtration method may be viewed as that many micropipette tests performed simultaneously on a single cell sample. Regardless of the agreement between the two approaches, the analogy between the filtration test and the capillary network is readily apparent. In previous studies of leukocytes (white blood cells, WBCs), the effect of size and deformability heterogeneity within a sample of cells with a narrow range of diameters (3–9 μm) was explored for transient filtration through filters with a 5 μm mean pore diameter with a ratio of cell diameter to pore diameter ranging $1 < \lambda < 2$ and a mean value of 1.4. To explore the properties of MSCs the present study employed a range of pore diameters from 7.3 to 15.4 μm . With a mean MSC diameter of 15.8 μm , the resultant range of mean λ was $1.0 < \lambda < 2.2$. It is readily apparent that the exponential rise of mean filtration pressure (Fig. 5) with average λ is contrary to the postulated cortical shell model of the MSC which embodies a linear relationship between $\langle P_Y \rangle$ and λ .

The broad range of average λ afforded the opportunity to explore the applicability of the cortical shell model to describe MSC properties. As shown in Fig. 5, the mean filtration pressure was reasonably linear with λ for $\lambda < 1.56$. Within this range, the mean filtration pressure (the pressure above which a cell flows through a pore) for MSCs averaged 0.87 ± 0.49 SD cmH_2O (Table 1) with average $\lambda = 1.39$. This result was about 60% greater than that of the much smaller WBCs ($\langle P_Y \rangle = 0.55$ cmH_2O)²¹ and insignificantly 30% greater than the 0.67 ± 0.10 SD cmH_2O found by Toma et al. for MSCs using a variation of the transient filtration method⁵⁶ with 10 μm pores. The differences between the current study and those of Toma et al. may have arisen from multiple methodological issues. In these latter studies, the pore diameter used was the nominal diameter specified by the filter manufacturer, and the reported mean and average of cell diameter was substantially larger than found here found for MSCs in a hanging drop (23.6 vs the present 15.8 μm). The lower filtration pressures reported therein may have arisen due to their method of taking the mean filtration pressure as the filter pressure drop at the point of 50% of the plugged pores cleared from the filter, in contrast to taking the first moment (expected value) of the log-Normal distribution used herein. Both studies are in reasonable agreement with the fraction of pores permanently plugged: 61% therein and 48% at $D_{\text{PORE}} = 10$ μm (Fig. 4B).

An analysis of the current methodology to illuminate departures from a linear ($\langle P_Y \rangle$ vs λ) cell model, comparisons with other studies, and pressure drops anticipated at the entrance to an *in vivo* microvessel, is presented in Fig. 7. Shown therein is the exponential fit of the mean filtration pressure ($\langle P_Y \rangle$) calculated from the mean of the log-normal distribution at each mean λ (from Fig. 5) and an exponential fit (broken line) of the median filtration pressure (P_Y MEDIAN) at each mean λ . The median value is the pressure at which half the filter pores become unplugged as flow and pressure are increased. Non-linear regression of median P_Y vs λ resulted in $P_Y = 0.032e^{2.1\lambda}$, $R^2=0.38$, $p < 0.05$. Due to the skewness of the log-normal distribution of P_Y , the median value is about 50% of $\langle P_Y \rangle$ at $\lambda = 1.56$ and 30%

of $\langle P_Y \rangle$ at $\lambda = 2.1$. Thus, the lower filtration pressures obtained by Toma et al.⁵⁶ may be the result of using the filtration pressure at 50% clearance of the filter.

The effect of skewness of cell diameter in the population of MSCs presented to each filter was estimated by applying the exponential correlation of $\langle P_Y \rangle$ vs λ (Fig. 5) to calculate P_Y for the median λ ($= \lambda_{\text{MEDIAN}}$) of each sample. This trend is shown in Fig. 7 as the dashed curve and reveals a P_Y that is 80% of the mean filtration pressure ($\langle P_Y \rangle$) at $\lambda = 1.56$ that increases to 40% of $\langle P_Y \rangle$ at $\lambda = 2.1$. Thus within the linear range of $\langle P_Y \rangle$ vs λ (i.e. λ 1.56) the larger diameter cells that skew the diameter distribution contribute to at most about 20% of the mean filtration pressure.

The exponential relationship between $\langle P_Y \rangle$ and mean λ (Fig. 5) averages the dependency of $\langle P_Y \rangle$ over all cell diameters in a sample for a given pore size. Thus the heterogeneity of mechanical properties in the cell sample is not readily apparent. That heterogeneity could be explicitly demonstrated if cells were separated according to cell size prior to filtration. However, doing so would require additional cell manipulations. Alternatively, the effect of heterogeneous cell properties can be demonstrated by calculating the P_Y at each value of D_{CELL} in the diameter distribution (Fig. 3A) from the exponential fit (Fig. 5) for a given sample run with a specific D_{PORE} . For a filter with a uniform pore size D_{PORE} , the resultant filtration pressure then takes on the frequency distribution of λ within the sample. This approach was taken by normalizing (to unit area) the frequency distribution of λ for each filter to obtain its probability density distribution (pdf) and then using that pdf as the frequency distribution of P_Y computed from the mean filtration pressure (Fig. 5) for a given value of λ in the sample. This procedure resulted in the exponential variation of P_Y (dotted line in Fig. 7) that would be the hypothetical value of filtration pressure if all cells had mechanical properties equal to that of the mean population. These trends were well represented by the exponential fit (dotted line, Fig. 7) of $P_Y = 5 \times 10^{-4} e^{6.1\lambda}$. Hence with increasing values of λ (i.e. decreasing pore size), the disparity between P_Y from the pdf of λ and the mean filtration pressure ($\langle P_Y \rangle$) appears to increase dramatically from 5-fold at $\lambda = 1.56$ to 200-fold at $\lambda = 2.1$. This difference reflects the non-uniform distribution of P_Y for a given cell sample, as suggested by the log-normal fits of filtration pressure for a given D_{PORE} .

Based upon the linearity of P_Y vs λ for $1 < \lambda < 1.56$, the best estimate of the cortical tension τ_0 was 0.99 dyn/cm. Using this value, P_Y was calculated over the entire range of λ , as shown by the dashed line in Fig. 7. Interestingly, a comparable value of 0.89 dyn/cm was found by Toma et al.⁵⁶. In contrast, substantially smaller values of τ_0 were found for WBCs; 0.28 dyn/cm for hamster WBCs ($D_{\text{CELL}} = 6.4 \mu\text{m}$) examined by transient filtration²¹, and 0.035 dyn/cm for human granulocytes ($D_{\text{CELL}} = 8.5 \mu\text{m}$) determined by micropipette aspiration²². These cortical tensions are significantly higher than that found to resist separation of the cell membrane from the cortical cytoskeleton, which was about 0.0015 dyn/cm as determined by laser tweezers⁵⁵. The much greater apparent cortical tension found by filtration may thus reflect the contribution of the MSC nucleus to resisting deformation upon entry into a pore, as suggested by the micropipette studies of Ribeiro and Dahl⁴⁷. As shown therein, the creep function representative of a cell entering a pore under constant pressure loading initially rises rapidly as the membrane is aspirated and then much more

slowly as the stiffer nucleus enters the pore. Although no studies are shown as a function of pore diameter, this behavior is consistent with the exponential rise in $\langle P_Y \rangle$ as λ exceeds 1.56 (Fig. 5). The size of the MSC nucleus relative to cell diameter found here was consistent with the observations of Ribeiro and Dahl. The current measurements of the ratio of cell to nucleus diameter equaled 1.62 ± 0.23 SD. Thus, the exponential rise in yield pressure beginning at $\lambda_{\text{cutoff}}=1.56$ (Fig. 5) occurs when the cell nucleus must begin to deform.

To address the *in vivo* significance of these data on the potential for microvascular entrapment of MSCs, presented in Fig. 7 is the distribution of microvascular pressures estimated across the capillary bed in cremaster muscle (P_{CAP}). These pressures correspond to a conservatively high estimate of the average pressure drop expected across a cell lodged in the entrance to a microvessel. This distribution of pressures was calculated from the measurements of Bohlen et al. in cremaster muscle of the normotensive rat⁶. Presented therein are measurements of intravascular pressures in arterioles feeding the true capillaries as a function of arteriolar diameter, and venular pressure draining the true capillaries. The average pressure drop across a sequestered cell (P_{CAP}) was estimated as the difference between arteriolar pressure in a microvessel of a given diameter and venular pressure draining the capillaries. The value of λ was taken as the ratio of average MSC diameter to arteriolar diameter, hence implying a bifurcating arteriole with three equal sized branches. As noted in Fig. 7, all mean filtration pressures ($\langle P_Y \rangle$) were less than the estimated P_{CAP} , which suggests that 100% of the cells would pass through the capillary bed for a mean λ 2.1 (equivalent microvessel diameter of 7.5 μm). For $\lambda > 2.1$, less than 100% of the cells would pass through the capillaries. Based upon the exponential rise of $\langle P_Y \rangle$, for an average capillary diameter⁶ of 5.4 μm ($\lambda=3.0$) mean filtration pressure would exceed capillary pressure drop by a ratio of 97:7. Direct measurements of the *in situ* distribution of capillary pressures and diameters reveal a substantial coefficient of variation (SD/mean) that ranges from 0.25 in cremaster muscle⁶ to 0.45 in tenuissimus muscle²⁴. Thus for λ less than the intersection of $\langle P_Y \rangle$ and P_{CAP} , passage of MSCs through the capillary bed may be substantially less than 100%, and above it sequestration may be mitigated. It has been reported that on the order of 90% of MSCs injected into arterioles of cremaster muscle became trapped in the capillary network, which resulted in a 27% reduction of flow through the network⁵⁶. It has been shown previously, for WBCs infused into cremaster muscle, that the effect of cell sequestration on the resistance to flow, and hence throughput of the network, is strongly dependent upon the cumulative number of cells infused¹⁹. As shown therein, flow resistance across the capillary bed rose 2.5-fold with successive bolus injections of activated WBCs as the capillary network became plugged. These activated WBCs had an estimated mean filtration pressure comparable to MSCs of about 1 cmH_2O with $\lambda_{\text{CAP}} = 1.3$ ²¹.

Studies of the passage of WBCs through cremaster muscle have suggested that the correspondence between cell deformability and microvascular entrapment depends on network topography and flow conditions²⁰. As shown therein, in response to bolus infusions of WBCs sequestration was mitigated by redistribution of WBCs through pathways of lesser resistance. Based upon the mean transit time of WBCs and plasma, it was demonstrated that effective cell concentration in the cremaster muscle was not significantly different for WBCs

vs red cells, until pressure gradients in the network were reduced by hemorrhagic hypotension. Preliminary experiments in this laboratory of bolus infusions of fluorescently labeled MSCs into mouse cremaster muscle (unpublished data) revealed a 50% greater ratio of MSC transit time to plasma compared to WBCs, with about 25% sequestration of the MSCs calculated by conservation of mass flow.

In summary, the current studies have applied the transient filtration method to quantitate MSC deformability. It appears that the cortical shell model of the MSC may be applicable to describing MSC mechanical properties for values of cell to pore diameter $\lambda < 1.56$. For a given sample of MSCs, it is apparent that the distribution of filtration (yield pressures) required for cell passage through a pore (or capillary) is well represented by a log-normal distribution that accounts for the distribution of cell size and deformability within the sample. Ignoring the tail of the yield pressure distribution by taking the median value of filtration pressure leads to a significantly lower estimate of filtration pressures. It is clear that although the transient filtration method can delineate the effects of heterogeneity of size and deformability on the mean filtration pressure ($\langle P_Y \rangle$) of a sample additional studies are needed to assess these differences for fractions of cells separated according to size. Doing so may not be straight-forward since harvested MSCs rapidly undergo morphological changes with and without differentiation⁵³ as well as, presumably, changes in their mechanical properties. It is evident that further studies are warranted and the transient filtration method may be a useful tool in establishing a quantitative foundation for these observations.

The current findings also suggest that the heterogeneity in MSC mechanical properties may significantly affect the number of cells sequestered at a desired site, which may in turn influence the regenerative outcome. Less deformable cells may be sequestered upstream and impair flow and convective transport of cells to the targeted site. Conversely, more deformable cells may pass through the microvascular network without entrapment, and lessen the total accumulation of cells. Hence, separating the cell population according to deformability may serve to optimize the fraction of cells trapped. The specific site of sequestration will of course depend on the topography of the network. In the context of cardiac tissue regeneration, the present analysis of potential entrapment has utilized hemodynamic data from the rat cremaster muscle, mainly due to a lack of similar data for the beating heart and human subjects. While there are, however, similarities between the cardiac microvasculature and cremaster muscle, the branching patterns and microvessel diameters are quite different. Nonetheless, the basic cell mechanics described herein may guide the development of future approaches to MSC delivery in cardiac tissue regeneration.

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Figure 1.

Transient filtration test procedure using Nuclepore track-etched filters. (A) Underlying basis for the test. A cell of diameter D_{CELL} lodged in the entrance of a filter pore (with diameter D_{PORE}) remains in equilibrium until the pressure drop (ΔP) across the filter exceeds the yield pressure (P_Y) dictated by the cortical shell model with cortical tension τ_0 . (B) A filter is loaded under low flow until 80–100% of pores are plugged. (C) The flow through the filter is increased in stepwise increments using a computer controlled syringe pump. (D) As pressure is increased, cells are washed out of pores, dependent on cell deformability and the ratio $\lambda = D_{\text{CELL}}/D_{\text{PORE}}$.

Figure 2.

Representative filtration test for sample of MSCs loaded into a filter with 5.9×10^4 pores and $9.7 \mu\text{m}$ mean pore diameter. (A) Resultant non-linear trend of total flow through the filter vs the equilibrium filtration pressure at each step depicted in Fig. 1. (B) Normalized fraction of pores plugged (f) during increasing pressure. (C) Cumulative density distribution (CDF) of the probability that a pore will be washed out as pressure exceeds the filtration pressure (abscissa) was obtained by fitting $(1 - f)$ to a log normal density distribution. (D) Corresponding probability density distribution of yield pressures (P_Y) give the mean filtration pressure ($\langle P_Y \rangle$, first moment) and standard deviation (SD, second moment) of the distribution of P_Y characteristic of the cell population for a given λ and cell deformability.

Figure 3.

(A) Pooled distribution of MSC cell diameters for 2090 cells from 9 inflow samples. Diameter was measured in a hanging drop and averaged 16.2 ± 3.0 SD with a range from 6.1 to 30.6 μm . The average of the means of each sample was 15.8 ± 0.73 μm , which was not significantly different, $p < 0.05$. (B) Ratio of Inflow to outflow mean MSC diameters obtained for six filters with the indicated pore diameter. The average ratio was not significantly difference from 1, and the regression slope was not significantly different from 0.

Figure 4.

(A) Fraction of the total number of pores in each filter initially plugged with MSCs. The fraction of plugged pores averaged 0.81 ± 0.12 SD and did not increase significantly with increasing ratio of mean cell diameter to pore diameter, λ . (B) The fraction of pores permanently plugged rose significantly from 20 to 70% as λ increased from 1.1 to 2.2, as indicated by the regression with $R^2 = 0.38$, $p < 0.05$.

Figure 5.

Mean filtration pressure ($\langle PY \rangle$) vs ratio of mean cell diameter to pore diameter, λ , for 31 samples of MSCs. Solid curve is a non-linear fit of $\langle PY \rangle = 0.019e^{2.85\lambda}$. Linear regressions are shown above and below λ_{CUTOFF} , which was iteratively determined as the intersection of the best linear fits.

Figure 6.

Estimates of the cortical tension τ_0 using Eq. 1. (A) Cortical tension calculated from the ratio of $\langle P_Y \rangle : \lambda - 1$ (Eq. 1) for 31 samples. Linear regression (solid line) revealed no significant correlation with λ , $R^2 = 0.086$, $p = 0.122$. (B) Regression of $\langle P_Y \rangle$ vs $\lambda - 1$ (forced through the origin) revealed a significant slope, $R^2 = 0.608$, $p < 0.05$, from which $\tau_0 = 1.55 \pm 0.72$ SD dyn/cm. This value was significantly greater than that computed from the slope of the linear regression of $\langle P_Y \rangle$ vs $\lambda - 1$ (forced through origin) for $\lambda < \lambda_{\text{CUTOFF}}$ (Fig. 5), which equaled 0.99 ± 0.42 SD dyn/cm, $p < 0.05$.

Figure 7.

Mean filtration pressure vs ratio of mean cell to pore diameter, λ . The exponential fit of the mean filtration pressure (Fig. 5) is shown (solid curve). A curve of the average pressure drop at a microvessel entrance was constructed from *in vivo* pressure measurements⁶. The dotted curve (P_Y from pdf(λ)) is the filtration pressure computed assuming all cells in the population had a filtration pressure given by the exponential fit of Fig. 5. The short dashed curve (λ_{MEDIAN}) corresponds to the variation of P_Y vs λ calculated from the fit of the experimental data (Fig. 5) for λ at 50% (median) of the cell diameter distribution. The broken curve ($P_{Y \text{ MEDIAN}}$) represents an exponential fit of the median value of P_Y in each distribution for measurements at a given λ , and represents the pressure at which 50% of pores are cleared from each filter (e.g. CDF=0.5 in Fig 2C). The dashed straight line represents the relationship between P_Y and λ for $\tau_0 = 0.99$ dyn/cm (Table 1). These curves define the bounds of the anticipated filtration pressure for a given λ , relative to the average microvascular pressure drop.

Table 1

Summary of MSC Characteristics, Filter Dimensions, and Results

Parameter	All λ (n = 31)	Range (min – max)	λ 1.56 (n = 20)	$\lambda > 1.56$ (n = 11)
D_{PORE} (um)	10.5 ± 2.0	7.3 – 15.4	11.5 ± 1.7	8.54 ± 0.74
D_{CELL} (um)	15.8 ± 0.73	6.0 – 23.0	15.8 ± 0.73	15.8 ± 0.73
<λ> = <D_{CELL}>/D_{PORE}	1.52 ± 0.24	1.1 – 2.2	1.39 ± 0.18	1.86 ± 0.17
N_{PORES} (10³)	62.7 ± 22.4	29.6 – 85.6	52.8 ± 21.2	80.5 ± 10.7
Fraction Perm Plugged	0.47 ± 0.16	0.09 – 0.84	0.42 ± 0.13	0.56 ± 0.17
Fraction Washed Out	0.41 ± 0.23	0.08 – 0.58	0.47 ± 0.23	0.27 ± 0.16
<P_Y> (cmH₂O)	1.74 ± 1.67	0.02 – 6.7	0.87 ± 0.49	3.32 ± 1.92
Std Dev of P_Y Dist	1.88 ± 2.31	0.19 – 12.3	1.85 ± 2.71	1.96 ± 1.26
μ Log Normal Mean	−0.49 ± 1.09	−4.9 – 1.6	−0.95 ± 0.92	0.34 ± 0.87
σ Log Normal Std Dev	0.96 ± 0.51	0.2 – 2.2	0.92 ± 0.53	1.05 ± 0.49
R² of Log Normal Fit	0.966 ± 0.028	0.884 – 0.996	0.97 ± 0.02	0.95 ± 0.03
τ_0 (dyn/cm)	1.55 ± 0.72 [†]	0.25 – 2.8 [*]	0.99 ± 0.42 [†]	1.79 ± 0.72 [†]

Shown are mean ± SD for measured and calculated parameters for all measurements and those divided into two groups with λ less or greater than $\lambda_{\text{cutoff}} = 1.56$ determined from the variation of λ with mean yield pressure (<PYIELD>). μ and σ are the mean and standard deviation of $\ln(PY)$, respectively. R^2 is the coefficient of determination of the log normal cumulative distribution fits.

[†] From slope of PY vs $\lambda-1$.

^{*} From ratio of PY/($\lambda-1$).