Microcarrier-Based Assay for *in vitro* 3D Quantification of Angiogenesis in Fibrin Gels

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Statement of Purpose:

Angiogenesis plays an important role in nearly every tissue regenerative process in the body. Tissue engineering methods including cell and growth factor delivery as well as the use of porous or hydrogel scaffolds primarily rely on the host vasculature to provide a positive environment for growth[1]. A number of factors have been shown to improve angiogenesis, but quantification of these methods *in vitro* has not been predictive of *in vivo* behavior. The majority of current methods fail to adequately represent the complex nature of angiogenesis because they utilize models that are effectively two dimensional or characterize growth in a qualitative manner[2]. We propose a new method for quantifying angiogenesis that can reliably quantify the extent of growth in three dimensions. By adhering human mesenchymal stem cells (hMSCs) onto collagen coated polystyrene microcarrier beads (MCs), and embedding these within fibrin gels, outgrowths were created spontaneously. After staining with Calcein AM and imaging with confocal microscopy, quantification of angiogenesis can be assessed using in-house Matlab™ programs and Imaris® software.

Methods:

hMSCs (Lonza) were cultured until confluent on maintenance hMSC medium (Lonza). The cells were enzymatically lifted with trypsin-EDTA (Lonza) before being added to MCs (Sigma). MCs were autoclaved in PBS and then washed with hMSC medium before being acclimated inside the cell incubator. The hMSCs and MCs were added to 15 ml Petri dishes that were treated with Sigmacote® before being sterilized by UV overnight. 153µl of 20 mg/ml MC solution was combined with $1.5x10^6$ cells in 15 ml medium giving a ratio of 625 cells/bead. The dish was gently agitated, to prevent aggregation of cells, every 10 mins for one and a half hours—at which time cells completely coated the MCs. These hMSC loaded MCs were added to tissue culture plastic for one hour—to remove free cells from the solution—then added to fibrin gels. Gels were prepared with porcine Fib (Sigma) and thrombin (Sigma). 40 mg/ml solution of Fib in TBS was added to the MCs (1:1 vol). One ml of this solution was added to each well of a 2 well chamber slide (Corning) before adding 1 ml of thrombin (25 U/ml). Gels were gently shaken before being placed in an incubator for 10 minutes. Gels were washed with hMSC medium at t=0, 30, and 1 hour after gelling occurred, then three times a day for two days, and once a day after two days. At one week after gelling, gels were washed repeatedly with PBS with Ca and Mg, to remove all dye from the medium. Calcein AM (Sigma) was then added to each gel to stain living cells, after which the gels were fixed with formaldehyde and imaged with a Leica® confocal microscope.

Results / Discussion:

MCs cultured in fibrin gels showed the outgrowth and networking of cells in three dimensions. Confocal microscopy was used to demonstrate cellular activity and three dimensional outgrowth from beads, as seen in Figure 1, but was more importantly used to quantify the extent of angiogenesis.

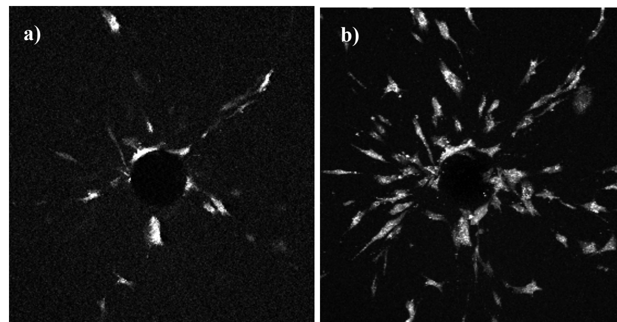

Figure 1: Confocal image 7 days after insertion into gel. a) One image captured from confocal b) Max projections of all images taken of that bead--shows the presence of cells on various planes surrounding the bead.

In-house Matlab™ programs were used to locate the MC in each of the confocal z-sections. The bead was then replaced with a specified threshold, as seen in Figure 2. Providing a 3D-threshold allows the series to be analyzed in Imaris®—which can quantify the length and amount of branching given a defined starting point (or the MC).

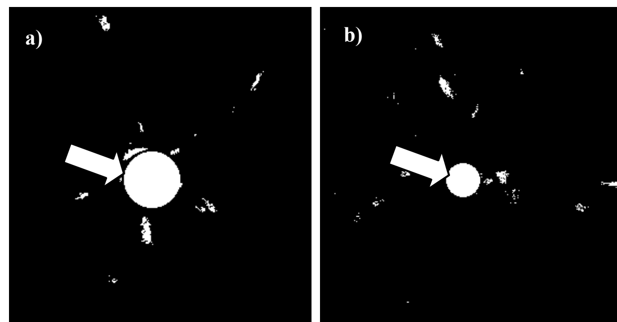

Figure 2: Confocal image 7 days after insertion into gel, analyzed by Matlab™ to provide a threshold where the bead would be. a) and b) represent the same bead at different z depths. Arrows denote the location of the simulated beads.

Conclusions:

Culturing cells in fibrin gels provides a three dimensional environment for cells to grow. Using Matlab™ in conjunction with Imaris® software, three dimensional angiogenesis can be accurately quantified. This technique was used to show that fibrin gels provide some level of angiogenesis *in vitro*, but it can be used to analyze the effects of numerous techniques on angiogenesis.

References:

1. Langer R, Vacanti JP. *Science* 1993;260(5110):920-926.
2. Nehls V, Drenckhahn D. *Microvascular Research* 1995;50(3):311-322.