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Enhanced reseeding of decellularized rodent lungs with mouse embryonic stem cells

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ABSTRACT

Repopulation of decellularized lung scaffolds (DLS) is limited due to alterations in the repertoire and ratios of the residual extracellular matrix (ECM) proteins, characterized by e.g., the retention of type I collagen and loss of glycoproteins. We hypothesized that pre-treatment of decellularized matrices with defined ECM proteins, which match the repertoire of integrin receptors expressed by the cells to be seeded (e.g., embryonic stem cells) can increase the efficacy of the reseeding process. To test this hypothesis, we first determined the integrin receptors profile of mouse embryonic stem cells (mESCs). Mouse ESCs express α3, α5, α6, α9 and β1, but not α1, α2 and α4 integrin subunits, as established by Western blotting and adhesion to laminin and fibronectin, but not to collagens type I and IV. Reseeding of DLS with mESCs was inefficient (6.9 ± 0.5%), but was significantly enhanced (2.3 ± 0.1 fold) by pre-treating the scaffolds with media conditioned by A549 human lung adenocarcinoma cells, which we found to contain ~5 μg/ml laminin. Furthermore, pre-treatment with A549-conditioned media resulted in a significantly more uniform distribution of the seeded mESCs throughout the engineered organ as compared to untreated DLS. Our study may advance whole lung engineering by stressing the importance of matching the integrin receptor repertoire of the seeded cells and the cell binding motifs of DLS.

1. Introduction

Lung diseases are a significant cause of morbidity and mortality worldwide. Approximately 3500 lung transplantations per year are performed worldwide [1]. The shortage of suitable donor lungs for transplantation emphasizes the need for other therapeutic approaches, such as cell-based regenerative therapies [2]. Amongst the possible solutions is the transplantation of engineered decellularized/repopulated lungs [2]. Different decellularization methodologies have been developed for efficient lysis and removal of cells, ranging from physical methods such as repeated freezing and thawing, to the use of chemical (e.g., detergents) and biological (e.g., enzymes) agents as single approaches or in combination [3]. Decellularized scaffolds then serve as matrices for recellularization using a variety of cell types e.g., repopulating the decellularized lung scaffolds (DLS) using whole lung cell suspensions, endogenous progenitor cells, embryonic/adult stem cells and induced pluripotent stem cells at different stages of differentiation [4]. Decellularization of the lung results in a cell-free, acellular, 3-dimensional (3-D) extracellular matrix (ECM) scaffold, which retains the gross anatomy of the original tissue/organ, but differs from the native lung ECM, in terms of protein composition, ultrastructure and mechanical properties [5]. It is becoming increasingly evident that the altered physicochemical properties of the DLS are in part responsible for the limited efficacy of recellularization [2]. The lack of efficiency and ability to fine-tune the recellularization process is of concern for translating this decellularization/recellularization technology of whole functional organ engineering into future clinical use.

Integrin receptors provide the primary functional link between cells and ECM components, thus mediating specific adhesion, physical and topographic sensing of the substratum [6]. Integrin receptors are heterodimers of α and β subunits, which responsible for the receptoral specificity through varying combination of currently identified 18 α and 8 β subunits [7]. In general, each cell type expresses a unique profile of integrin receptors that undergo dynamic changes e.g., during ESCs differentiation [8]. Therefore, a potential approach to improving the recellularization process is to

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characterize the integrin receptors profile of reseeded cells and in parallel, to restore some of the components of the decellularized lung ECM, which were lost during the decellularization process in order to enhance adhesion, spreading and site-specific differentiation of the reseeded cells. We hypothesized that pre-treatment of DLS with defined ECM proteins that match the repertoire of integrin receptors expressed by the cells to be seeded (e.g., embryonic stem cells) can increase the efficacy of the reseeding process. This approach has not yet been quantitatively addressed in the context of cell–biomaterial interactions in lung tissue engineering.

To test our working hypothesis we pre-treated decellularized lung scaffolds with conditioned media (CM) of A549 cells, a human alveolar adenocarcinoma cell line, which contains a number of lung-specific ECM proteins, such as laminin (LM) and fibronectin (FN) [9]. The A549-CM is also of particular interest, because it induces pulmonary differentiation of mouse embryonic stem cells (mESCs), as previously shown by our laboratory [10] and others [11].

2. Materials and methods

2.1. Embryonic stem cell culture

Mouse embryonic stem cells, E14tg2a (ATCC CRL-1821) were originally purchased from ATCC (Manassas, VA). For comparison, another batch of the same cells was kindly provided by Prof. Athanasios Mantalaris (Biological Systems Engineering Laboratory, Imperial College, London, UK). No differences were noted between the two sets of cells. The cells were maintained in a feeder-free culture system in T-75 tissue culture flasks coated with 0.1% gelatin (Millipore, Billerica, MA) in a humidified incubator at 37 °C in 5% CO_2. The maintenance media was composed of Dulbecco's Modified Eagle Medium (DMEM, Cellgro, Manassas, VA) supplemented with 10% stem-cell grade FBS (Biowest, France), 100 IU/ml penicillin and 100 μg/ml streptomycin (Cellgro), 2 mM L-Glutamine (Invitrogen, Carlsbad, CA), 10 ng/ml human recombinant BMP-4 (R&D Systems, Minneapolis, MN), 1000 U/ml ESGRO® mouse LIF (Millipore) and 0.15 mM 1-Thioglycerol (Sigma, St. Louis, MO), as previously described [11]. The maintenance media was changed every day and cells were split every 2–3 days using trypsin (TrypLE Express, Invitrogen) upon reaching 80% confluence at a 1:6 ratio. The cell cultures were routinely visually evaluated and microphotographs were taken using a Nikon Eclipse TE 2000-U (Nikon, Melville, NY) connected to a Hitachi KP-D50 digital camera (Woodbury, NY).

2.2. Preparation of conditioned media

A549 cells (ATCC CCL-185) were cultured in T-150 tissue culture flasks in a humidified incubator at 37 °C in a 5% CO_2 atmosphere. The cells were maintained in DMEM (Cellgro) supplemented with 10% FBS (Gemini BenchMark, West Sacramento, CA), 100 IU/ml penicillin and 100 μg/ml streptomycin (Cellgro), and 2 mM L-Glutamine (Invitrogen). The maintenance media was changed every other day and the cells were split upon reaching 80% confluence at a 1:8 ratio using trypsin (TrypLE Express, Invitrogen) as previously described [10]. In order to collect serum-free conditioned media (CM), 90% confluent flasks were quickly washed three times with Hank's Balanced Salt Solution (HBSS, Cellgro) containing calcium and magnesium followed by three additional washes for 10 min each with serum-free DMEM supplemented with antibiotics. To initiate CM collection each flask was supplemented with 30 ml of serum-free DMEM containing antibiotics and left for 48 h in the incubator. Upon completion of the conditioning step, the CM was collected and filtered through sterile low protein binding 0.2 μm syringe filters (Corning, Corning, NY). The filtered CM (considered as 100% CM) was aliquoted and kept at −20 °C. Aliquots were thawed only once immediately before use.

2.3. Immobilization of proteins for adhesion and ELISA assays

For the adhesion assays, compounds of interest (select ECM proteins, purified disintegrins, integrin receptor-specific antibodies, DMEM and CM) were immobilized in the wells of 96 well ELISA plates with high binding surface capacity (Cat. No.: 3590; Corning) and incubated overnight at 4 °C. The following ECM proteins were immobilized in a volume of 100 μl/well at a concentration of 10 μg/ml in phosphate buffered saline (PBS, Cellgro): bovine collagen type IV (Millipore); human collagen type IV (Millipore); human pro-collagen type I (kind gift from Dr. Andrzej Fertala, Thomas Jefferson University, Philadelphia, PA, USA); human collagen type I (Millipore); rat collagen type I (BD Biosciences, San Jose, CA); human plasma-purified fibronectin (Millipore); thrombospondin-1 (purified from human platelets [12]); VCAM (R&D Systems, Minneapolis, MN); vitronectin (R&D Systems); bovine elastin (sigma); Matrigel-purified laminin 111 (Sigma); and human recombinant laminins 111, 211, 332, 411, 421, 511, 521 (BioLamina, Sweden). The following disintegrins were purified from lyophilized snake venoms as previously described [7] and immobilized in a volume of 100 μl/well at a concentration of 20 μg/ml in PBS: viperistatin; VP-12; VLO-4, VLO-5 and echistatin. The antibodies were immobilized in a volume of 100 μl/well at a concentration of 10 μg/ml in PBS (listed in S-Table 1). Different concentrations of CM diluted with DMEM were immobilized at a final volume of 100 μl/well overnight at 4 °C. As negative controls PBS-only, DMEM-only and 10 μg/ml of BSA (Sigma), were immobilized overnight at 4 °C in a final volume of 100 μl/well.

In order to co-immobilize multiple ECM proteins, following the first immobilization step, as described above, the wells were washed three times with 200 μl Hank's Balanced Salt Solution (HBSS) without Ca^{2+} and Mg^{+2}, and incubated with 200 μl/well of 1 mg/ml BSA (1% BSA blocking solution) for 1 h at room temperature (RT). Thereafter, the wells were washed three times with 200 μl HBSS without Ca^{2+} and Mg^{+2}, and incubated with 1 μg/well of fibronectin or laminin, or 100 μl/well of 100%-CM for 1 h at 37 °C. As negative controls, immobilized BSA (1 μg/well) was incubated with 1 μg/well fibronectin or laminin, or 100%-CM for 1 h at 37 °C at a final volume of 100 μl/well.

2.4. Adhesion assay

The adhesion assay was based on a previously published protocol [13] with specific adjustments for mESCs. Upon completion of the immobilization step, as detailed above, the wells were quickly washed three times with 200 μl HBSS without Ca^{2+} and Mg^{+2}, and incubated for 1 h at RT with 200 μl/well of 1% BSA in HBSS (blocking solution) to block non-specific binding sites. In parallel, subconfluent mESCs cultures were washed three times with HBSS without Ca^{2+} and Mg^{+2}, trypsinized and centrifuged for 5 min at 1000 RPM. The cell pellet was resuspended in 1 ml of HBSS with Ca^{2+} and Mg^{+2}, The vital fluorescent probe CellTracker™ Green 5-Chloromethylfluorescein Diacetate (CMFDA, Invitrogen) was added at final concentration of 5 μg/ml and incubated for 30 min at 37 °C. Only viable cells enzymatically cleave the non-fluorescent molecule to produce a cell membrane impermeable fluorescent product. The stock solution of CMFDA was prepared in dimethyl sulfoxide (DMSO; Sigma) and the final concentration of DMSO in the cell suspension never exceeded 0.1%. Thereafter, the labeled cell suspension was diluted with blocking solution and centrifuged for 5 min at 1000 RPM. The pellet was resuspended, counted and diluted in blocking solution to a final cell concentration of 1×10^6 cells/ml. To initiate the adhesion assay, 100 μl of cell suspension were added to each well and incubated for 1 h at 37 °C. Thereafter, the wells were washed quickly three times with 200 μl/well of blocking solution. The specifically attached cells were solubilized in 100 μl/well of 0.5% Triton X-100 (Sigma). In order to estimate the number of attached cells, a calibration curve was prepared by solubilizing increasing volumes of the cell stock suspension (containing known cell numbers) with 100 μl/well of 0.5% Triton X-100. For each experiment a new calibration curve was generated, which included at least six different points in duplicates and resulted in linear correlation coefficient ($R^2 > 0.95$) between cell-associated fluorescence and cell number. Upon solubilization, the fluorescence of the well was quantified using a fluorescence plate reader (Synergy-4, Biotek, Winooski, VT) at λ_{Ex} = 485 nm/λ_{Em} = 528 nm. The number of attached cells was calculated from the calibration curve and expressed as percentage of adhesion to 1 μg/well of fibronectin, which was considered as positive control, unless mentioned otherwise.

2.5. Western blotting

Isolation of total cellular proteins, protein quantitation and Western blotting were performed essentially as previously described [14]. Briefly, subconfluent cultures of mESCs were scraped on ice and lysed with RIPA lysis buffer (Teknova, Hollister, CA). The amount of total soluble protein was quantified using the BCA protein assay (Pierce, Rockford, IL). For Western blotting, aliquots of protein solutions were mixed with β-mercaptoethanol-containing Laemmli sample buffer (Bio-Rad, Hercules, CA) at a ratio of 2:1, denatured for 5 min at 95 °C and a final amount of 35 μg protein was loaded onto 4–15% precast mini-polyacrylamide gradient gels (Bio-Rad). The samples were separated by SDS-PAGE (100 V for 1.5 h) and transferred for 30 min at 25 V using a semi-dry blotting system (Bio-Rad) to PVDF membranes (Millipore). The non-specific binding was blocked for 2 h at RT using 5% non-fat powdered milk (Santa-Cruz, Dallas, TX) and incubated with primary antibodies (listed in S-Table 1) at 4 °C overnight. The next day, detection was performed using horseradish-peroxidase-conjugated secondary antibodies (listed in S-Table 1) and visualized by chemiluminescence using an ECL 2 Western Blotting Substrate (Pierce). Subsequently, the membranes were stripped using Restore Western Blot Stripping Buffer (Pierce) and incubated with an anti-actin antibody as a control for equal protein loading. Films were exposed between 5 and 60 s, developed and scanned in a flat-bed scanner in transmission mode (Epson, Long Beach, CA). Positive controls for integrin receptors included 35 μg of total protein from K562 cells for α5 (ATCC CCL-243), K562 cells overexpressing α2 or α6 (kind gift from Dr. Martin E. Hemler, Dana-Farber Cancer Institute, Boston, MA, USA), A549 cells for α1 and α3, and LN229 cells for α9 and αV (ATCC CRL 2611).

For detection of proteins in the CM, aliquots of three independently collected batches were mixed with β-mercaptoethanol-containing Laemmli sample buffer at a 3:1 ratio and denatured for 5 min at 95 °C. A final volume representing 30 μl of CM was loaded on precast mini-polyacrylamide 4–15% gradient gels, separated, transferred and processed as described for total cellular protein. As positive controls for identifying ECM proteins in the CM, the same purified proteins that were used during immobilization for the adhesion assay were also loaded on gels at 1 μg/lane.