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Extracellular matrix deposition of bone marrow stroma enhanced by macromolecular crowding

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ABSTRACT

Decellularized extracellular matrices (ECM) from *in vitro* cell cultures can serve as *in vivo*-like matrix scaffolds for modulating cell-ECM interactions. Macromolecular crowding (MMC), the supplementation of synthetic or naturally occurring molecules resulting in excluded volume effects (EVE), has been demonstrated to provide valuable options for recapitulating the physiological environment of cells during matrix secretion. Human mesenchymal stem cell (MSC)-derived ECM was produced upon supplementation of standard culture medium with three different macromolecules of various size (10–500 kDa). Matrix secretion, ECM morphology and composition were compared for matrices obtained from crowded and non-crowded MSC cultures. In the context of generating functional stem cell niches, the MSC-derived bone marrow mimetic ECM scaffolds were tested for their supportive effect to maintain and expand human hematopoietic stem and progenitor cells (HSPC) *in vitro*. MMC in combination with metabolic stimulation of MSC was found to result in tissue-specific, highly organized ECM capable of retaining glycosaminoglycans and growth factors to effectively build *in vitro* microenvironments that support HSPC *expansion*.

1. Introduction

Decellularized extracellular matrix (ECM) scaffolds offer valuable options for investigating ECM signaling and remodeling *in vitro* [1–5]. However, culture conditions strongly influence how cells deposit and organize their ECM, and to which extent they secrete a certain ECM profile. Under physiological conditions cells are typically surrounded by a dense biopolymer meshwork that modulates the molecular assembly of the ECM [6–8], but standard cell culture settings do not sufficiently address the physiological crowding of molecules. Culture media are often very dilute nutrient solutions and are merely supplemented with various sera, small metabolites or growth factors. Beyond the presence of such dilute supplements, the concept of macromolecular crowding (MMC) becomes increasingly interesting for cell culture applications in order to mimic the natural *in vivo* environment more closely. Standard culture media are supplemented with polymeric macromolecules of synthetic or natural origin to occupy a given volume and thereby reduce the available space for other molecules – the so-called excluded volume effect (EVE) – and thus strongly influence reaction kinetics and molecular assembly of enzymes, proteins, and biopolymers [7–9]. MMC and EVE have been extensively studied over the past decades in the context of protein folding, association, and stability [10–15], and more recently have been explored for extracellular matrix enhancement by Raghunath et al. to support cell culture and tissue engineering applications [12,16]. For example, lung fibroblasts cultured under MMC were observed to build up more collagen in their environment due to the enhanced enzymatic conversion of *de novo* synthesized pro-collagen into mature collagen [6,10,17,18]. Recent studies using human mesenchymal stem cells (MSC) demonstrated that MMC enhanced collagen I deposition and alignment [19,20]. Cell sheets rich in ECM from cell types such as human lung and skin fibroblasts, tenocytes, or osteoblasts were obtained by MMC within a short culture period [12,21,22]. MMC also enabled amplified MSC differentiation into adipocytes when co-administered with adipogenesis-inducing

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supplements [23–25].

Previous own work on decellularized ECM scaffolds from human bone marrow MSC allowed the effective dissection of the matrix biology of MSC under osteogenic differentiation and ascorbic acid stimulation (known to enhance collagen synthesis [26,27]) [24,28]. Considering the potential of MMC to recapitulate physiological matrix conditions *in vitro*, the current study was designed to explore the effect of combined media supplementation of osteogenic supplements or ascorbic acid with macromolecules to emulate the excluded volume effect (Fig. 1a, Table 1). Physical, structural, and compositional properties of the ECM scaffolds were investigated.

To validate the biologic advantages of MMC modulation on ECM secretion the cell-free matrix scaffolds were applied as stem cell microenvironments for the expansion of hematopoietic stem and progenitor cells (HSPC). In the human body HSPC reside within the bone marrow, where they are regulated by multiple factors of the stem cell niche – namely cell–cell contacts, growth factors, physical cues, and the ECM [29,30]. Within the bone marrow MSC are the main cell type that secrete large amounts of ECM [31] and they were therefore used within this study to generate ECM scaffolds to mimic the HSPC niche. Although the ECM creates and modulates all of the niche exogenous signals, its role is still underappreciated when trying to recapitulate the stem cell microenvironment. To overcome the unmet need of HSPC expansion for therapeutic or regenerative purposes various materials and culture conditions have already been developed. Attempts to mimic the bone marrow niche for HSPC expansion range from growth factor supplemented suspension cultures [32] to co-cultures with MSC or osteoblast feeder-layers [33–35], or bioengineering approaches using synthetic and biomolecular components in biohybrid systems [36]. However, to date there is no sufficient tissue-mimetic niche system available that reliably expands functional HSPC without substantial stem cell differentiation. Our previous work on decellularized MSC-derived matrices demonstrated that cell-free ECM substrates had an improved potential to expand HSPC populations *ex vivo*, providing larger cell quantities for transplantation without a loss of long-term engraftment capacity into immune-comprimised mice, compared to HSPC expanded on classical plastic carriers [28]. The current work was aiming to further optimize these bone-marrow mimetic ECM microenvironments by macromolecular crowding and probe their potential for HSPC support and expansion.

2. Materials and methods

2.1. Culture carrier preparation [37]

Covalently immobilized thin films of maleic anhydride copolymers and fibronectin were generated on circular glass coverslips (Menzel-Glaeser). Freshly oxidized glass coverslips were aminosilanized and spin-coated with a 0.16% (w/v) poly(octadecene-alt-maleic anhydride) solution (POMA; molecular weight, 40,000 g/mol) in tetrahydrofuran (Polysciences) to create covalently bound polymer thin films (~5 nm). Fibronectin (Roche) solution at 50 μg/ml in PBS was applied to freshly annealed POMA-coated surfaces for 1 h at 37 °C, which resulted in a covalent bond between the protein and the polymer substrate. The surface was rinsed with PBS to remove excess protein and to achieve a homogeneous coating. Fibronectin-POMA surfaces were used immediately or stored under PBS at 4 °C for up to 48 h.

2.2. MSC culture

Human primary bone marrow MSCs were isolated from bone marrow aspirates of healthy male donors aged 20–40 years after obtaining informed consent. The institutional review board of the Medical Faculty at the University Hospital Dresden approved the study. Primary MSCs were expanded and characterized as described previously [38]. Cells were maintained in a humidified atmosphere of 5% carbon dioxide and cultured in low-glucose Dulbecco's modified Eagle medium (DMEM, Gibco) supplemented with 10% FBS (FBS, Gibco), referred to as standard medium.

2.3. Preparation of ECM layers

To create decellularized ECM layers from early passage (p2) MSCs, we seeded cells at ~1 × 10^4 cells per cm^2 onto POMA-FN surfaces and cultured to confluence. At the onset of confluence the medium was changed to start the different culture parameters (Table 1). Crowding and non-crowding culture conditions were applied to i) standard MSC culture medium, ii) osteogenic differentiation medium (to create an osteogenic microenvironment of bone marrow ECM, and iii) ascorbic acid medium (to create collagen-rich ECM). The respective media were then changed every second day. To yield cell-free ECM structures, we decellularized the cultures at day 10 using warm double distilled water supplemented with 20 mM ammonium hydroxide (Sigma). Specifically, decellularization was done by gentle agitation on a rotating platform for 10-min at room temperature. The resulting ECM layers were washed with an excess of deionized water three times and PBS (with calcium and magnesium) twice. Next, all ECM samples were treated with DNase (Roche or AppliChem) at a concentration of 1000 U/ml for 20–30 min at 37 °C. The DNA-free ECM layers were washed with an excess of PBS three times and stored under PBS (with calcium and magnesium) supplemented with penicillin and streptomycin at 4 °C for up to 4 weeks.

2.4. HSPC culture

G-CSF mobilized human HSPCs were isolated from leukapheresis products of healthy donors via magnetic-activated cell sorting (MACS, Miltenyi Biotec) of CD34-positive cells. The purity of the selected cell population was verified to range between 92 and 99% CD34 + cells. Cells were cultured in serum-free CellGro medium (CellGenix) supplemented with 10 ng/ml stem cell factor (SCF) (Miltenyi, Bergisch Gladbach, Germany), thrombopoietin (TPO) and FMS-like tyrosine kinase 3 ligand (Flt-3) (both R&D Systems) for one week without medium change. The culture was carried out as suspension culture in 12 well plates containing the previously decellularized MSC-derived matrices.

2.5. Flow cytometry

After one week in culture, suspension and ECM-bound cells were harvested from the matrices by three washes in PBS and 10 min incubation in Accutase (PAA) and were subsequently analyzed by flow cytometry for the following surface markers: CD34, CD133, CD45 (all Miltenyi, used according to the manufacturer's instructions). Non-specific Fc binding was prevented by adding polyclonal human immunoglobulin. Labeled samples were washed once with PBS/5% FCS before measurement. Doublet discrimination was carried out and non-viable cells were excluded by propidium iodide staining. All measurements were performed on a MACSQuant Analyzer (Miltenyi, Bergisch Gladbach, Germany). Data analysis was done using FlowJo-software (Tree Star, Ashland, OR, USA).

2.6. Collagen quantification

Amounts of total collagen and sulfated GAGs in decellularized