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Revealing cytokine-induced changes in the extracellular matrix with secondary ion mass spectrometry

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ABSTRACT

Cell-secreted matrices (CSMs), where extracellular matrix (ECM) deposited by monolayer cell cultures is decellularized, have been increasingly used to produce surfaces that may be reseeded with cells. Such surfaces are useful to help us understand cell–ECM interactions in a microenvironment closer to the in vivo situation than synthetic substrates with adsorbed proteins. We describe the production of CSMs from mouse primary osteoblasts (mPObs) exposed to cytokine challenge during matrix secretion, mimicking in vivo inflammatory environments. Time-of-flight secondary ion mass spectrometry data revealed that CSMs with cytokine challenge at day 7 or 12 of culture can be chemically distinguished from one another and from untreated CSM using multivariate analysis. Comparison of the differences with reference spectra from adsorbed protein mixtures points towards cytokine challenge resulting in a decrease in collagen content. This is supported by immunocytochemical and histological staining, demonstrating a 44% loss of collagen mass and a 32% loss in collagen I coverage. CSM surfaces demonstrate greater cell adhesion than adsorbed ECM proteins. When mPObs were reseeded onto cytokine-challenged CSMs they exhibited reduced adhesion and elongated morphology compared to untreated CSMs. Such changes may direct subsequent cell fate and function, and provide insights into pathological responses at sites of inflammation.

1. Introduction

The extracellular matrix (ECM) is known to be a key director of the fate, behavior and function of cells and tissues. Cells may sense surface composition, topology and mechanical properties of the ECM. Through complex signaling pathways, these may influence cell activities as diverse as cell adhesion, contractility, morphology and gene expression. These, in turn, drive phenotypic characteristics and regulate the remodeling of the ECM itself. In many inflammatory disorders, changes to ECM composition and structure drive both disease progression and severity [1,2]. For example, in inflammatory environments the action of cytokines such as interleukin (IL)-1β, tumor necrosis factor (TNF)-α and interferon (IFN)-γ disrupt matrix homeostasis by reducing ECM deposition and enhancing ECM resorption. Simultaneously, altered ECM deposition in inflamed tissues can actively influence immune responses at such sites [3].

The influence of the ECM on cell behavior can be challenging to investigate experimentally in a biologically relevant manner. Adsorbed protein surfaces have been widely used, although they only provide a flat, adhesive surface that does not approach the complexity of native ECM. Many developments have been made in the production of decellularized tissues and organs [4–6], their characterization [5,7,8] and developing use in the clinic [9–11]. However, whilst matrices may be produced from different tissue sources, anatomical locations, disease models or species, given their in vivo source, little control over matrix composition and structure at the point of deposition is possible. Therefore, the production of in vitro cell-secreted matrices (CSMs), where cells of interest are stimulated to deposit an ECM in situ before decellularization, providing a surface that may mimic in vivo niche environments, have been increasingly reported, with the aim of more closely recapitulating the in vivo environment.

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CSMs, also referred to in the literature as cell-derived matrices, have been produced from a range of cell types, including mesenchymal stem cells [12–16], hematopoietic stem/progenitor cells [17], epithelial and endothelial cells [18], fibroblasts [19,20], hepatic cells [21] and osteoblasts, both primary [22,23] and from cell lines [24]. They have found a number of applications, from early studies to understand cell proliferation and migration [19,25], to later studies investigating how the ECM directs stem cell differentiation [14,16,26]. CSMs have also been used to understand a number of disease states, notably examining cancer cell migration and metastasis [23,27–30], and genetic bone diseases exhibiting abnormal matrix deposition [22]. Recent studies report the use of CSMs in a broad range of studies, including those of vascularization [31], mesenchymal stem cell (MSC) differentiation [32] and cell migration [33]. A key advantage of CSMs is that they may be manipulated or modified at multiple points during their deposition. This may be through selection of cell types [22,26], culture conditions [14], differentiation protocols or post-decellularization modification [22,26].

Most commonly, CSM surfaces have been studied in situ by immunocytochemical and histological techniques [15,22], or ex situ, solubilizing the ECM before analysis by immunolabeling techniques such as enzyme-linked immunosorbent assay (ELISA) or Western blotting. For example, Bhat et al. [22] used immunohistochemistry to reveal increased levels of ECM components in CSMs from osteoblasts from donors with the genetic bone disease craniosynostosis compared to healthy donors. Whilst these approaches may be used to identify specific components, prior knowledge is required to select and identify appropriate targets. Spectroscopic and spectrometric techniques are useful as they collect data representative of the sample as a whole, rather than targeting specific components. Recently, proteomic methods employing mass spectrometry have been used to study CSMs and decellularized tissues [34], including the identification of novel matrix components from CSM secreted by a fibrotic liver cell line [21]; a comparative analysis of CSMs that support or inhibit the maintenance of pluripotency in human embryonic stem cells [35]; and proteomic analysis of decellularized human vocal fold mucosa [36]. However, such approaches require solubilizing the sample, removing one of the advantages of CSM systems – that the ECM proteins are presented at the surface in their native "as-secreted" orientations and structures. Therefore in situ surface analytical methods are advantageous.

Time-of-flight secondary ion mass spectrometry (ToF-SIMS) is a surface analytical technique that has been widely used to study a range of biological samples, including proteins [37–40], lipids [41], DNA microarrays [42] and cultured cells [43,44], along with "soft" [45–47], "hard" [48] and decellularized tissues [7,8]. In ToF-SIMS a primary ion beam (in this study Bi_3^+) is used to impact the surface of interest, yielding characteristic fragments from the top few nanometers of the surface. A proportion of these fragments are charged ions and may be analyzed using a time-of-flight detector to yield mass/charge spectra characteristic of the surface. From these spectra information may be extracted about the chemistry [49], structure [50] and molecular orientation [51] of the surface. ToF-SIMS provides certain advantages in the analysis of biological materials over both other proteomic mass spectrometry based methods and traditional biochemical techniques. Notably, ToF-SIMS allows surfaces to be analyzed in situ without digestion or disruption of the surface; and does not require a priori selection of targets of interest. When proteins are analyzed by ToF-SIMS, the positive ion spectra are dominated by their amino acid building blocks and associate molecular fragments [37]. The study of amino acid homopolymers [52,53] has allowed characteristic peaks for each amino acid residue to be identified, and this information can be used to probe the nature and orientation of proteins at interfaces [37].

Multivariate analysis techniques are useful to aid the interpretation of data from complex, multicomponent surfaces [54]. These mathematical methods can be used to identify distinct groupings and subtle spectral differences in a large number of complex samples. Principal components analysis (PCA) is one multivariate technique that has been widely used to study protein-rich samples [7,37,55,56]. PCA has previously been used to distinguish spectra from adsorbed protein mixtures [37], decellularized tissues [7,8] or residual ECM proteins following cell lift-off [56,57].

With carefully decellularized surfaces and powerful analytical techniques available, this study sets out to: (i) use ToF-SIMS to identify differences in the composition of CSMs stimulated by exposure to the pro-inflammatory cytokines IL-1β and TNF-α and IFN-γ; (ii) compare findings from ToF-SIMS analysis with those from complementary biochemical techniques; and (iii) relate the structure of these CSMs to the behavior of cells reseeded on them.

2. Materials and methods

2.1. Cell culture

Mouse primary osteoblast cells (mPObs) were isolated from the calvariae of 1- to 3-day-old CD1 mice as described previously [58]. Briefly, calvariae were dissected and digested in a solution of 0.5 mg ml^{-1} trypsin II S (Sigma) and 1.4 mg ml^{-1} collagenase IA (Sigma). Cells released during the first two digestion periods (10 min each) were discarded. Cells collected from the subsequent three digestion periods (20 min each) were pooled and cultured at a density of 6.6×10^3 cells cm^{-2} in a basal medium consisting of minimum essential medium-α (Lonza) containing 10% fetal bovine serum (Sigma), 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 2 mM L-glutamine. Cells were passaged once before cryopreservation and used experimentally before passage 4.

2.2. CSM production

The mPOb cells were plated in basal medium on 12-well tissue culture treated polystyrene (TCP) multi-well plates or 13 mm diameter Thermanox coverslips (Thermo) at a density of 10,000 cells cm^{-2}. Cells were allowed to adhere overnight before culture in osteogenic medium consisting of basal medium supplemented with 50 mM beta-glycerophosphate, 50 μg ml^{-1} L-ascorbate-2-phosphate and, when cytokines were not present, 10 μM dexamethasone (all from Sigma, Inc.).

After 14 days, cultures were decellularized, but retained an intact ECM surface on the substrate. Cultures were washed gently with phosphate-buffered saline (PBS) before treatment with 20 mM ammonium hydroxide in water for 20 min at 37 °C with intermittent shaking, followed by a further gentle wash in PBS before treatment with 50 U ml^{-1} DNase I in PBS for 1 h at 37 °C. Subsequent to decellularization, CSMs were washed gently in PBS and allowed to soak overnight in PBS. CSMs were stored in PBS at 4 °C for no more than 2 weeks before use.

2.3. Cytokine challenge

Cytokine challenge medium was prepared by supplementation of osteogenic medium (without dexamethasone) with 1 ng ml^{-1} recombinant human IL-1β, 10 ng ml^{-1} human TNF-α and 100 ng ml^{-1} mouse IFN-γ (all R&D systems). Both IL-1β and TNF-α have cross-species reactivity between mouse and human, but IFN-γ does not. Control medium was not supplemented with