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Decellularized human colorectal cancer matrices polarize macrophages towards an anti-inflammatory phenotype promoting cancer cell invasion via CCL18

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ABSTRACT

Macrophages are frequently identified in solid tumors, playing important roles in cancer progression. Their remarkable plasticity makes them very sensitive to environmental factors, including the extracellular matrix (ECM). In the present work, we investigated the impact of human colorectal tumor matrices on macrophage polarization and on macrophage-mediated cancer cell invasion. Accordingly, we developed an innovative 3D-organotypic model, based on the decellularization of normal and tumor tissues derived from colorectal cancer patients' surgical resections. Extensive characterization of these scaffolds revealed that DNA and other cell constituents were efficiently removed, while native tissue characteristics, namely major ECM components, architecture and mechanical properties, were preserved. Notably, normal and tumor decellularized matrices distinctly promoted macrophage polarization, with macrophages in tumor matrices differentiating towards an anti-inflammatory M2-like phenotype (higher IL-10, TGF-β and CCL18 and lower CCR7 and TNF expression). Matrigel invasion assays revealed that tumor ECM-educated macrophages efficiently stimulated cancer cell invasion through a mechanism involving CCL18. Notably, the high expression of this chemokine at the invasive front of human colorectal tumors correlated with advanced tumor staging. Our approach evidences that normal and tumor decellularized matrices constitute excellent scaffolds when trying to recreate complex microenvironments to understand basic mechanisms of disease or therapeutic resistance.

1. Introduction

The tumor microenvironment has been widely studied in the last decades. The recognition that tumors are comprised by extracellular matrix (ECM) components and different cell populations, including immune cells, fibroblasts and endothelial cells, opened new perspectives for the understanding of cancer biology and the development of more efficient therapies [1,2].

In this context, macrophages emerged as key players in cancer progression, with the capacity to modulate cancer cell migration, invasion and metastasis [3]. These highly plastic immune cells may adopt a myriad of distinct polarization phenotypes, according to the

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microenvironment context. In a rather simplistic vision, macrophages were initially described as being either pro-inflammatory (M1-like) or anti-inflammatory (M2-like). M1-like macrophages are induced by factors such as LPS, interferon-gamma (IFN-γ) or TNF-α. They exhibit high antigen presenting capacity, produce high levels of IL-6, IL-12 and IL-23 and toxic intermediates, and are known for their tumoricidal properties. Conversely, M2-like macrophages are induced by factors such as IL-10, IL-4, IL-13 or glucocorticoid hormones. They produce low levels of pro-inflammatory cytokines and high levels of anti-inflammatory cytokines, e.g. IL-10, having an important role in tissue repair, angiogenesis and in tumor progression [4]. In the last decade, macrophage polarization was recognized to be more complex and a continuum of polarization status between these two extreme populations was proposed [5,6].

Together with these cell populations, the ECM was described as being of pivotal importance for cancer progression [7,8], modulating the behavior of both cancer cells and other populations within the tumor microenvironment, namely immune and endothelial cells. The ECM is a highly dynamic and complex network of macromolecules, consisting of a reservoir of numerous bioactive domains and arrested growth factors [9]. It has critical functions, both in disease and homeostasis, development, morphogenesis and stem cell fate [10,11].

Since it has been clearly demonstrated that cells behave differently in 2D or 3D cultures [12], efforts have been made to develop new models that recapitulate as accurately as possible the native tumor microenvironment. These include organotypic explant cultures, multicellular tumor spheroids, or the use of engineered scaffolds composed of natural and/or synthetic components [13,14].

In the last decade, tissue decellularization, a process that enables cell removal without affecting the ECM structure and composition, emerged as an alternative technique in the field of tissue engineering and regenerative medicine [15]. Organs such as heart [16], lung [17] and liver [18] have been successfully decellularized and re-colonized by cells, constituting a promising solution for end-organ failure. Decellularization has also been recently applied in an attempt to unravel the complex role of ECM on metastasis and tumor progression [19–21].

In the present work, we developed a model that mimics the native tumor microenvironment, aiming to elucidate the role of tumor ECM on macrophage polarization and how this impacts cancer cell invasion. To achieve this goal, we used paired samples of human colorectal cancer (CRC) and non-neoplastic mucosa, which were efficiently decellularized and repopulated with primary human monocytes. Notably, although derived from the same patient, normal and tumor matrices distinctly modulated macrophage polarization. In tumor decellularized matrices, macrophages differentiated towards an anti-inflammatory phenotype, stimulating CRC cell invasion through a mechanism involving the CCL18 chemokine. In agreement, we observed that CCL18 was highly expressed at the invasive front of more advanced CRC cases. Overall, this 3D-organotypic approach, using tumor decellularized matrices as native human ECM source, provided new insights into the intricate crosstalks established at the tumor microenvironment, and contributed to the discovery of a putative new target for therapeutic intervention.

2. Materials and methods

2.1. Study approval

In the present study, both normal and tumor human samples were obtained from the Pathology Department from Centro Hospitalar São João (CHSJ, Porto, Portugal). Human monocytes were isolated from buffy coats from healthy blood donors, also from CHSJ. All studies using these human samples were approved by CHSJ Ethics Committee for Health (References 259 and 260/11), in agreement with the Helsinki declaration. Informed consent was obtained from all subjects.

2.2. Clinical samples

Fresh colorectal surgical specimens were collected directly from the Pathology Department from the Pathology Department from CHSJ within 1 h after surgery and transported in HBSS (Sigma-Aldrich), at 4 °C, to the laboratory where they were processed. Briefly, fragments were cut in smaller samples, placed in plastic containers and covered with mounting medium for cryotomy (OCT compound, Thermo Scientific). Samples were rapidly frozen in liquid nitrogen cooled 2-methylbutane and stored at −80 °C until further use.

2.3. Decellularization

Normal and tumor frozen colorectal specimens, from the same patient, were washed in PBS, cut in similar sizes, weighed and placed in a 24-well plate. Colorectal samples were decellularized based on the protocol described elsewhere [22]. After incubation with hypotonic buffer A (10 mM Tris, 0.1% EDTA, pH 7.8) for 18 h, tissue fragments were washed with PBS and decellularized for 24 h with 0.1% SDS. Following three washes with hypotonic buffer B (10 mM Tris, pH 7.8), a 3 h digestion, at 37 °C, was performed using 50 U/mL DNase (Applichem) prepared in 20 mM Tris and 2 mM $MgCl_2$, pH 7.8. The protocol was performed under constant agitation (165 rpm) in presence of 10 μg/mL of gentamicin (Gibco). Two different controls were considered: the “Native”, a fragment stored at −80 °C immediately after sample collection, and the “Non-decellularized”, a fragment incubated with PBS and refreshed every time a new solution was added to the samples undergoing decellularization. The experimental setup is represented in Supplemental Fig. S1.

2.4. DNA evaluation

After decellularization, samples were formalin-fixed, processed to paraffin blocks and sliced into 3 μm-thick sections. These were counterstained with Vectashield containing DAPI (VectorLaboratories) for fluorescent staining of nucleic acids. To assess total DNA content within the native tissue and the decellularized and non-decellularized matrices, DNA was extracted using PureLink Genomic DNA Mini Kit (Invitrogen) according to the manufacturer's instructions. DNA was quantified using Quant-iT™ PicoGreen® dsDNA kit (Invitrogen) and the fluorescence was measured with excitation at 480 nm and emission at 520 nm. Results were presented as ng of DNA per mg of tissue.

2.5. Histological analysis

3 μm-thick sections from paraffin-embedded samples were processed and stained with Hematoxylin and Eosin (H&E) and Masson's Trichrome for histomorphological analysis.

2.6. Immunohistochemistry

Imunohistochemistry analyses were performed for ECM proteins fibronectin, laminin and collagens type I and IV. Antigen retrieval was performed in a water-bath at 98 °C for 35 min with citrate buffer pH6, following blocking using Ultra V Block (Thermo Fisher Scientific) for 30 min. Tissue samples were then incubated