Communication

Bioactive Factors Isolated and Purified from Bovine Colostrum Can Restore Extracellular Matrix under Degradation by Metalloproteinases

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Bioactive Factors Isolated and Purified from Bovine Colostrum can Restore Extracellular Matrix under Degradation by Metalloproteinases

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Abstract: The ECM is composed of a considerable number of biochemically and structurally diverse constituents. ECM is a highly dynamic system that constantly receives and sends biological, chemical and mechanical signals. Several studies suggest that mechanical signals derived from the extracellular microenvironment regulate skin regeneration and wound healing. We evaluated effects of a mixture of biomolecules extracted and purified from bovine colostrum on restoring the mechanical properties of ECM. Test measuring collagen contraction showed a significant difference in contraction activation in samples treated with the 2% colostrum derivative mixture compared to the control. The analysis of the supernatant showed an inhibition of metalloproteinase-2 expression and an increase in collagen secretion by fibroblasts in treatment samples. Our hypothesis is that the molecules extracted and purified from bovine colostrum restore the ECM environment qualitative and quantitative characteristics, thus guaranteeing through a mechanical action the restoration of the wound due to the transduction of the signal activated by the integrins.

Keywords: mechanical signals; wound healing; collagen; integrin; mechanotransducer; ELISA test

1. Introduction

The cells within tissues are inserted in a highly structured microenvironment and are very sensitive to the geometric and mechanical constraints of the latter [1]. The cellular microenvironment, formed by the extracellular matrix (ECM) and neighboring cells, influences not only cellular architecture and mechanics, but also the polarity and functions of the cell [1,2]. Mechanical properties of the ECM depend on a complex protein network that forms a fibrous 3D scaffold whose structural components are collagen fibers, proteoglycans, and glycosaminoglycans [3]. Cells adhere to this network and act as a reservoir for nonstructural components such as growth factors, cytokines and proteolytic enzymes [4]. ECM proteins, by moving under the influence of forces, can act as mechanotransducers by exposing specific sites and growth factors [5]. The ECM is a dynamic structure, constantly undergoing a remodeling process, an important mechanism whereby cell differentiation can be regulated wound repair. In physiological conditions, the balance between processes of destruction and regeneration of the constituents of the extracellular matrix is regulated by specific tissue inhibitors of metalloproteinases (MMPs) [7]. An alteration of this balance, as in the case of wounds, is related to a catalytic hyperactivity of MMPs that play a critical role in wound healing. Their main function is degradation with removal of the damaged ECM during the inflammatory phase. The presence of these enzymes is necessary for effective wound healing, but they can play a harmful role at high concentrations, causing excessive tissue degradation and slow wound healing [7].

Knowledge of the cellular response to mechanical stimuli coming from the cellular microenvironment can be fundamental for applications in regenerative medicine to de-sign new and more effective scaffolds or biomaterials [8].

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Scaffolds should mimic the properties of the target tissue [9], ensure the restoring of anisotropy (one of the fundamental characteristics of most tissues) [10–12] and through mechanotransduction activate integrins and induce the cascade of growth factors (TGF-beta 1, CTGF, IGF-1, etc.) necessaries, among other things, to produce new collagen by the fibroblast [13]. The response of integrins to force involves three mechanochemical steps. Integrins bind to ECM molecules transmitting forces into the cell that are converted into biochemical signals (mechanotransduction). Finally, integrins connect to the cytoskeleton to transmit forces throughout the cell and strengthen adhesions to resist forces. Mechanical signals that are transmitted through structural components of the cytoskeleton play a key role in events that regulate cell migration, polarity, and proliferation [14].

The aim of our research is to demonstrate that bioactive factors purified from bovine colostrum are like molecules normally present in the extracellular matrix and that if supplied to stressed tissues (such as in chronic wounds), they can provide the scaffolding necessary to activate the signal transduction mechanisms that activate the response of integrins. Bovine colostrum is a nutrient milk secretion containing bioactive compounds that support calf nutrition and immune development. Colostrum is profuse in bioactive compounds like immunoglobulins, growth factors, lysozymes, lactoferrin, lactoperoxidase, but also possesses elevated levels of fats, proteins, minerals and vitamins. Bovine colostrum is also rich in extracellular nanovesicles, such as exosomes, which protect bioactive components from degradation [15]. These bioactive molecules are also components of the ECM, and their mechanical role is fundamental in its remodeling [16–18]. In order to demonstrate our hypothesis, we carried out also a test to evaluate collagen contraction and measured the expression of collagen production and metalloproteinases-2, crucial elements for wound healing [7,19,20].

2. Materials and Methods

2.1. Colostrum Derivative Mixture Preparation

The colostrum derivative mixture (CDM) preparation was processed according to the procedure described by [21]. Bovine colostrum was collected from Holstein cows from 1 up to 6 h after parturition. This methodology, through micro and nano filtration, allows the elimination of casein, fats and other non-functional macromolecules including bovine immunoglobulins from the colostrum, and essentially isolates growth factors and cytokines identified with ELISA tests [21]. In all experiments, we used 2% colostrum derivative mixture because, our unpublished data, we have seen that this corresponds to concentration growth factors and cytokines normally present in the extracellular matrix.

2.2. Cell Lines

The Human Dermal Fibroblasts (HDF) (106-05A, Merck) were grown in Dulbecco's Modified Eagle Medium (DMEM). Medium were supplemented with 10% fetal bovine serum. The medium was replaced every 2 days, starting when the cells reached 80% confluence. Cultures were maintained at 37°C in a humidified environment containing 5% CO_2.

2.3. Collagen Contraction

To assess matrix contraction free-floating collagen lattice models were used according to [19]. The lattices were detached before addition of test substances. The reduction of the lattice area due to contraction was evaluated at 24 hours intervals for up to 96 hours.

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2.4. Collagen Production

Matrix synthesis was determined over 7 days in the presence or absence of colostrum derivative mixture 2%. Secreted C-terminal propeptide of collagen type I (CICP) was measured in culture supernatant using an enzyme immunoassay kit (Metra Biosystem, Quidel Corporation).

2.5. Determination of MMP-2 Concentration

MMP-2 expression, constitutively secreted from fibroblasts, was determined with a commercially available quantitative ELISA test (Amersham Pharmacia Biotech). All procedures were performed according to the instructions of the manufacturer.

2.6. Statistical Analysis

Data were analyzed using the GraphPad Prism 5.0 program (GraphPad Software, La Jolla, CA, USA), using the analysis of variance (ANOVA) test and Tukey's test. A p value ≤ 0.05 was used to identify statistically significant differences. Parametric correlation was calculated using Pearson correlation coefficient.

3. Results and Discussions

Tissues are not only made up of cells, but a significant part of their volume is formed by the extracellular space, occupied by an intricate network of macromolecules, whose three-dimensional organization is represented by the extracellular matrix. Biochemical analysis of the ECM reveals that it is composed of several proteins and polysaccharides, which aggregate in a compactly organized network connected to the surface of the cells that produced it and the surrounding ones [6,16,22]. ECM forms the bio-scaffold of our body and adapts to the environmental variables. The biomechanical properties of ECM are negatively affected by aging, but also during wound healing or restore skin in burning injuries [23]. Many computational models and experimental studies have revealed the important effects of cell-generated mechanical forces, forces acting upon cells, and mechanical characteristics of the extracellular matrix on cell morphology and function [23]. The microenvironment surrounding cells play a large role in directing cell behavior and the mechanical functions are important for both understanding cell behavior and excellent scaffolds designed. Cell can actively sense the mechanical properties of their surroundings by exerting contractile force, which can transmit between cell–matrix or cell–cell. Cells produce and can modify the organization of this ECM, which can vary widely in both composition and mechanical proprieties. Thus, these mechanical properties are a direct result of cellular activity, leading to the principle of dynamic reciprocity between the cell and its environment. But on cells can act mechanical forces exerted from ECM if as tissues are deformed, such as in wounds [24–26]. Our hypothesis is that the molecules extracted from colostrum (Table 1), identified with ELISA test according to [21], restore chemical/physical ECM environment, thus guaranteeing through a mechanical action the restoration of the wound due to the transduction of the signal activated by the integrins. Our hypothesis is supported by the results obtained with collagen contraction test that demonstrated exposure of fibroblast-populated collagen lattices to concentrations of colostrum derivative mixture significantly has activated contraction by day 7, compared with the control (Figure 1). These results are of great interest especially if correlated to the simultaneous results of ELISA test made during collagen contraction. Tests demonstrated that fibroblasts actively have secreted CICP during lattice contraction up to 7 days (Figure 2) and inhibited MMP-2 expression (Figure 3) in the samples treated with the 2% colostrum derivative mixture.

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Table 1. Growth factors and cytokines present in bovine colostrum identified with Elisa test.

Growth factors and cytokines present in bovine colostrum	
Transforming Growth Factors β (TGF-β)	EOTAXIN-CCL11
Insulin-Like Growth Factor 1 (IGF-1)	Tumor Necrosis Factor α (TNF-α)
basic Fibroblast Growth Factor (bFGF)	Nerve Growth Factor (NGF)
Vascular Endothelial Growth Factor (VEGF)	Gamma Interferon (INF–γ)
Epidermal Growth Factor (EGF)	Bone Morphogenetic Protein 2 (BMP-2)
Platelet-Derived Growth Factor (PDGF)	Stromal Cell-Derived Factor 1α (SDF1-α)
Keratinocyte Growth Factor (KGF)	Interleukin-2 (IL-2)
Hepatocyte Growth Factor (HGF)	Interleukin-4 (IL-4)
Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF)	Interleukin-6 (IL-6)
Granulocyte-Colony Stimulating Factor (G-CSF)	Interleukin-17A (IL-17A)

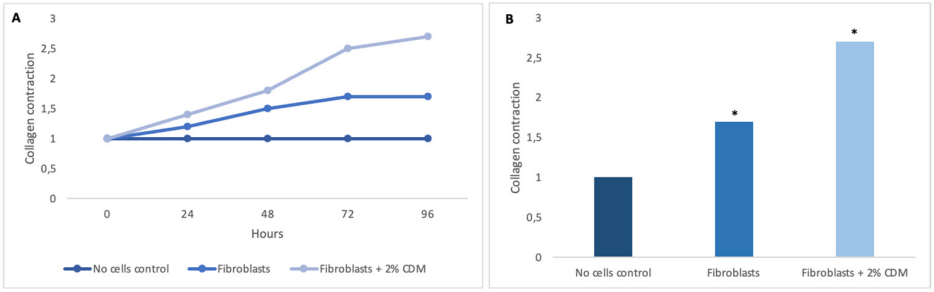

Figure 1. Graphical representation of collagen contraction. A. Collagen gel contraction over 96 h at 24 h time intervals. B. Fold change in collagen gel contraction at 96 h. Asterisk denotes the degree of significance between results: *$p < 0.01$.

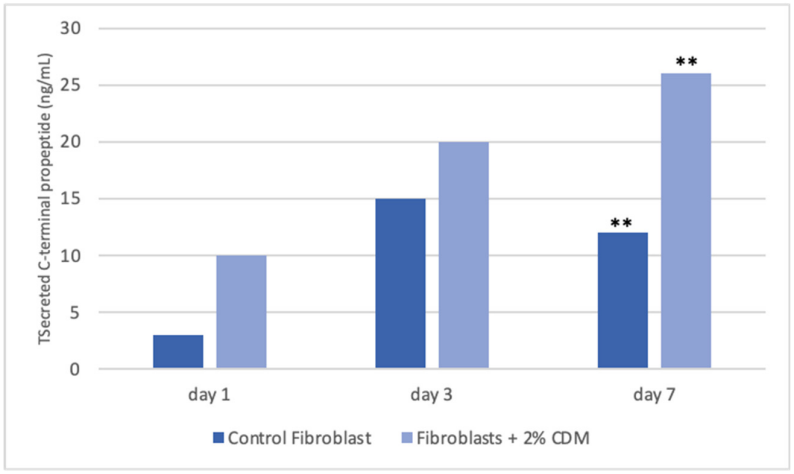

Figure 2. Graphical representation of collagen production during collagen contraction. Fibroblasts actively has secreted CICP during lattice contraction up to 7 days. In control samples, secretion of CICP has increased up to 3 days and after was reduced. Asterisk denotes the degree of significance between results: **$p < 0.001$.

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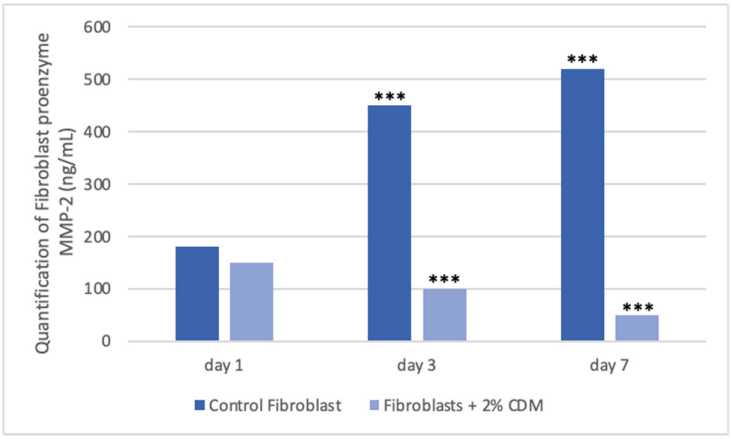

Figure 3. Graphical representation of proenzyme MMP-2 production during collagen contraction. Asterisk denotes the degree of significance between results: ***$p < 0.0001$.

Statistical analysis using Pearson correlation coefficient, confirmed a significant positive linear correlation between collagen contraction and collagen production during collagen contraction from fibroblasts ($r = 0.98$, $p < 0.001$) and a significant negative linear correlation between collagen contraction and proenzyme MMP-2 production during collagen contraction ($r = 0.99$, $p < 0.001$) (Figure 4).

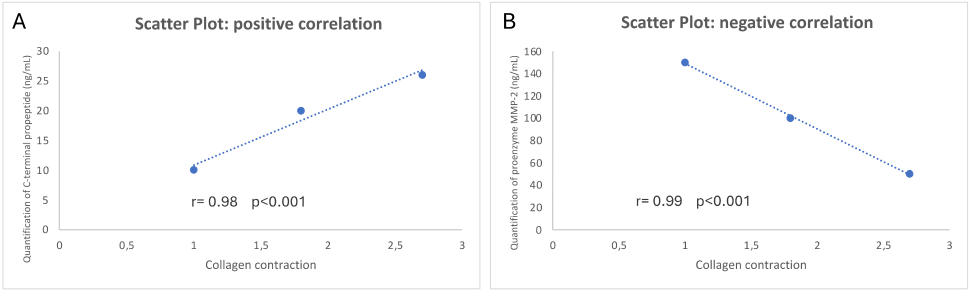

Figure 4. Scatter Plot. **A**, strong positive linear correlation and **B**, strong negative linear correlation, indicated by Pearson coefficient values (r).

Contraction and remodeling of the extracellular matrix are essential processes during wound healing [27,28]. At the center of these two phenomena are fibroblasts, which not only produce and secrete extracellular matrix proteins but can also reorganize them through mechanical interactions [29,30]. Collagen is the predominant structural protein in the ECM providing not only tensile strength but also play a role in cells adhesion and migration [31]. Matrix metalloproteinases (MMPs) are a family of proteolytic enzymes capable of degrading various structural components of the matrix [32–37]. They therefore play a fundamental role in various physiological and pathological processes including wound healing [17,28]. It is now known that the biomechanical properties of the ECM contribute to physiological development of cells and tissues [24].

The elasticity of the ECM allows to perceive external forces and, therefore, provides an important environmental input signal that determines the cell's response. Indeed, the adhesion complex, which consists of integrins and a multicomplex of adapters and signaling proteins, can be considered as a mechanosensor that connects the actomyosin cytoskeleton with the ECM [38]. Many of the adhesion components undergo conformational changes that determine functional consequences in the response to applied force. Cytoskeleton together with nuclear matrice, nuclear envelope and chromatin, they constitute a mechano-sensing system that determines how cells react to forces transmitted by the ECM [38–42]. Many of the focal adhesion components undergo conformational

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changes that determine functional consequences in the response to applied force. Together with the cytoskeleton and nuclear matrices, nuclear envelope, and chromatin, they constitute a sophisticated mechanosensing machinery that determines how cells react to forces transmitted by the ECM, which regulates various essential cellular behaviors, including cell fate determination, differentiation, and cell function [38–42]. The different properties of the ECM are not independent of each other but influence each other. Therefore, when the ECM increases in stiffness, as, for example, in pathological conditions, its biomechanical properties change, and cells respond by exerting markedly different types of force. Furthermore, the stiffening of the matrix also determines a change in the other physical properties of the ECM and, consequently, directly modifies the cellular ability to migrate. ECM, constantly undergoing restructuring in different tissues, is highly dynamic [42]. ECM dynamics can arise from changes in the amount or composition of ECM, due to altered synthesis or degradation of one or more components. Alternatively, ECM dynamics may show no changes in the composition of its components, but instead involve only the transformation of its components, into spatially organized structures, thanks to covalent and non-covalent bonds [6,22].

Finally, one of the most important features of cell-ECM interactions is reciprocity [43]. Cells constantly make, degrade or rearrange components of the ECM to modify it one or more properties; but, since the ECM regulates the behavior of cells, any change in it, as result of cellular activities, will in turn influence adjacent cells and modify their behaviors. This feedback regulatory mechanism between cells and the ECM allows cells and tissues to rapidly adapt to the changes of environment [6].

4. Conclusions

Wounds heal through coordinated action of fibroblast-mediated extracellular matrix (ECM) deposition, ECM remodeling, and wound contraction. For these processes, are fundamental the mechanical signals between ECM and cells. In this paper, we demonstrated that biomolecules extracted and purified of bovine colostrum can restore chemical/physical ECM environment, reestablish the mechanical properties of ECM and the anisotropy characteristics of the damaged tissue. This process activates transduction of the signal by the integrins and reactivates the ability of the fibroblast to synthesize new collagen, inducing the autologous mechanisms of repair and remodeling of damaged connective tissue. Fibroblasts, in fact, can be able to generate tensile forces as well as receive them; these contraction forces of the fibroblast are indispensable for wound resolution processes.

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