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Matrix metalloproteinases as reagents for cell isolation

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ABSTRACT

Cell isolation methods for therapeutic purposes have seen little advancement over the years. The original methods of stem cell and islet isolation using bacterial collagenases were developed in the early 1980s and are still used today. Bacterial collagenases are subject to autodegradation, and isolates obtained with these enzymes may be contaminated with endotoxins, reducing cell viability and contributing to toxicity in downstream applications. Here we describe a novel method for isolation of mesenchymal stem cells from adipose tissue (ADSC) utilizing recombinantly produced matrix metalloproteases (MMPs). The ADSCs isolated by MMPs displayed essentially identical morphological and phenotypical characteristics to cells isolated by bacterially-derived collagenase I and Liberase™. Samples isolated with MMPs and Liberase™ had comparable levels of CD73, CD90, and CD105. The adipogenic and osteogenic potential of the ADSCs isolated by MMPs was retained as compared to cells isolated with Liberase™. However, ADSCs isolated by Liberase™ displayed 6% contamination with other cells as per negative markers revealed by PE staining, as opposed to <1% for all MMP-treated samples. MMP-based cell isolation may contribute to optimization of transplantation technology.

1. Introduction

Proteases comprise over 2% of the human genome and regulate a wide range of cellular processes, including cell proliferation and differentiation, angiogenesis, neurogenesis, digestion, DNA replication and transcription, protein processing, homeostasis, development, immunity, blood coagulation, wound repair, senescence, necrosis, and apoptosis [1,2]. The human degradome is comprised of 569 proteases distributed into five broad classes, with metalloproteases being the most abundant, followed by serine, cysteine, threonine, and aspartyl family members. Under normal conditions, protease expression and actions are controlled spatially and temporally and the substrate repertoire is highly specific. Nearly all proteases are expressed as inactive precursors (zymogens), which are transported to the site of final intra- or extra-cellular activity. These zymogens must be activated, frequently by other proteases in a sequential protease pathway. In addition, the activity may be regulated at transcriptional and post-translational levels, or by native protease inhibitors. However, dysregulation of the proteolysis frequently leads to pathological processes resulting in cancer growth and metastasis, inflammatory diseases, and neurodegenerative and cardiovascular disorders [1,2].

Given the important role proteases play in disease development and progression, there has been considerable effort in the development of protease inhibitors for various diseases. However, there are very few uses of proteases themselves for clinical applications. The few FDA-approved uses for proteases are limited to applications in stroke, acute myocardial infarction, muscle spasms, sepsis, traumatic bleeding, and blood clotting and digestive disorders. Most marketed proteases are serine proteases, except for botulinum neurotoxin (Botox®) and *Clostridium histolyticum* collagenase, which are zinc metalloproteases [1,3,4].

The matrix metalloproteinases (MMPs) are a family of zinc-dependent enzymes capable of catalyzing the degradation of virtually all extracellular matrix (ECM) components, including collagens, laminin, fibronectin, and elastin [5–7]. MMPs also selectively cleave cell surface receptors, cytokines, chemokines, and cell–cell adhesion molecules [8]. Although necessary for tissue

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Table 1
Primers and vectors used for the production of the CAT domains of MMP-1 (S1C), MMP-3 (S3C), MMP-12 (S12C), MMP-19 (S19C), MMP-25 (S25C), or full-length MMP-19 (S19FL).

Enzyme	Primers used	Restriction enzyme used	Vector used	Protein sequence
S1C	Fwd: GCCGATCATATGTTTGTCCTCACTGAAGGC Rev: CCGATAAGCTTTCACTGGACAGGATTTTGGGAACG	NdeI HindIII	pET28	F^{101}-Q^{270}
S3C	Fwd: GCCGATCATATGTTCAGAACCTTTCCTGGCATCC Rev: GCCGATGGATCCTTATGGTGTCTCAGGTGAGTCAGG	NdeI BamHI	pET28	R^{101}-V^{275}
S12C	Fwd: GCCGATCATATGTTCAGGGAAATGCCAGG Rev: GCCGATGGATCCCTACAAGCGTTGGTTCTCTTTTGG	NdeI BamHI	pET21	F^{99}-L^{271}
S19C	Fwd: GCCGATCATATGCTTAAATACCTGTTGCTGGGC Rev: GCCGATAAGCTTCAACTCTTCTTGCCATAGAGAGC	NdeI HindIII	pET28	L^{96}-S^{259}
S19FL	Fwd: GCCGATCATATGAACTGCCAGCAGCTGTGG Rev: CCGATAAGCTTTCAGTATTCAAACGTGGTTTCTGTGG	NdeI HindIII	pET21	M^{1}-Y^{508}
S25C	Fwd: GCCGATCATATGTACGCTCTGAGCGGCAGCGTG Rev: GCCGATAAGCTTTCACCCATAGAGTTGCTGCAGGCC	NdeI HindIII	pET21	Y^{107}-G^{280}

homeostasis under normal physiological conditions, MMP dysregulation has been found in many disease states such as cancer development and survival, metastatic tumor growth, angiogenesis, invasion, modulation of the immune response, and pathological degradation of extracellular matrix (ECM) components, such as collagen and laminin. While MMP inhibition has been explored for many years in an effort to treat various diseases, to our knowledge the use of these proteases towards clinical applications, such as cell isolation or tissue engineering, has not been explored.

Adipose tissue ECM has been described as "loose connective tissue." Immunofluorescence staining of bovine adipose tissue ECM revealed types I, III, IV, V, and VI collagen, laminin, and fibronectin [9]. More detailed proteomic analyses have shown some variation based on species, but the collagens are consistent [10]. Decorin or other proteoglycans may be important in adipose tissue ECM [10]. Quantitation of human adipose tissue ECM showed significant levels of acid-soluble collagen and elastin, but only low levels of sulfated glycoaminoglycans (GAGs) [11].

Broad-spectrum inhibition of MMPs impairs adipose tissue growth, while MMP-3 and MMP-11 deficient mice develop more adipose tissue than wild-type mice [12]. These results indicate that MMPs participate in adipose tissue remodeling. Due to that fact, as well as due to the composition of the fatty tissue and the known substrate repertoire of the MMPs, we hypothesized that MMPs may be used to isolate adipocyte-derived stem cells.

In the present study we applied various recombinantly produced MMPs (MMP-1, MMP-3, MMP-8, MMP-9, MMP-12, MMP-19, and MMP-25) towards isolation of mesenchymal stem cells (MSCs) from adipose tissue and compared the isolation efficiency to collagenase I and Liberase™. These latter proteases are routinely utilized for the isolation of MSCs from various tissues. Collagenases I and II are obtained from the extremely dangerous bacillus *Clostridium*, an agent of gas gangrene. Crude preparations from *Clostridium histolyticum* contain not only collagenases but also a sulhydryl protease, clostripain, a trypsin-like enzyme, and an aminopeptidase. Liberase™ is an enzyme mixture composed of collagenases I and II and thermolysin [13]. A significant problem is that collagenase I is the most unstable component of Liberase, as the Ia form is rapidly autocatalytically degraded to the Ib form. Degraded collagenases have an adverse effect on islet viability [14]. There is a wide range of endotoxin contamination of traditional collagenase preparations compared with the endotoxin level of Liberase. However, regardless of the source, all purified collagenases and neutral proteases from bacterial bullion are contaminated with endotoxin [13,15,16]. Prior studies have investigated the relative amount of endotoxin in different collagenase preparations and the impact on isolated cell health [16–18] and observed that the presence of endotoxin is harmful for adipose tissue-derived stem cell (ADSC) viability. In addition, success in cell transplantation is directly proportional to the quality of cells isolated, cultivated, and allografts prepared. Thus, other, more stable enzymes need to be considered for isolation of stem cells and islets.

ADSCs isolated by protease treatments were propagated and characterized morphologically and immunophenotypically. To assure the ADSC differentiation potency, adipocyte and osteocyte cell differentiation was induced. We also demonstrate the recombinant production and characterization of full-length MMP-25, which has not been previously reported.

2. Methods and materials

2.1. Commercial enzyme activation

Buffer reagents, chymotrypsin, and Oil Red O solution (0.5% in isopropanol) were obtained from Sigma (St. Louis, MO). Liberase™ was obtained from Roche (San Francisco, CA). Collagenase I was obtained from Worthington Biochemical (Lakewood, NJ). Trypsin-3, MMP-1, MMP-3, MMP-8, and MMP-12 were obtained from R&D Biosciences (San Diego, CA). Activated MMP-9 and the catalytic domain of MMP-3 (MMP-3 CAT) were obtained from Calbiochem (La Jolla, CA). All enzymes except MMP-9 and MMP-3 CAT were activated at 20 ng/μL concentration with 5 ng chymotrypsin/5 ng trypsin mixture for 30 min at 37 °C. The reaction was stopped by addition of 2 mM PMSF (Biosynth, Itasca, IL). Enzyme activity was tested using 5 μM Knight single-stranded peptide (SSP) [Mca-Lys-Pro-Leu-Gly-Leu-Lys(Dnp)-Ala-Arg-NH_2] in TSB (50 mM Tris, 150 mM NaCl, 0.02% NaN_3, 0.01% Brij-35, 10 mM $CaCl_2$, 1 μM $ZnCl_2$, pH 7.5). The Knight SSP was synthesized by methods described previously [19,20]. The activity of full-length MMP-25 was also tested against $(Gly\text{-}Pro\text{-}Hyp)_5$-Gly-Pro-Lys(Mca)-Gly-Pro-Gln-Gly∼Cys(Mob)-Arg-Gly-Gln-Lys(Dnp)-Gly-Val-Arg-$(Gly\text{-}Pro\text{-}Hyp)_5$-$NH_2$ (where Mob = 4-methoxybenzyl, Hyp = 4-hydroxy-L-proline, Mca = (7-methoxycoumarin-4-yl)acetyl, and Dnp = 2,4-dinitrophenyl), a collagen-model fluorogenic triple-helical MMP substrate previously described [21]. Activated enzyme stocks were frozen at −80 °C for further use.

2.2. Recombinant expression and production of MMP-1, MMP-3, MMP-12, MMP-19, and MMP-25

Recombinant catalytic (CAT) domains of MMP-1, MMP-3, MMP-12, and MMP-25 and the CAT domain and full length MMP-19 were produced as follows. cDNA of the CAT domain of MMP-1, MMP-3, MMP-12, and MMP-19 were amplified from commercial clone sources (Thermo Scientific, Waltham, MA) by PCR using primers containing NdeI and HindIII or BamHI restriction sites (Table 1). cDNA of the CAT domain of MMP-25 was amplified