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Transforming growth factor-β3 (TGF-β3) loaded PLGA-b-PEG nanoparticles: Efficacy in preventing cardiac fibrosis induced by TGF-β1

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1. Introduction

Cardiac fibroblasts (CFs) have vital structural and functional role in providing structural support and homeostasis to extracellular matrix (ECM). Production and secretion of connective tissue components such as collagens, elastin and fibronectin by CFs have direct contribution to homeostasis of ECM. Proteolytic enzymes called matrix metalloproteinases (MMPs) which mediate degradation of ECM involved in the turnover of ECM by counterbalancing this homeostasis [1–3]. In addition, endogenous inhibitors of MMPs which are known as tissue inhibitors of metalloproteinases (TIMPs), are important regulators of ECM turnover [4].

A remodeling process, a pathological state characterized by accumulation of connective tissue proteins, is observed when the homeostasis of ECM is disturbed. This is the major leading event for development of fibrosis. CFs are the principle cells mediating fibrosis by having role in orchestrating ECM remodeling. In fibrosis, CFs transdifferentiate into activated state termed as cardiac myofibroblasts (CMFs). This transdifferentiation may be stimulated with various factors such as exposure to certain drugs, aging and heart diseases [5]. Regardless of the stimuli leading to differentiation of fibroblasts to myofibroblasts, the carefully regulated balance of the ECM will be disturbed and fibrosis will be observed due to the inhibition of MMPs, activation of TIMPs and excess accumulation of collagen [6]. As a consequence, reduction in heart contractility and impairment in heart performance will occur.

It has been reported that during different acute and chronic heart diseases such as obstructive hypertrophic cardiomyopathy and myocardial infarction activated platelets secrete some factors that may participate in inflammation and cardiac remodeling observed in cardiac injury [7,8]. During cardiac injury, storage granules of activated platelets releases the stored contents into the microenvironment. Transforming growth factor β1 (TGF-β1), which is known to be stored in granules and released into the environment with this activation, has been reported to be involved in the development of cardiac fibrosis [9,10].

TGF-β is a multifunctional growth factor that have been reported to be involved in tissue repair process; scar tissue formation by regulating cell proliferation and migration, differentiation, ECM production, and immune modulation [11]. In mammals TGF-β has three isoforms as TGF-β1, β2, and β3 that are part of TGF-β superfamily. Isoforms are encoded by three distinct genes, and show significant homology (60–80%) [11]. *In vitro* data suggest that all isoforms compete for the same receptors [12], showing that the isoforms may have different, possibly antagonizing functions. TGF-β1 has a role in pathological fibrosis [13] by stimulating over expression of many matrix proteins such as collagens, laminin, fibronectin … etc. and by inhibiting the degradation of these matrix proteins [14]. This action of TGF-β1 has also been observed in fibrotic heart and kidney diseases [15–19]. On the other hand, the other isoform TGF-β3, suppresses the dense collagen production and prevents the scar formation [11]. Although they belong to the same protein family, TGF-β1 leads to fibrosis by stimulating excess ECM protein production while TGF-β3 acts as an antifibrotic agent by preventing excess collagen synthesis.

In addition to traditional cardiovascular treatments used in heart failure models, new and promising antifibrotic treatment strategies have been developed. The purpose of these strategies focuses on reducing the local concentration of TGF-β1 via using directly TGF-β1 antibodies or antisense oligonucleotides or gene silencing [20]. Although TGF-β3 has the ability to reverse the fibrotic effects of TGF-β1, TGF-β3 has never been used to prevent or suppress cardiac fibrosis due to the possibility of rapid removal from target tissue or enzymatic degradation. To be able to use this growth factor as a therapeutic, antifibrotic agent new drug transport systems are needed due to biological instability, proteolysis and short half-life.

The development of safe and effective delivery system containing TGF-β3 is of paramount importance. To address this problem, we aimed to develop TGF-β3 loaded poly(lactic-co-glycolic acid)-b-poly(ethylene glycol) (PLGA-b-PEG) delivery system to be used as an effective treatment strategy to prevent fibrosis observed in cardiac tissue healing after injury. PLGA-b-PEG polymer has degradation products called lactic and glycolic acid units that are naturally found in the body and these units can be removed by normal metabolic pathways and therefore is widely used as a biocompatible and biodegradable drug transport system [21].

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Besides, this polymer is a good candidate to have a protection against proteolytic degradation of encapsulated agent, TGF-β3 [22]. In this study, TGF-β3 loaded PLGA-b-PEG nanoparticles were successfully prepared and characterized. Fibrosis was induced by TGF-β1 in human cardiac fibroblast cell line and effects of TGF-β3 loaded PLGA-b-PEG nanoparticles on progression/prevention of fibrosis were evaluated and discussed in detail.

2. Materials and methods

2.1. Chemicals

Poly(ethylene glycol) methyl ether-block-poly(lactide-co-glycolide), bovine serum albumin (BSA, Mw 66 kDa) which was used as model protein and Transwell polycarbonate membrane cell culture inserts (Corning) were from Sigma-Aldrich Co. Pluronic F-68 was from Gibco. Phosphate-buffered saline (PBS) was purchased from Invitrogen. Acetonitrile (HPLC grade) was from Fisher Scientific (Pittsburgh, PA). The active ingredient, TGF-β3, in nanoparticle formulations was purchased from R&D systems. Human cardiac fibroblasts (CFs), FGM-3 Fibroblast Growth Medium-3 (FGM) and media supplements were purchased from Lonza (Basel, Switzerland). Recombinant Human TGF-β1 was from Peprotech (London, UK). cDNA synthesis kit and SYBR Green were obtained from Bioline (Toronto, Canada). Total RNA isolation kit was from Zymo Research (California, USA). ELISA kits were purchased from Invitrogen (USA). All other chemicals were analytical grade, used without further purification and were obtained from Sigma-Aldrich unless otherwise indicated.

2.2. Preparation of blank, BSA or TGF-β3 loaded PLGA-b-PEG nanoparticles

BSA (as a model protein) or TGF-β3 loaded nanoparticles were prepared by the nanoprecipitation method which is widely used to prepare polymeric nanoparticles of approximately 160–170 nm [23]. Briefly, organic phase was prepared by dissolving PLGA-b-PEG (10, 20 or 40 mg) in 5 mL of acetone. This organic phase was drop-wise added by a syringe to 10 mL distilled water containing Pluronic F-68 (0.1%, 0.2% or 1% w/v) and BSA (1 μg/mL) or TGF-β3 solution (1–2 μg/mL) under a magnetic stirrer. The solution was stirred over a magnetic stirrer for 24 h in order to remove the organic phase completely. The final nanoparticle suspension was centrifuged at 50,000 × g for 30 min. The supernatant was discarded and nanoparticles were resuspended in distilled water and further washed with distilled water for two times in order to remove the excess of surfactant. Also, blank PLGA-b-PEG nanoparticles were prepared using a similar method, nanoprecipitation, without incorporating either BSA or TGF-β3. The formulations of blank nanoparticles were presented in Table 1.

2.3. Characterization of prepared nanoparticles

2.3.1. Particle size, polydispersity index (PDI) and zeta potential

Malvern Zetasizer Nano-ZS was used to determine the particle size and zeta potentials of the nanoparticle formulations. Clear disposable zeta cells were used and measurements were taken after dispersing the precipitated nanoparticles in PBS (pH ~ 7.4). Blank, BSA or TGF-β3 nanoparticle formulations were subjected to the particle size, polydispersity index (PDI) and zeta potential measurements for at least six different batches. Results were presented as mean ± SD.

2.3.2. Morphology

Morphology of nanoparticles was visualized by using scanning electron microscopy (SEM, Nova NanoSEM, FEI, Eindhoven, The Netherlands) at 20 kV. Prior to SEM analysis nanoparticles were placed on sample holder, freeze-dried and coated with a thin gold layer (150-250 Å) for 40 s using a sputter coater.

2.3.3. Nanoparticle encapsulation efficiency

Indirect method was used to determine the encapsulation efficiency of nanoparticle formulations. This method requires the quantification of free, unencapsulated or adsorbed protein in the water phase. For this purpose, the amount of unloaded BSA or TGF-β3 in the water phase was determined by BCA Protein kit and TGF-β3 ELISA kit respectively. After centrifugation step at 14,000 × g for 20 min the obtained supernatant was filtered through polytetrafluoroethylene (PTFE) filters (pore size: 0.22 μm). The eluent was used to quantify free BSA or TGF-β3. The following formula was used to calculate the encapsulation efficiency:

$$\textit{Nanoparticle encapsulation efficiency}\ (\%) = \frac{K_1 - K_2}{K_1} \times 100$$

where K_1 is the total amount of BSA or TGF-β3 used in nanoparticle preparation, and K_2 the amount of free BSA or TGF-β3 in the water phase.

2.3.4. In vitro release studies

To study the *in vitro* BSA or TGF-β3 release from nanoparticles 10 mg of nanoparticles were resuspended in 2 mL of PBS solution (pH 7.4) and incubated in a water bath at 37 ± 0.5 °C under light agitation. At appropriate time intervals, individual samples were centrifuged at 14,000 rpm for 20 min and the amount of BSA or TGF-β3 in the supernatant phase was determined by BCA Protein kit and TGF-β3 ELISA kit respectively. The *in vitro* release profiles were determined by plotting the amount of released BSA or TGF- β3 versus time. The release experiments were carried out as triplicates and the results were expressed as mean ± standard deviation (SD).

2.4. Cell culture

Unless otherwise stated CFs were cultured in FGM medium supplemented with 10% fetal bovine serum, 100U/ml penicillin and

Table 1
Formulations, Particle size (nm), Polydispersity Index (PDI) and Zeta Potential (mV) of Blank Nanoparticle Formulations (NF 1–9).

Nanoparticle Formulation[a] (NF)	Amount of PLGA-b-PEG	Amount of Surfactant	Particle size[b] (nm)	Polydispersity Index[b] (PDI)	Zeta Potential[b] (mV)
NF 1	40 mg	1% Pluronic F68/10 mL	73.02 ± 1.22	0.139 ± 0.014	−28.1 ± 0.723
NF 2	40 mg	0.2% Pluronic F68/10 mL	101.6 ± 0.55	0.161 ± 0.002	−26.3 ± 1,.46
NF 3	40 mg	0.1% Pluronic F68/10 mL	104.7 ± 0.65	0.152 ± 0.008	−26.8 ± 0.557
NF 4	20 mg	1% Pluronic F68/10 mL	71.12 ± 0.74	0.169 ± 0.011	−23.6 ± 1.17
NF 5	20 mg	0.2% Pluronic F68/10 mL	78.22 ± 2.15	0.135 ± 0.01	−28.2 ± 0.462
NF 6	20 mg	0.1% Pluronic F68/10 mL	86.7 ± 1.72	0.172 ± 0.012	−21.9 ± 0.223
NF 7	10 mg	1% Pluronic F68/10 mL	65.89 ± 1.75	0.146 ± 0.012	−13.1 ± 0.351
NF 8	10 mg	0.2% Pluronic F68/10 mL	84.1 ± 0.87	0.191 ± 0.007	−21.6 ± 0.56
NF 9	10 mg	0.1% Pluronic F68/10 mL	85.2 ± 1.03	0.221 ± 0.019	−29.2 ± 0.89

[a] For all formulations, 5 mL acetone was used as an organic solvent.
[b] Results were presented as mean ± SD.