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Preparation of an adipogenic hydrogel from subcutaneous adipose tissue

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ABSTRACT

The ability to generate controlled amounts of adipose tissue would greatly ease the burden on hospitals for reconstructive surgery. We have previously shown that a tissue engineering chamber containing a vascular pedicle was capable of forming new fat; however, further refinements are required to enhance fat formation. The development and maintenance of engineered adipose tissue requires a suitable source of growth factors and a suitable scaffold. A hydrogel derived from adipose tissue may fulfil this need. Subcutaneous fat was processed into a thermosensitive hydrogel we refer to as adipose-derived matrix (ADM). Protein analysis revealed high levels of basement membrane proteins, collagens and detectable levels of growth factors. Adipose-derived stem cells exposed to this hydrogel differentiated into adipocytes with > 90% efficiency and in vivo testing in rats showed significant signs of adipogenesis after 8 weeks. ADM's adipogenic properties combined with its simple gelation, relatively long shelf life and its tolerance to multiple freeze–thaw cycles, makes it a promising candidate for adipose engineering applications.

1. Introduction

Congenital, traumatic or post-surgical deformities such as mastectomy often require restoration of contour, usually involving adipose tissue. Not only does adipose tissue act as a reservoir for lipids, it also provides insulation and physical protection to the underlying tissue. Being vascularized, it makes an excellent graft bed for other tissues and can be used in complex reconstructive scenarios for which no appropriate donor tissue exists. Adipose tissue engineering has recently received much attention as it promises enhanced efficacy, reproducibility and predictability, compared with the contemporary methods used to treat disfiguring contour imperfections. Autologous free fat grafting with processed lipoaspirate has unpredictable results due to post-graft resorption with sometimes as little as 10% of the original fat volume retained [1–3]. While the use of vascularized fat flaps generally has more favourable results, complications such as flap failure, infections and pulmonary embolisms exist, along with morbidity relating to the donor site [4].

Once removed from their native environments, stem cells differentiate less efficiently [5,6]. Therefore, when designing a suitable replacement three-dimensional (3-D) scaffold for thick tissues such as fat, it is important to incorporate characteristics of the native cellular environment to maintain optimal tissue development. For adipose tissue engineering, the ideal scaffold would be a self-gelling injectable material capable of inducing adipogenesis. Such a scaffold should contain the necessary extracellular matrix (ECM) components to initiate angiogenesis and subsequently induce resident stem cells to undergo adipogenesis. Of particular importance is the regulatory and structural role of the native extracellular matrix and associated factors [7]. The mechanotransduction between the ECM and cells plays a critical role in the regulation of angiogenesis [8] and in directing cells towards specific differentiation pathways [9]. Not only does the extracellular matrix provide structural support for the cells, it is also a reservoir for tissue-specific growth factors and signalling molecules that an entirely synthetic scaffold lacks.

Chemical crosslinking agents are successful in creating scaffolds from soluble proteins; however, the introduction of artificial linkages risks converting an otherwise native protein into something that may hinder cell infiltration and maturation [10].

Naturally derived scaffolds and hydrogels have been used for some years [11–22] and have shown great potential in supporting cell growth whilst maintaining their volume. Sharma et al. demonstrated that an adipocyte-derived ECM extract supported hepatocytes with a higher metabolic activity than with Matrigel,

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a commercially available ECM hydrogel [14], whereas Flynn et al. showed that matrices prepared from acellular placental ECM and hyaluronic acid support the differentiation of adipose-derived stem cells (ADSCs) [18–22]. In another study, freeze-dried, injectable powders were prepared from human lipoaspirate [11]. This scaffolding material combined with ADSCs resulted in well-vascularized adipose tissue after implantation into nude mice. When prepared as a hydrogel, adipose-derived extracts have been shown to not only support the growth of seeded ADSCs [17], but also showed signs of inducing neoadipogenesis when implanted along the rat epigastric artery vascular pedicle [15]. However, many of the current formulations of adipose-derived hydrogels either require additional steps to initiate gelation or have inconsistent gelation. Many collagen-based extracts require acid solubilization, which may adversely affect some proteins in the extract.

From what can be gathered in the literature, it is clear that adipose-derived products are capable of promoting cellular infiltration and have the potential to help form new vascularized adipose tissue once implanted in animals. In this study our aims were twofold: first, to produce a hydrogel from adipose tissue containing intact, bioactive proteins, displaying consistent gelation under physiological conditions; and second, to determine the adipogenic potential of this product both in vitro and in vivo.

2. Methods

2.1. Preparation of adipogenic hydrogels

Frozen porcine subcutaneous adipose tissue was shaved into 1–2 g pieces and homogenized with an equal volume of phosphate-buffered saline (PBS) until it reached a smooth consistency. After centrifugation (1942*g*, 4 °C, 10 min), the tissue was treated with 2 U ml^{-1} dispase II (Roche, Australia) for 30 min in a shaking 37 °C incubator to help with decellularization. The tissue was then centrifuged (3000*g*, 4 °C, 10 min) and excess dispase removed. This was followed by washes with 2× volumes of salt buffer (3.4 M NaCl, 50 mM Tris–HCl pH 7.4, 2 mM NEM, 8 mM EDTA). After 5 min mixing, the tissue was centrifuged (1942*g*, 4 °C, 10 min) and the buffer removed. The uppermost layer of lipid was removed before subsequent washing after each step. This washing step was repeated until the majority of visible lipid had been removed. The tissue was then extracted with an equal volume of urea buffer (2 M urea, 50 mM Tris–HCl pH 7.4) for 24 h at 4 °C. After incubation, visible lumps of solidified lipid were removed and the extract (Extract 1) recovered by centrifugation (1942*g*, 4 °C, 10 min) and dialysed against tris-buffered saline (TBS: 50 mM Tris–HCl pH 7.4, 150 mM NaCl) at 4 °C. An equal volume of guanidine-HCl buffer (4 M GuHCl, 50 mM Tris–HCl pH 7.4, 5 mM dithiothreitol (DTT)) was then added to the remaining tissue and homogenized. This extract was mixed for at least 18 h at 4 °C and then dialysed against TBS at 4 °C to produce an extract made up of a solid and liquid component. The liquid extract (Extract 2) was recovered by centrifugation (1942*g*, 4 °C, 10 min) and combined with Extract 1. Residual lipid was removed from the extract via 0.2 μm filtration and high speed centrifugation (15,000*g*, 4 °C, 10 min) before being concentrated up to four times and stored at 4 °C.

The remaining pellet containing unextracted protein was combined with an equal volume of 1% pepsin (Sigma–Aldrich, Australia), and 0.5 M acetic acid, and mixed at room temperature until dissolved. After incubation, any undigested protein and residual lipid was removed by centrifugation (15000*g*, 4 °C, 10 min) and the remaining digest was dialysed against TBS at 4 °C to inactivate the pepsin. The neutralized digest was combined with the concentrated liquid extract to form adipose-derived matrix (ADM). The ADM was sterilized by dialysis against chloroform to a final concentration of 0.5% at 4 °C before being dialysed at 4 °C with 8 M urea overnight, followed by dialysis in chilled TBS or Dulbecco's modified Eagle medium (DMEM). The ADM (~3 mg ml^{-1}) was stored long-term at −20 °C or for up to 2 months at 4 °C.

Human ADM was prepared with the following modifications. After the tissue had been extracted for a second time with 4 M GuHCl, the soluble fraction (Extract 2) was collected by centrifugation and combined with Extract 1, then filtered and dialysed in TBS, and concentrated up to four times. The leftover 4 M GuHCl-extracted tissue was rinsed twice with two volumes of 70% ethanol and then incubated for 30 min at 37 °C in four volumes of 70% ethanol to help remove excess lipid. After incubation, the tissue was washed three times with two volumes of water. After removing the water, the tissue was pepsin-digested with 0.5% pepsin, 0.5 M acetic acid at room temperature until dissolved. The ADM was then produced as described above.

2.2. Protein analysis

2.2.1. SDS–PAGE and Western blot analysis

All SDS–PAGE was performed with NuPAGE Novex Bis-Tris 4–12% gradient gels (Invitrogen, Australia). Polyclonal anti-skeletal muscle actin (human/mouse/rat), anti-myosin (human/animal), and anti-pan-laminin (human/animal) antibodies were purchased from Sigma–Aldrich, Australia. Polyclonal anti-fibronectin (human/mouse), and anti-mammal collagen I, IV and VI antibodies were produced by Abcam (supplied by Sapphire Bioscience). All samples were reduced with DTT (20 mM) except when probed for collagen I, IV and VI. To visualize resolved proteins, gels were stained with 0.1% Coomassie brilliant blue R-250 and/or 0.05% silver nitrate. For Western blot analysis, resolved proteins were transferred to PVDF membrane and blocked with 3% BSA for 1 h at room temperature. Primary antibodies were used at 1:500 in PBS/0.1% Tween-20 and the membranes probed for at least 1 h at room temperature followed by washing with PBS/0.1% Tween-20. Fluorescent secondary antibodies: Alexa Fluor 680 goat anti-mouse or anti-rabbit (Invitrogen, Australia) were used at 1:10,000 in PBS/0.1% Tween-20. After a final wash, membranes were scanned with an Odyssey Infrared Imaging System (Li-Cor Biosystems) using the 700 nm channel.

2.2.2. Protein concentration assays

The protein concentration of adipose extracts was measured with a bicinchoninic acid (BCA) assay (Thermo Fisher Scientific, Australia) using bovine serum albumin (BSA) as a protein standard. The assay was performed following the manufacturer's instructions and absorbance was measured with a 96-well plate reader at 450 nm. Samples were diluted 1:10 in TBS for the BCA assay. For SDS–PAGE, protein concentrations were also estimated by absorbance at 280 nm prior to electrophoresis.

2.2.3. 1,9-Dimethylmethylene blue assay

The glycosaminoglycan concentration of adipose extracts was measured using the 1,9-dimethylmethylene blue (DMMB) dye-binding assay against chondroitin sulphate standards [23]. To reduce interference in protein-rich samples, concentrated ADM was proteinase K-digested prior to glycosaminoglycan (GAG) measurements at 595 nm with a reference wavelength of 655 nm.

2.2.4. DNA extraction

The DNA concentration of ADM and unprocessed porcine adipose controls were monitored using an AllPrep DNA/RNA/Protein mini kit (Qiagen). Briefly, samples were proteinase K-digested for up to 1 h (~60 mAU ml^{-1}, 50 °C), then heated at 95 °C for 5 min. Samples were then centrifuged (15000*g*, room temperature) and the supernatant applied to an AllPrep DNA column and processed