ABSTRACT SUMMARY

A gelatin-based system for delivery of macromolecules in cell culture is described. The mild cross-linking condition ensures stability of a wide range of soluble biological signals and controlled release of proteins up to two weeks with retention of biological activity.

INTRODUCTION

The development of tissue involves well-defined gradients and patterning of biological signals. In recent years, the signaling environment has been elucidated for several developmental processes. The formation of long bones involves the transformation of a cartilage callus into bone matrix through endochondral ossification (EO). The first step in EO is the proliferation and differentiation of mesenchymal stem cells (MSCs) into a cartilage matrix. While the key molecules involved in MSC differentiation have been identified, little is known about the spatiotemporal variability of these signals. Key among these is the pattern and gradients involving Wnts, FGF-2 and BMP-4 (soluble signals). To identify the physiological relevant signal gradients and patterns, a system for delivery of a plurality of molecules in cell culture is desired.

In order to study the signal effect in vitro and in vivo, a biocompatible matrix material is necessary that can provide storage and release thereof in a controlled manner. In this regard collagen derived gelatin was identified as a suitable candidate, as it has been widely used for medical delivery applications. Gelatin does not form a stable matrix in an aqueous environment but structural integrity can be introduced by cross-linking with glutaraldehyde (GA). The toxicity of GA has limited the use of this approach as unreacted GA has to be removed through extensive dialysis. This necessitates the compound of interest to be loaded osmotically after cross-linking. We have overcome this limitation by employing vapor phase cross-linking, which in addition to eliminating the dialysis step allows for the loading of proteins and small molecules prior to cross-linking.

EXPERIMENTAL METHODS

Fabrication of gelatin release system: In brief, gelatin (250 mg, type B from bovine skin) was dissolved in deionized water (2.5 mL) by heating to 60°C in a water bath. The soluble signal solution (Wnt agonist (100 µL, 10 mg/mL), Wnt3a (176 µL, 10 µg/mL), or purmorphamine (100 µL, 10 mg/mL)) was then added and the solution was transferred to a plastic dish and stored at room temperature (RT) until gelation. Following this, discs were punched out of the gel using a biopsy punch (diameter of 3 mm) and the discs were cross-linked by exposing them to GA vapors overnight. The cross-linked discs were stored at -20°C until further use. Physical characterization: The structural integrity of the fabricated gelatin devices were assessed by incubating them in deionized water at 37°C under constant agitation. At predetermined time points, the devices were removed, blotted dry, weighed, the heights measured using a caliper, and visually examined, and then further incubated. Release studies: In order to determine the amount of released signal, discs were separately placed in deionized water (0.5 mL) at 37°C under constant agitation. At predetermined time points, the supernatant was removed and replaced with fresh release medium and soluble signals quantified using HPLC or ELISA. To test the biological activity of the released Wnt3a protein, discs were thawed, sterilized under UV light, and placed in culture with hMSCs and Wnt pathway activation was followed by real time polymerase chain reaction (RT-PCR).

RESULTS AND DISCUSSION

The procedure described herein for the fabrication of cross-linked gelatin drug delivery systems yields devices that undergo less than 30 % swelling which occurs primarily during the first 1 h upon incubation. Furthermore, the gelatin discs demonstrate exceptional stability in aqueous media with a 30 % mass loss over three months of which 20 % occurs within the first 10 days of incubation at 37°C (Fig. 1). Typical devices as well as a cross-section of the cross-linked gelatin are shown in Fig. 2. Leachants, such as the free GA, are the primary contributor to cellular toxicity observed in implantable systems. Extracts of gelatin discs when incubated in escalating concentrations had no adverse effect on the proliferation of hMSCs, therefore making the devices suitable for use in direct contact with cultured human cells (Fig. 3).

We chose to implement molecules that can activate the Wnt pathway (2-Amino-4-(3,4-(methylenedioxy) benzylamino)-6-(3-methoxy phenyl)-pyrimidine, a small molecule Wnt agonist and the protein Wnt3a), and the Indian Sonic Hedgehog pathway (purmorphamine) as both of these pathways have been implicated in MSC differentiation in vivo. While the Wnt agonist exhibits a very rapid release, the release profile of purmorphamine shows a lag phase followed by an appreciable nearly linear release profile (Fig. 4). This may be attributed to differences in the molecular interactions between the gelatin matrix and the corresponding compound. As an example of the release of larger signaling molecules we assessed the release of Wnt3a protein (37.4 kDa, Fig. 4). A sustained linear release profile for 10 days was attainable. Since the swelling of the gelatin discs occurs early during
incubation, the long term release can be attributed to the steady state diffusion of the molecule, although the degradation of the matrix may play a contributing role.

Figure 1. Cross-linked gelatin discs were incubated in water at 37°C and the change in weight recorded (n = 6, ± SD).

Figure 2. a) Non-loaded disc, b) disc loaded with Rhodamine B dye, c) SEM micrograph of disc cross-section showing the homogenous core (scale bar: 20 µm).

Figure 3. Viability of hMSCs cultured for 7 days in α-MEM supplemented with different volume ratios of gelatin extracts in DMEM assessed by MTT assay (n = 3, ± SD).

Figure 4. Release of Wnt agonist, Wnt 3a and purmorphamine from gelatin discs (n = 3, ± SD) as assessed by HPLC and ELISA.

To assess if the released Wnt3a was biologically active, hMSCs were exposed to Wnt3a-loaded discs over a period of 7 days. An up-regulation of genes associated with the activation of the Wnt pathway was confirmed using RT-PCR (data not shown).

CONCLUSION
We have developed an easy to implement, cost effective and quick method to fabricate gelatin-matrices for the delivery of soluble signals involved in tissue developmental processes. One novel aspect of the method is a single step that allows simultaneous incorporation of molecules and cross-linking as well as choice of the matrix geometry. Using disc-shaped matrices, the long-term delivery of active Wnt3a to hMSCs has been demonstrated.

REFERENCES

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