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Enhanced ectopic bone formation using a combination of plasmid DNA impregnation into 3-D scaffold and bioreactor perfusion culture

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Abstract

The objective of this study is to enhance in vivo ectopic bone formation by combination of plasmid DNA impregnation into three-dimensional (3-D) cell scaffolds and a developed in vitro culture method. Gelatin was cationized by introducing spermine (Sm) to the carboxyl groups for complexation with the plasmid DNA. As the MSC scaffold, collagen sponge reinforced by incorporation of poly(glycolic acid) (PGA) fibers was used. A complex of the cationized gelatin and plasmid DNA of BMP-2 was impregnated into the scaffold. MCS were seeded into each scaffold and cultured by a static and perfusion methods. When MSC were cultured in the PGA-reinforced collagen sponge, the level of BMP-2 expression was significantly enhanced by the perfusion culture compared with static method. When the osteoinduction activity of the PGA-reinforced collagen sponges seeded with PBS, MSC, naked plasmid DNA-BMP-2, cationized gelatin-plasmid DNA-BMP-2 complex, and transfected MSC by static and perfusion method, were studied following the implantation into the back subcutis of rats in terms of histological and biochemical examinations, homogeneous bone formation was histologically observed throughout the sponges seeded with cationized gelatin-plasmid DNA of BMP-2 complex and transfected MSC by perfusion method, although the extent of bone formation was higher for the later one. The level of alkaline phosphatase activity and osteocalcin content at the implanted sites of sponges seeded with transfected MSC by perfusion method were significantly high compared with those seeded with other agents. We conclude that combination of plasmid DNA-impregnated PGA-reinforced collagen sponge and the perfusion method was promising to promote the in vitro gene expression for MSC and in vivo ectopic bone formation.

Keywords: In vitro culture system; Enhanced gene expression; Cationization; PGA-reinforced collagen sponge; Osteoinduction

1. Introduction

Bone defects and fracture non-union are common problems, affecting as many as thousand patients in the world every year, and are difficult to heal using current therapies [1]. Previously, these cases have been treated by surgery, using techniques such as autologous bone grafting or artificial bone grafting. However, autologous bone grafts have a number of problems including donor-site problems, the limitations of harvested bone or the weak strength of graft-bone, while artificial bone grafts also have associated problems caused by the use of biomaterials, including immunogenicity, biodegradation or strength limitations. It has been reported that bone morphogenetic proteins (BMPs) can induce bone formation in both ectopic and orthotopic sites in vivo [2]. BMPs belong to the transforming growth factor-β superfamily and play an important role in osteogenesis and bone metabolism [3]. Among them, BMP-2 has a very strong osteoinductive

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activity. Since recombinant human BMP-2 (rhBMP-2) has become available, many animal studies on the induction of bone formation by implantation of rhBMP-2 using various carriers have been performed [4–7]. However, the use of BMP alone requires large amounts of protein because of its short half-life. To overcome these problems and to reduce the amounts of BMP required, developments in new types of scaffold and combined treatments with other reagents which can enhance bone regeneration have been examined. As for bone regeneration using cells, the use of mesenchymal stem cells (MSC) has been mainly reported [1]. MSC mainly exist in bone marrow and can differentiate into osteoblasts, chondrocytes and adipocytes [8–10]. Implantation of MSC after combination with rhBMP has been suggested to lead to earlier bone formation than with MSC alone; however, large amounts of protein are also required for this method [11].

Gene transfection is a powerful and promising technique that involves the in vitro or in vivo introduction of exogenous genes into cells for experimental and therapeutic purposes. Efficiency of transfection is dependent on both the efficiency of DNA delivery (i.e., fraction of DNA molecules getting into the nucleus) and the efficiency of DNA expression (i.e., fraction of nuclear DNA molecules that undergo transcription). Most DNA delivery systems operate at one of three general levels: DNA condensation and complexation, endocytosis, and nuclear targeting/entry. These complexes are taken up by cells, usually through endocytosis, the route of uptake determining subsequent DNA release, trafficking, and lifetime in the cells. Endocytosis is a multiple process involving binding, internalization, formation of endosomes, fusion with lysosomes, and lysis. The extremely low pH and enzymes within endosomes and lysosomes usually bring about degradation of entrapped DNA and associated complexes. Finally, DNA that has survived both endocytotic processing and cytoplasmic nucleases must then dissociate from the condensed complexes either before or after entering the nucleus. Entry is though to occur through nuclear pores (which are $\sim$10 nm in diameter) or during cell division. Once inside the nucleus, the transfection efficiency of delivered DNA is mostly dependent on the composition of the gene expression system.

Bone regeneration by gene transfer into MSC has also been reported [12–14]. These reports have mainly used a retrovirus, or adenovirus vector carrying human BMP-2, -4, or -7 as the therapeutic gene and these were effective in the formation of new bone. However, considering the immunological and safety issues of viral vectors, necessity in the development of non-viral vector systems has been increasingly magnified. Recently, some new osteoinductive gene therapies have been developed, such as in vivo electroporation with a BMP-containing plasmid expression vector [15]. However, osteoinduction by electroporation of a BMP-containing plasmid did not induce enough ectopic bone for clinical application, so a more effective method of gene therapy is needed. Collagen matrices have been used for gene delivery and bone tissue formation [16,17].

Recently, a system using gene-engineered urothelial cells was developed for in vivo gene therapy [18]. Cells seeded onto three-dimensional (3-D) polymer scaffolds were genetically modified, which then formed an organ-like structure with stable expression of the transgene in vivo. This paradigm provided a proof of principle for the use of tissue engineering techniques as a means to improve methods of gene transduction. The 3-D transgene cell construct can be potentially used as therapeutic cell-based gene delivery or as an in vitro model system for testing of genetic manipulations in order to understand the effects of gene expression on tissue development.

However, a key question in 3-D culture is whether or not the composites of cells and biomaterials can be cultured in vitro while maintaining their specific phenotype and functions. To achieve this, it is important to mimic the physiological nutrient and metabolic environment as closely as possible. Conventional static culture method does not satisfy the requirements any longer. A perfusion culture method is able to guarantee the continuous exchange of medium and constant removal of metabolic waste, and has been used for a variety of cells and tissues [18]. The conventional culture method with static medium may provide insufficient nutrition to support the survival and differentiation of cells in scaffolds in which the cell density is high and more active viability is expected. The perfusion of medium ensures fresh medium to cells and avoid the accumulation of harmful metabolic products, providing a constant microenvironment suitable for cellular differentiation and better tissue development. Perfusion culture method enhanced the viability and function of murine osteosarcoma cells and murine bone marrow stromal cells and increased matrix synthesis by chondrocytes in 3-D collagen sponges [19]. All the results suggest that perfusion culture is a beneficial method for cell attachment, proliferation and differentiation on/in a polymer scaffold.

This study was undertaken to investigate the effect of different culture systems on the enhancement of gene transfection of MSC and in vivo bone formation. As a material of non-viral vector, spermine (Sm) was chemically introduced to gelatin to obtain a cationized gelatin capable for polyion complexation of plasmid DNA. Collagen sponge reinforced by incorporation of poly(glycolic acid) (PGA) fibers was selected as the cell scaffold. Different culture systems, such as static and perfusion culture methods, were used to evaluate effect of the culture system on the transfection efficiency of cationized gelatin-plasmid DNA encoding BMP-2 complex on MSC and osteoinduction activity following the implantation of scaffold into the back subcutis of rats in terms of histological and biochemical examinations.