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ORIGINAL ARTICLE

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Role of poly-L-lysine-coated plates and fetal calf serum concentration in sheep chondroprogenitor cell culturing

Abstract Conventional methods for differentiation of chondroprogenitor cells on plastic plates face several problems that hinder the application of this method for the treatment of chondrogenic injury. This work focused on the effect of poly-L-lysine (PLL)-coated plastic surfaces and fetal calf serum concentration on the chondroprogenitor cells. In the present study, cartilage was isolated from the articular cartilages of sheep and the cells were seeded on PLL-coated plates in various serum concentrations. Histochemical analysis was used to determine chondrogenic differentiation of the cells. According to our results, the cells formed three-dimensional masses and chondrogenic cells. In the present investigation, the best culture conditions for maximum proliferation of isolated cells were examined. Taken together, the results indicated that PLL may have some effect on the adhesive properties of chondroprogenitor cells and could be used for cartilage engineering.

Key words Poly-L-lysine · Chondroprogenitor cells · Fetal calf serum

Introduction

Cartilage is a very important tissue, serving specific functions in the human body. Once damaged, the regenerating response of cartilage to injury is very limited because of the avascular nature of the tissue.[1,2] To prevent early degenerative arthritis, "healing" of cartilage defects with in vitro generated bioartificial cartilage is desired. Tissue engineering approaches aimed at the regeneration of articular cartilage would profit from the easy availability of large numbers of cartilage-forming cells. To date, different cells derived from several tissues, including periosteum, bone marrow, spleen, thymus, skeletal muscle, adipose tissue, skin, and retina have been used for the regeneration of articular cartilage.[3–10] As a source for cartilage engineering, mesenchymal stem cells, which are capable of differentiating into bone, cartilage, tendon, or muscle, are favored by investigators.[11] However, for several reasons, such as the inaccessible nature of the tissue source, low cell frequency, and limited information, the use of these progenitors in tissue engineering has not always been straightforward.[3] Recently, a new population of chondroprogenitor cells isolated form the superficial zone of the articular cartilage has been identified and partially characterized.[3,12]

The conventional method for preparation of cartilage pieces in vitro is pellet formation by centrifugation of cells in polypropylene centrifuge tubes to establish aggregates, followed by resuspension in a defined chondrogenic medium. However, the aggregation and three-dimensional mass formation of cells on plastic plates is difficult. Here, we designed a novel method for three-dimensional mass formation of cultured chondroprogenitor cells, mainly based poly-L-lysine (PLL)-coated plastic surfaces. The present study also sought an optimal culture condition for the maximum proliferation of chondroprogenitor cells.

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Materials and methods

Tissue isolation and cell culture

Cartilage was isolated from the superficial zone of articular cartilage in ten scapula joints from sheep (1–2 weeks old). The cartilage pieces were digested in Dulbecco's modified

Eagle's medium (DMEM: Gibco-BRL, Grand Island, NY, USA) with 2.5 mg/ml pronase (Calbiochem, San Diego, CA, USA) for 3 h at 37°C with stirring, followed by 0.5 mg/ml collagenase type II (Sigma, St. Louis, MO, USA) for 3 h at 37°C with stirring. Cell suspensions were filtered, sedimented at 400 *g* for 10 min, and then resuspended in fresh DMEM 40% fetal calf serum (FCS) supplemented with 50 IU/ml penicillin/50 µg/ml streptomycin, 50 µg/ml insulin-transferrin-selenium (ITS: Gibco-BRL), 1 ng/ml fibroblast growth factor-2 (FGF-2; Peprotech), and 10 ng/ml transforming growth factor-β3 (TGF-β3: Sigma).[13] The cells were counted using a hemocytometer as describe by Digirolamo et al.,[14] seeded at 10^4 cell/cm^2 onto a six-well tissue culture plate that had been coated with 0.1 mg/ml PLL at room temperature for 15 min (Sigma). Cultures were kept at 37ºC in a humidified atmosphere containing 95% air and 5% CO_2. The culture medium was changed twice a week. In addition, some cells were cultivated on uncoated plates as the cell culture control.

Population doubling time (PDT)

The mean population doubling time (PDT) was calculated according to the equation: PDT = t log2/(logNt – logNo), where No is the number of seeded cells, Nt the number of harvested cells, and t is the time of the culture (in hours).[15]

Optimizing the culture condition for maximum proliferation

In the present investigation, the isolated cells were plated in 70-mm^2 dishes containing DMEM supplemented with 2 mM L-glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin (PAA Laboratories, Martinsried, Germany) at varying concentrations of FCS (10%, 20%, 30%, and 40%) for 10 days. Subsequently, the number of cells was calculated in each group and compared by means of a one-way analysis of variance.

Histological examination

The cells were fixed overnight at 4°C in 4% paraformaldehyde. Samples were then embedded in paraffin, sectioned to 8–10 µm, and affixed to microscope slides. Sections were then stained with toluidine blue.

Immunocytochemistry

The cells (aggregated cells in differentiation medium and the cells cultivated in a monolayer pattern) were cultured on sterile glass cover slips, fixed by incubation in 1% paraformaldehyde/Phosphate buffered saline for 3–5 min, and sectioned for immunocytochemical evaluation. The extracellular staining patterns of collagen type II proteins were analyzed by immunostaining with mouse anti-collagen type II antibodies (Millipore, Temecula, CA, USA). The fluorescent isothiocyanate (FITC)-conjugated anti-mouse IgG (Sigma) was used as the secondary antibody.

Results

Cell culture

The isolated cells exhibited high potential to proliferate in medium after 24 h: we cultured these cells for eight passages. Figure 1 shows the monolayer growth of chondroprogenitor cells in culture.

Three-dimensional mass formation

After cell seeding in a PLL-coated plate, we showed that without application of extrinsic agents, cells tended to aggregate and form three-dimensional masses similar to embryoid bodies (EBs). The aggregated cells tended to incorporate into one another and form larger masses during the culture period (Fig. 2). In the control culture, the cells expanded in a monolayer pattern (Fig. 1B).

Population doubling time (PDT)

According to the PDT calculation, cells seeded in a PLL-coated plate underwent 12.6 ± 1.3 population doublings during the experiment culture period (10 days), and the population doubling time was 21.2 ± 0.5 h.

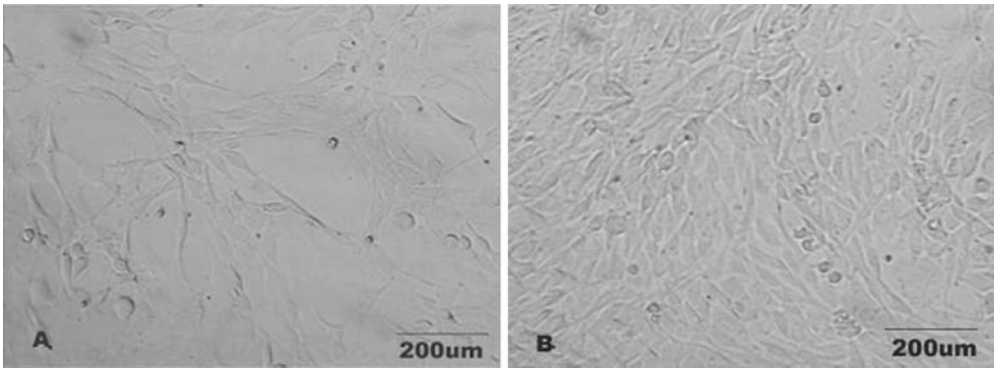

Fig. 1. Morphological characteristics of chondroprogenitor cells in an uncoated plastic culture dish. **A** the cells in days 2, **B** the cells in days 5

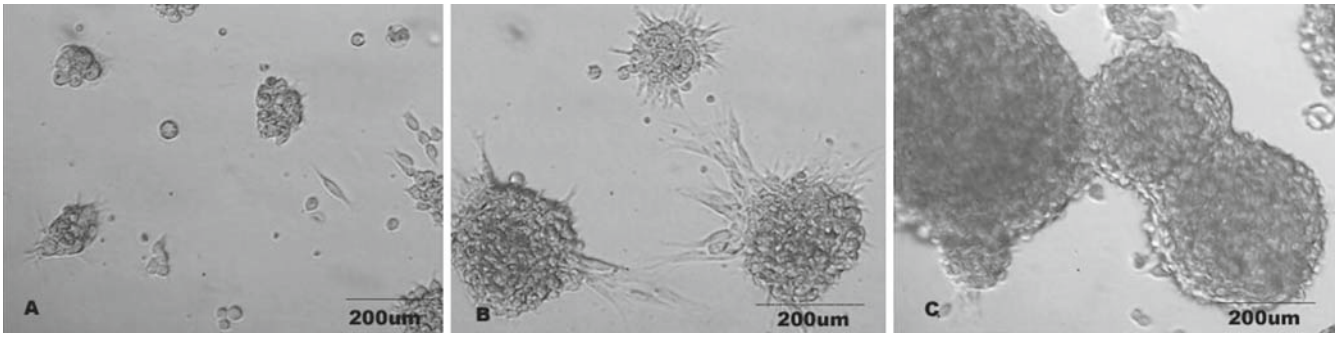

Fig. 2. Three-dimensional aggregated cells in a poly-L-lysine (PLL)-coated plate after 2 (**A**), 4 (**B**), and 7 (**C**) days. The size of cell aggregates increases during the culture period

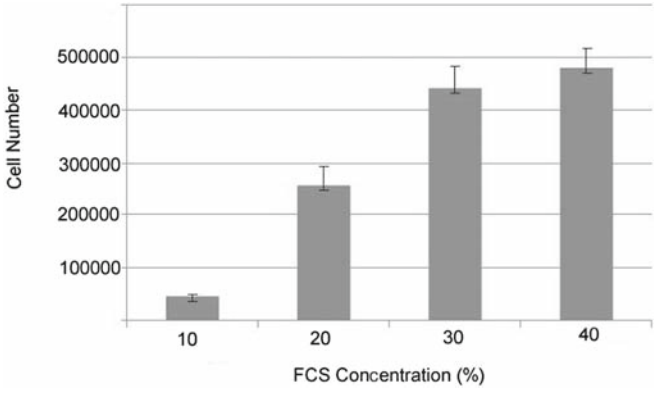

Fig. 3. Effect of different fetal calf serum (*FCS*) concentration on chondrogenic cells

Serum concentration

Cells seeded in a PLL-coated plate with 40% FCS had a significantly larger increase in cell numbers compared with those with 10% and 20% FCS; however, the difference with the 30% FCS concentration group was not statistically significant (Fig. 3).

Histological examination

Figure 4A shows the section of aggregated cells stained with toluidine blue. The accumulation of cartilaginous proteoglycans was demonstrated by the toluidine blue staining method.

Immunocytochemistry

Chondrogenic potential was also confirmed by immunohistostaining for collagen type II. Figure 5A shows the collagen type II protein present in the sections. However, this protein was not expressed in the cells cultivated using the monolayer method (Fig. 5B–D).

Discussion

Cartilage has very little capacity for spontaneous healing because of the avascular nature of the tissue. To resolve defects in articular cartilage, researchers have isolated bone marrow and have also employed synovial membranes and periosteal tissues as cell sources for use in cell-based therapies.[16,17] The periosteum harbors a large quantity of progenitor cells that directly differentiate into chondrocytes.[11,18] Three-dimensional cell aggregate culture has been used to study in vitro chondrogenesis using polypropylene centrifuge tubes to establish the aggregates. The current study explores the effect of PLL-coated plates and FCS concentration (10%–40%) on aggregated and cultured chondroprogenitor cells.

The periosteum consists of a fibrous layer and a cambium layer that contains progenitor cells. In native articular cartilage, the extracellular matrix (ECM) is primarily made up of a network-like structure consisting of proteoglycans and type II collagen. Chondrocytes in the ECM are round and exist within small pockets throughout the tissue. Although the articular cartilage matrix has some mechanical strength, both the surface and the subchondral bone can be damaged by the imposition of substantial mechanical loads. Unfortunately, few attempts to treat injuries to this joint have been made.[19]

The micromass culture technique is the conventional method for differentiating cells to achieve chondrogenesis. The utilization of this method for the differentiation of cells to achieve in vivo chondrogenesis faces problems such as small numbers of aggregates that hinder the application of this method to the treatment of chondrogenic injury. Although, such difficulties can be overcome by culture conditions utilizing suspension or pellet cultures, the problems persist on plastic surfaces.[20,21] We report how adult auricular chondrocytes can be aggregated and maintained in the differentiated state on PLL-coated plastic surfaces.

Our results showed that the cultivated chondroprogenitor cells on PLL-coated plates tended to aggregate and form large three-dimensional masses, whereas on untreated plastic plates they acquired a fibroblast-like phenotype. Many researchers have reported that chondroprogenitor

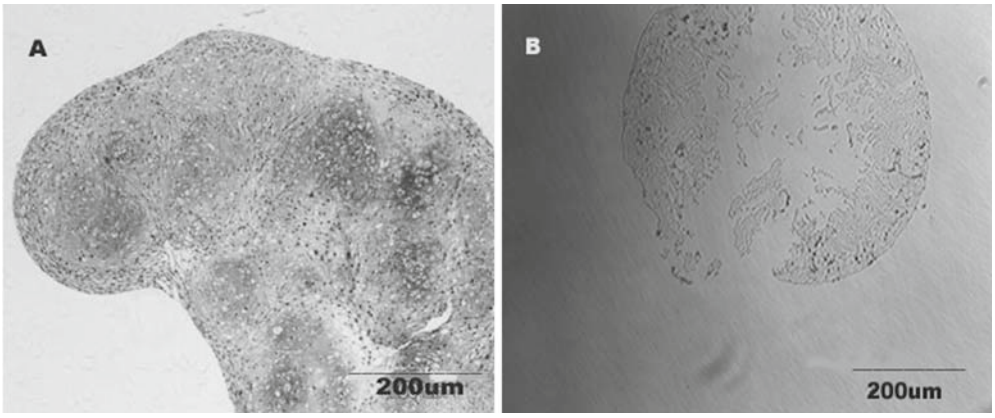

Fig. 4. The toluidine blue staining of cells after (**A**) and before (**B**) cultivation in a PLL-coated plate

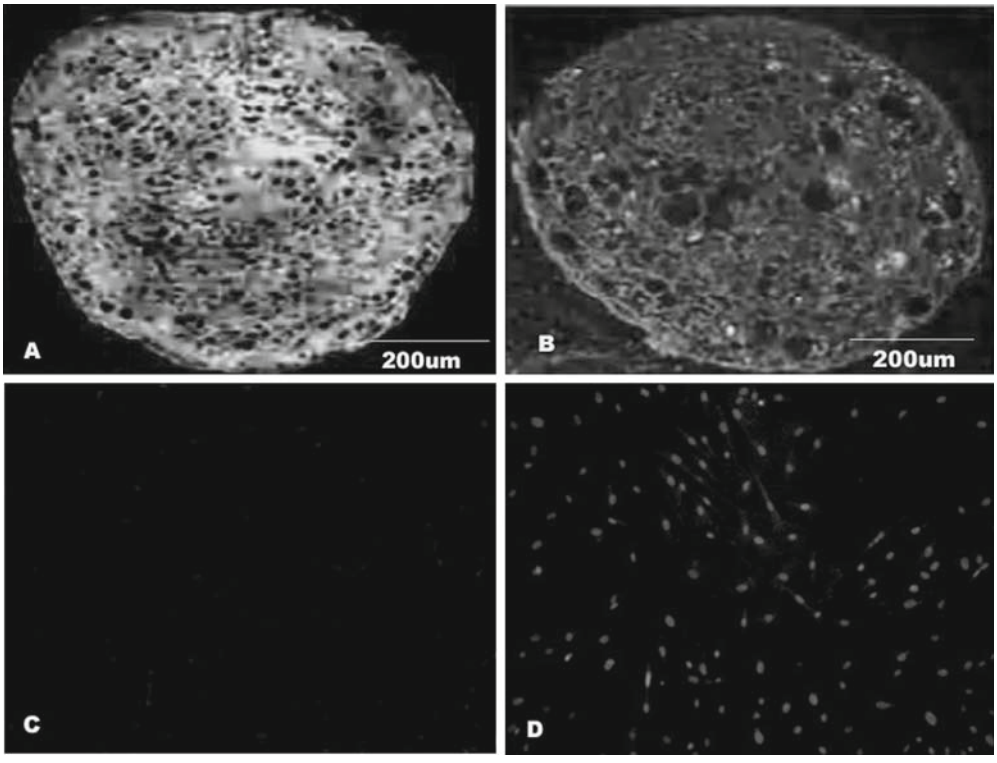

Fig. 5. Histological and immunocytochemical analysis of the aggregated cells (**A**) and the control cells (**B–D**). The control cells showed that collagen type II protein is not expressed (**C**). Cells were co-stained with 4,6-diamidino-2-phenylindole to visualize nuclei related to collagen type II (**D**)

cells seeded on plastic surfaces lose their round shape and acquire a fibroblast-like phenotype.[22–24] The cells do not adhere to the PLL-coated plate because PLL induces cell–cell rather than cell–substrate interaction. PLL interacts with the phospholipids of the cell membrane to create stationary structures. This synthetic molecule influences the differentiation and functionality of cells via the induction of cytokines.[25] In the present study, the cells differentiated into chondroprogenitors without the use of pellet-formation methods. A previous study showed that ECM plays a major role in the regulation of cell differentiation in vivo. Some studies have established that when isolated chondrocyte cells are seeded onto articular cartilage disks in vitro, they retain their phenotype and synthesize an appropriate cartilage matrix.[26] It has been reported that the addition of hyaluronan to human articular chondrocyte cultures simultaneously promotes their proliferation and redifferentiation both on plastic surfaces and in a type-I/III collagen sponge.[27] With the addition of hyaluronan, the cells form three-dimensional nodules as opposed to the monolayer control cells. In the present study, chondroprogenitor cells from the scapula joints of sheep were isolated and cultivated on PLL-coated plates. PLL, with its positive charge, prevented the chondrogenic cells adhering to the plastic culture dish. Furthermore, previous researchers indicated that chondrogenesis is stimulated by cationic materials such as PLL.[28] It seems that a PLL-coated scaffold may be used for differentiating cells into chondrogenic cells in vivo. However, the mechanisms by which PLL exerts this effect need to be investigated further.

A previous study described a protocol to promote chondrogenic differentiation of human mesenchymal stem cells using growth factors and cytokines.[29] In the present investigation, we also examined the effect of serum concentration

on proliferation of chondroprogenitor cells. The results showed that serum concentration affects the proliferation of the cells, with the highest proliferation seen when using 40% FCS.

Although articular cartilage is more interesting for tissue engineering and constitutes a simpler model for the study of chondrocyte culture to obtain a functional substitute, it has been much less studied. To our knowledge, there have been no reports on the preparation of three-dimensional mass formation of chondroprogenitor cells on PLL-coated plates. In contrast to the conventional method (pellet formation), this method has some advantages such as being a shorter and less expensive procedure for the preparation of large numbers of aggregates.

Histochemical analysis indicates that the aggregate cells were differentiated into chondrogenic cells. The toluidine blue staining showed that cartilaginous proteoglycans were accumulated in aggregated cells. According to the results, PLL-coated plates are seemingly an advantageous extracellular matrix choice for the preparation of aggregates for chondrogenesis differentiation.

In conclusion, the results presented in this work suggest that PLL may have some effect on the adhesive properties of chondroprogenitor cells and may be useful for cartilage engineering. We have effectively formed chondrogenic aggregates using a PLL-coated plate culture system. This work might have implications for the development of future transplantation strategies for the treatment of degenerative diseases of cartilage by tissue engineering.

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