[HSM01] The effects of various growth factors on the proliferation of human nasal septum chondrocytes and reconstruction of human cartilage via tissue engineering technology

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Introduction

Tissue loss and organ failure are one of the costly health care management in all over the world. One of the recent strategies for the treatment of organ failure and damaged tissues is via tissue engineering technology. The primary goal of all approaches in tissue engineering is to repair and restore tissue functions through the delivery of living elements which become integrated into the patient (Langer and Vacanti, 1993).

Cartilage is an avascular tissue that has limited self-repair ability once damaged. A cartilage graft is needed for any attempt to restore or repair the damaged cartilage. Autologous cartilage is the best candidate and far more superior compared to allograft, xenograft or synthetic materials for cartilage repair. However, there is only limited amount of cartilage can be harvested without causing deformity and morbidity at the donor sites. The main objective of this study was to investigate the possibility of using tissue engineering technology to construct an autologous cartilage tissue for the repair of damaged cartilage.

Materials and Methods

Strategies and research approach

The first stage of the study was to investigate the individual dose effects of Basic fibroblast growth factor (bFGF), insulin like growth factor-I (IGF-I) and transforming growth factor-beta 2 (TGF-β2) on human nasal septum chondrocytes. The dose effects were evaluated by measuring the chondrocytes growth rate, population doubling time, viability, cumulative cell doubling, phenotype gene expression and the ability of cultured chondrocytes for cartilage regeneration. After the optimum dose of each factor has been determined, the second stage of the study was to investigate the combination effects of growth factors on human nasal septum chondrocytes. The effects of insulin-transferrin-selenium and serum concentration were also evaluated in this stage. The third stage of the study was to investigate a neocartilage formation technique without using any scaffold material.

Human nasal septum chondrocyte isolation and culture expansion

Human nasal septum cartilages were obtained from consented adult patients after elective septoplasty. These specimens would usually be discarded and their used in this study has been approved by the Ethical Committee of Medical Faculty, Universiti Kebangsaan Malaysia. Each cartilage was cleaned from perichondrium, minced into small pieces ($1mm^3$) and digest with 0.6% collagenase II (Gibco) at 37^0C for 12 hours. After digestion, total cell quantification was done with hemocytometer. Viability of chondrocytes was determined with trypan blue vital dye exclusion test (Gibco).

Isolated chondrocytes were seeded in tissue culture plate (Falcon) at a cell density of 5,000 cells/cm^2 in an equal volume mix of Ham's F12 medium and Dulbecco's Modified Eagle Medium (Gibco) added with 1%(v/v) 200mM L-glutamine (Gibco), 1%(v/v) antibiotic-antimycotic (Gibco) and 1%(v/v) ascorbic acid (Sigma). This culture medium was then added with different test components such as fetal bovine serum (FBS; Gibco), pooled human serum (HS), insulin-transferrin-selenium (ITS), basic fibroblast growth factor (bFGF), transforming growth factor-beta2 (TGF-β2) and insulin-like growth factor-I (IGF-I) for individual dose effects investigation and combination study. All

cultures were maintained in 5% CO_2 incubator at 37^0C with culture media changed twice a week. Cultures were examined everyday under inverted light microscope to monitor the growth of culture.

When the primary culture (P0) reached confluence, it was trypsinized with pre-warmed (37^0C) 0.05% trypsin-0.53mM EDTA (Gibco). Cultured chondrocytes were then passaged three times (P1, P2 and P3) with the same cell seeding density, culture medium and culture environment. Total cell count and cell viability were recorded at every passage. Chondrocyte growth rate, population doubling time and cumulative cell doubling in each media at every passage (P0, P1, P2 and P3) was then calculated for statistical analysis (ANOVA and Student's *t* test).

Chondrocyte phenotype gene expression evaluation

Total RNA from cultured chondrocytes in different experiment groups were extracted using TRI Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instruction. Polyacryl Carrier (Molecular Research Center) was added in each extraction to precipitate the total RNA. Extracted RNA pellet was then washed with 75% ethanol and dried before dissolved in Rnase and Dnase free distilled water (Invitrogen, Carlsbad, CA). Yield and purity of the extracted RNA was determined by spectrophotometer (Bio-Rad, Hercules, CA). Total RNA was stored at -80^0C immediately after extraction.

Expression of type I and type II collagen genes was evaluated by one step reverse transcriptase-polymerase chain reaction (One Step RT-PCR; Invitrogen). Expression of human β-actin gene was used as control. The specific sense and antisense primers used in the reaction were designed from listed NIH GenBank database and has the following sequences: type I collagen, 5′-AAGGCTTCCAAGGTCCCCCTGGTG-3′ and 5′-CAGCACCAGTAGCACCATCATTTC-3′; type II collagen, 5′-CTGGCAAAGATGGTGAGACAGGTG-3′ and 5′-GACCATCAGTGCCAGGAGTGC-3′; β-actin, 5′-CCGGCTTCGCGGGCGACG-3′ and 5′-TCCCGGCCAGCCAGGTCC-3′. One Step RT-PCR reaction mix was prepared according to the manufacturer's instruction. Each reaction consisted of 100ng total RNA and 10pmol of each sense and antisense primers. One step RT-PCR was performed in a 9700 thermal cycler (Perkin Elmer, Norwalk, CT) with reaction profile of; cDNA synthesis for 30 min at 50^0C; pre-denaturation for 2 min at 94^0C; PCR amplification for 38 cycles with 30 sec at 94^0C, 30 sec at 60^0C and 30 sec at 72^0C. This series of cycles was followed by a final extension of 72^0C for 2 min. Subsequently, the PCR products were separated by electrophoresis on a 1.5% agarose gel stained with ethidium bromide and visualized by UV transillumination.

In vivo cartilage regeneration and evaluation

Cultured chondrocytes from different growth factors experiments were suspended into a 30% (wt/vol) of a co-polymer of polyethylene oxide and polypropylene oxide, Pluronic F-127 (BASF, Mount Olive, NJ) at 4^0C with a cell density of 3×10^7 cells/ml. The resulted admixer was then injected subcutaneously at the dorsal part of the nude mice under general anesthesia (ketamine, xylazil and zoletil). Care of the nude mice was carried out following the animal facility guideline of the Animal Unit, Institute Medical Research Malaysia.

The regenerated/engineered tissues were harvested after 8 weeks of *in vivo* implantation. The nude mice were sacrificed by anesthetic overdose and engineered tissues were carefully dissected free from surrounding soft tissue. The engineered tissue was divided into three parts with one part fixed in 10% phosphate buffered formalin and processed into paraffin embedded block. Paraffin blocks were then sectioned and the slides sections were stained with standard histological staining; Hematoxylin & Eosin (H&E staining) and Safranin O staining. Another part of the excised tissue was digested with Collagenase II enzyme and total RNA was extracted for one-step RT-PCR analysis as mentioned earlier.

Part of the engineered tissues was also evaluated with electron microscopy technique in Universiti Putra Malaysia. The tissues were cut into $1mm^3$ blocks, fixed in 2.5% glutaraldehyde (Sigma) for 24 hours. Tissues blocks were then post-fixed in 1% osmium tetraoxide for 2 hours, dehydrated, infiltration with resin, embedding, ultra-thin sectioned,

stained with 2% uranyl acetate and examined with CX-2000 transmission electron microscope (JEOL, Tokyo, Japan).

Technique of tissue engineered human cartilage formation without scaffold material

Chondrocytes were seeded onto 175cm^2 flask and culture in medium consists of 10% serum. Cultures were allowed to be over grew, formed multilayer and then chondrocyte mass within 3 weeks. The chondrocyte mass was further maintained until the constructs established stable size and shape. These constructs were then implanted subcutaneously on the dorsal part of nude mice for 8 weeks. After 8 weeks of implantation, the *in vivo* engineered constructs were removed and evaluated by histological staining, one step RT-PCR and transmission electron microscopy for its quality.

Results

Human nasal septum chondrocytes Morphologic Feature in Monolayer Culture

Human nasal septum chondrocytes cultured in different growth factors supplementation exhibited different morphologic features in monolayer culture. Chondrocytes were different in size, shape and density under influence of various growth factors.

Human nasal septum chondrocytes growth kinetic and phenotype gene expression

The optimum dosage of Basic fibroblast growth factor (bFGF), insulin like growth factor-I (IGF-I) and transforming growth factor-beta 2 (TGF-β2) were obtained in dose response graph (data not shown). Chondrocytes growth rate in culture medium supplemented with different combination of optimum growth factors dosage was demonstrated in Figure 1. The combination of 10μg/ml insulin + 5.5μg/ml transferrin + 6.7ng/ml sodium selenite (ITS) + 5ng/ml IGF-I + 3ng/ml bFGF and 1ng/ml TGF-β2 provided the highest chondrocyte growth rate (Figure 1). This combination of various growth factors was also capable to retain the cartilage phenotype of cultured chondrocytes by maintaining type II collagen gene expression until passage 3 (Figure 2).

Engineered/regenerated tissues evaluation

After 8 weeks of *in vivo* implantation, the Safranin O staining on the engineered tissue sections showed strong orange-red staining demonstrating abundant proteoglycans production (Figure 3A). Hematoxylin & Eosin staining demonstrated engineered tissues consisted of evenly spaced lacunae cells embedded in basophilic matrix (Figure 3B). Transmission electron micrographs showed cells in the engineered tissues were cuboidal in shape with cytoplasm processes extended into the extracellular matrix (Figure 3C). The cells were rich with mitochondria and endoplasm reticulum for protein production. The presence of abundant collagen fibrils was noted as the major component in the extracellular matrix (Figure 3C). One step RT-PCR analysis on the total RNA extracted from engineered tissues display high level expression of type II collagen gene (Figure 3D).

Formation of tissue engineered human cartilage without scaffold material

In this technique, multilayer chondrocyte culture was detached away from the edge of the flask and aggregated to form chondrocyte mass which appear like jellyfish (Figure 4A). At the end of third week of *in vitro* culture, the chondrocyte mass reached its steady stage (refer as neocartilage) and was mechanically strong enough for implantation (Figure 4B). At this stage, Safranin O staining on the neocartilage section demonstrated no orange-red staining which indicate negative production of cartilage proteoglycans (Figure 4C). After 8 weeks of *in vivo* implantation, this neocartilage was able to develop into a mature cartilage demonstrated by positive staining of Safranin O (Figure 4D). The removed tissues were glistening white in color and firm in consistency, resemble the native cartilage (Figure 4E).

Discussion

Human nasal septum chondrocyte growth kinetic data showed basic fibroblast growth factor (bFGF) is a powerful mitogen (Ruszymah *et al*., 2002) among other tested growth factors. The mitogenic effects of bFGF come into saturation at 3-5ng/ml concentration in the culture medium (Chua *et al*., 2002b). Further increase in bFGF

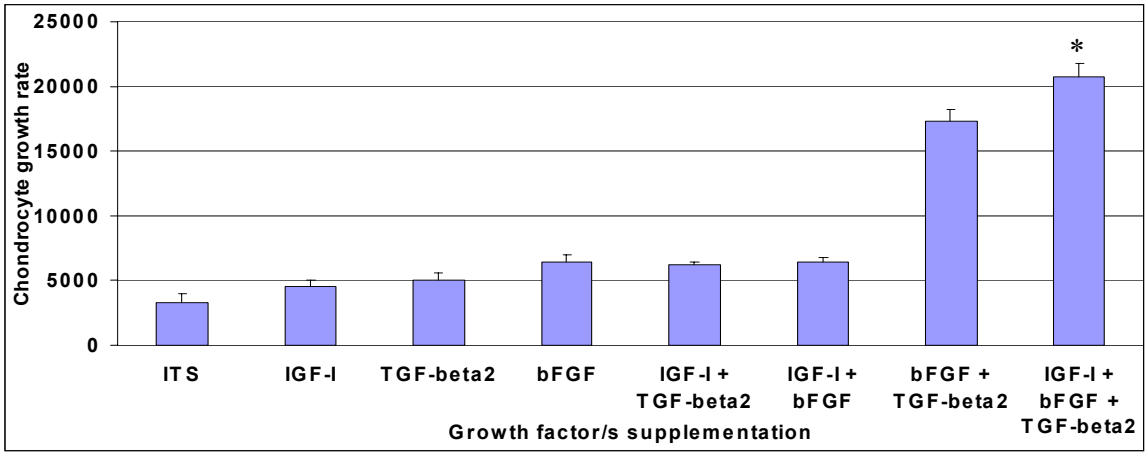

FIGURE 1 Human chondrocyte growth rate (Increase in number of cells/days/cm^2) in medium Supplemented with various growth factors at primary culture. Values were showed as mean ± SEM (n = 6). ∗ Medium supplemented with ITS + 5ng/ml IGF-I + 3ng/ml bFGF + 1ng/ml TGF-β2 showed significant higher growth rate than other groups. $p<0.05$. ITS = Insulin-transferrin-selenium. IGF-I = 5ng/ml insulin like growth factor-I. bFGF = 3ng/ml fibroblast growth factor. TGF-beta2 = 1ng/ml transforming growth factor-beta 2.

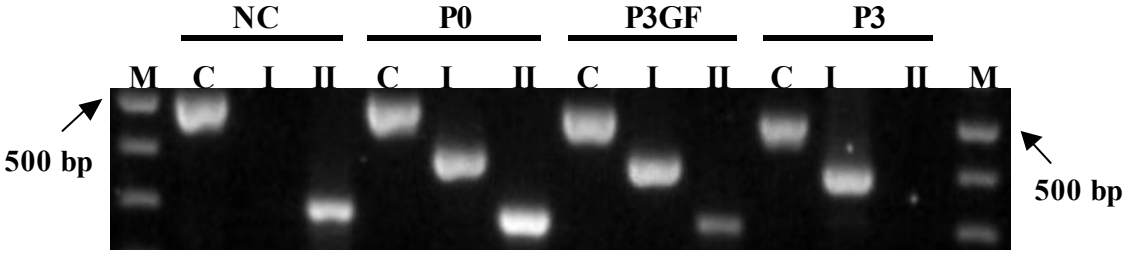

FIGURE 2 One step RT -PCR analysis on the gene expression of chondrocytes from native cartilage (**NC**), primary culture (**P0**), passage 3 in various growth factors supplemented medium (**P3GF**) and passage 3 in medium without growth factor (**P3**). Chondrocytes cultured in medium supplemented with growth factors retained type II collagen gene expression. Lane M: 100bp DNA marker. Lane C: β -actin gene fragment (Control; 495bp). Lane I: T ype I collagen gene fragment (396bp). Lane II: Type II collagen gene fragment (294bp).

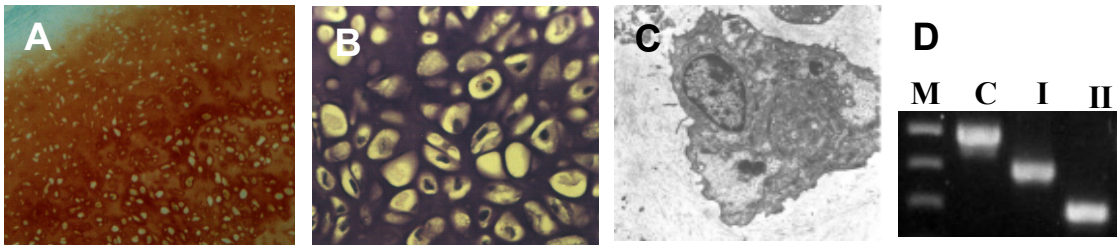

FIGURE 3 Evaluation on engineered/regenerated tissue. Safranin O staining showed positive orange -red staining indicate abundant proteoglycans production, x 40 (**A**). Hematoxylin & Eosin staining demonstrated lacunae cells embedded in basophilic matrix, x 400 (**B**). Transmission electron micrographs showed cell in engineered tissue was cuboidal in shape, rich with mitochondria and endoplasm reticulum. Abundant collagen fibril was noted in the extracellular matrix, x 4000 (**C**). One step RT -PCR analysis showed high expression of type II collagen gene in the engineered tissue (**D**).

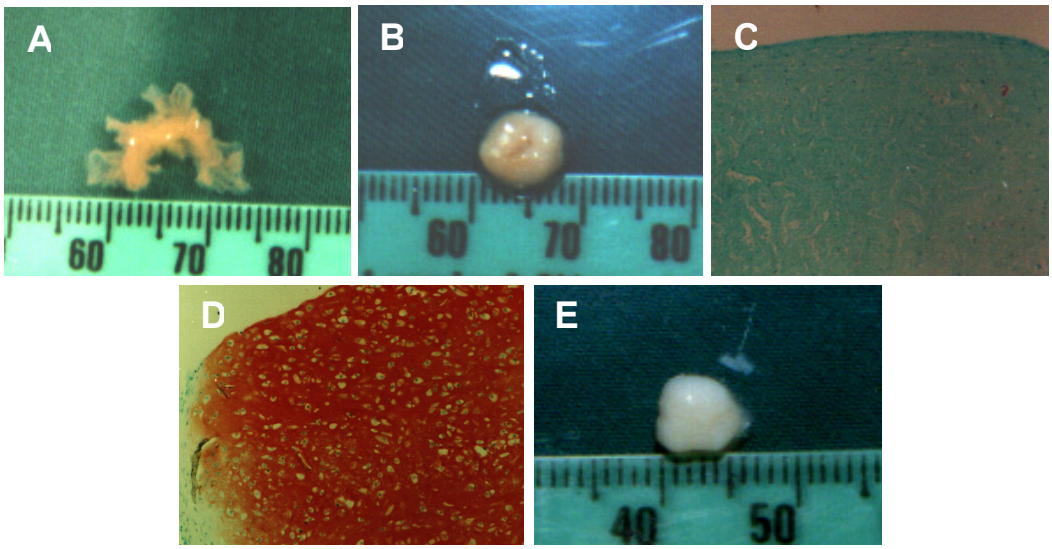

FIGURE 4 Early formation of chondrocyte mass in the form of jellyfish like (**A**). Chondrocyte mass reached its steady stage (neocartilage) (**B**). Safranin O staining showed negative staining on the neocartilage, x 40 (**C**). Safranin O staining showed positive staining on engineered tissue after 8 weeks of *in vivo* implantation, x 40 (**D**). The removed tissues were glistening white in color and firm in consistency at physical appearance (**E**).

concentration will only cause unnecessary addition of cost and may result in longer period of time for cartilage regeneration. In growth factor combination study, bFGF reacts synergistically with transforming growth factor-β2 to promote human nasal septum chondrocyte proliferation. The optimum combination of bFGF at 3.0ng/ml with TGF-β2 at 1.0ng/ml concentration was able to promote good quality engineered cartilage tissue formation (Chua *et al*., 2003a). Addition of Insulin-transferrin-selenium (ITS) into the culture medium promoted higher chondrocyte proliferation and cartilage regeneration (Chua *et al*., 2003b). Insulin like growth factor-I was later proven to be beneficial of retaining the cartilage phenotype expression in the culture medium (Chua *et al*., 2004b). In order to overcome problems on using animal sera, such as viral-cause disease transmission, the danger of prion contamination and immune reaction against animal serum proteins, the effects of human serum were also investigated in the study and was shown that it provided additional values of supporting the growth and differentiation of human nasal septum chondrocytes (Ruszymah *et al*., 2003; Chua *et al*., 2004a).

This study managed to formulate a human chondrocytes growth medium resulted from the combination of various growth factors in a specific concentration. The medium formulation was found novel and managed to fail for a patent (PI20041917) in the name of UKM-MECC. UKM-MECC is capable to support 178,000 fold increases in chondrocyte number and maintained the cartilage phenotype during expansion. Culture-expanded chondrocytes were able to form high quality cartilage tissue in nude mice model after 8 weeks of implantation. UKM-MECC was also showed to be cost effective and valuable for clinical application of cartilage tissue engineering.

The novel technique on constructing tissue engineered human cartilage without scaffold material is able to generate high quality cartilage after implantation. This new technique is potential for the repair of various cartilage defects since it has eliminated the problem of immune-reaction (Chua *et al*., 2002a; Ruszymah *et al*., 2002; Aminuddin *et al*., 2003).

Acknowledgements

The authors express sincere thanks to the Ministry of Science, Technology and Innovation (MOSTI), Malaysia for the National Science Fellowship awarded to Chua Kien Hui and for IRPA grants of 06-02-02-003 BTK/ER/022 and IRPA 06-02-02-0037-EA189. Special thanks also to Yayasan Sultan Iskandar of Johore, Hospital Universiti Kebangsaan Malaysia and Faculty of Medicine, Universiti Kebangsaan Malaysia

for their funding. The authors were also grateful to Dr. Fuzina N. Hussein for nude mice facility in Institute Medical Research Malaysia and Asso. Prof. Dr. Fauziah Othman from Microscopy and Microanalysis Unit, Institute of Bioscience, Universiti Putra Malaysia for her expertise in the electron microscopy interpretation.

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