COMBINATION OF BASIC FIBROBLAST GROWTH FACTOR, TRANSFORMING GROWTH FACTOR BETA-2 AND HUMAN SERUM ENHANCE HUMAN CARTILAGE TISSUE ENGINEERING

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ABSTRACT

Growth factors are used in human chondrocytes culture in order to increase its number while preserving its differentiated phenotype. However, the optimum combination of basic fibroblast growth factor (bFGF) and transforming growth factor beta-2 (TGFB-2) with human serum has not yet been determined for human chondrocytes culture and cartilage tissue engineering. The objective of this study was to determine the optimum combination of bFGF and TGFB-2 in 2% human serum supplemented medium to increase the chondrocyte yield while maintaining its differentiated phenotype. Various combinations of bFGF and TGFB-2 with human serum were tested for human chondrocytes growth, gene expression and ability for cartilage formation. The results show among various compositions tested; 3ng/mL bFGF + 1ng/mL TGFB-2 scores the highest chondrocytes growth rate compared to the other groups. It results in 50 folds higher cell number compare to the control group. However, the expression level of collagen type II and chondrocytes differentiation index is the highest in the combination of 1ng/mL bFGF + 1ng/mL TGFB-2. In cartilage engineering, the cultured chondrocytes from 3ng/mL bFGF + 1ng/mL TGFB-2 are able to form glistening white cartilage tissue after 8 weeks of implantation. Histological evaluation shows the tissue is rich in proteoglycan matrix and contained lacunae cells embed in basophilic matrix. Quantitative gene expression evaluation shows the engineered cartilage express high collagen type II and aggrecan mRNA with high differentiation index. Conclusions: Combination of 3ng/mL bFGF + 1ng/mL TGFB-2 with 2% human serum can provide high cell yield during monolayer expansion and good quality of engineered cartilage formation in vivo.

1.0 Introduction

One of the most important issues in cartilage tissue engineering is finding good combination of growth factor in the suitable medium that can efficiently expand the number of chondrocytes while retaining its differentiated phenotype. In clinical practice, expanding the scanty number of chondrocytes that are isolated from donor site to a sufficient number for transplantation is the main challenge in cartilage tissue engineering [1].
Approach to increase the proliferation capacity of chondrocytes can be achieved with the addition of mitogenic growth factors. Previously we have shown that basic fibroblast growth factor (bFGF or FGF-2) is a good mitogen for human chondrocytes and it worked synergistically with both animal serum as well as human serum [2]. However, the use of animal sera for the generation of new cartilage for human implantation should be avoided since animal sera may cause viral transmission and immune rejection. Study has shown immune response against bovine serum proteins in burn patients receiving keratinocytes graft cultured using foetal bovine serum [3]. Production of antibodies and immune response against animal sera was also detected in patients receiving dendritic cells and T cells grew in culture medium containing animal serum [4,5]. In our previous studies, we have demonstrated the benefits of using human serum (either pooled or autologous source) for human chondrocytes culture-expansion that can be replaced the animal sera [6-8].

Although bFGF has shown strong mitogenic effect but chondrocytes cultured with bFGF alone can easily dedifferentiated. On the other hand, transforming growth factor beta 2 (TGFβ-2) was able to maintain the phenotypes of cultured human articular chondrocytes [9]. Previous studies had reported conflicting results on the actions of both bFGF and transforming growth factor beta (TGFβ) in chondrocytes culture. Bradham & Horton (1998) showed bFGF and TGFβ promoted rabbit and human articular chondrocytes proliferation. However, these chondrocytes lost type II collagen expression as early as passage 2 [10]. The same outcome was also reported by Jakob et al. (2001) on human articular chondrocytes [11]. In cartilage reconstruction, bFGF and TGFβ supplementation in the culture medium was shown to increase the cell proliferation in in vitro construct consisting of bovine articular chondrocytes seeded onto polyglycolic acid [12]. Recently, reports showed that human septum chondrocytes can proliferated greatly in serum reduced medium with supplementation of bFGF and TGFβ-1 [13]. In contrast, Arevalo-silvaand colleagues (2000, 2001) showed TGFβ antagonized the growth promotion activity of bFGF in porcine and human pediatric auricular chondrocyte culture [14,15]. TGFβ also reduced the quality of engineered cartilage that formed. Quatela and colleagues (1993) showed that bFGF and TGFβ-1 did not give any positive action on human nasal septum chondrocyte growth [16]. Besides these contradicting results, no report was found on the study to evaluate the combination effects of bFGF and TGFβ-2 with human serum. Thus, the main objective of this project was to investigate and identify the optimum dose for the combination of bFGF with TGFβ2 when human chondrocytes were cultured in human serum supplemented medium. Human serum may contain various growth factors such as EGF, VEGF and PDGF that can interfere and mask the effects of bFGF and TGFβ2- in the experiment. Therefore, we reduced the amount of supplementation to 2% human serum in this study [17]. In our previous study, we had shown the valuable effects of Insulin-transferrin-selenium (ITS) in human chondrocytes culture when 2% serum was used in the culture medium [18]. Thus, ITS was added in the current culture medium to promote chondrocytes expansion and prevent dedifferentiation.

### 2.0 Materials and Methods

#### 2.1 Chondrocytes Isolation

This study was approved by the Research and Ethical Committee of Universiti Kebangsaan Malaysia (Approval number: D-010-2002). Human septum cartilage was obtained from six adult patients after elective septoplasty. Patient consent was obtained prior to the tissue collection. Each specimen was cleaned from surrounding perichondrium before minced into small pieces and digested with 0.6% collagenase type II (Invitrogen, Carlsbad, CA) at 37°C for 12 hours in an incubator shaker. After digestion, the chondrocytes suspension was centrifuged at 600xg for 5 minutes to collect the cell pellet. Chondrocytes were then resuspended in phosphate buffer saline (PBS; Invitrogen) for total cell quantification with hemocytometer (Weber Scientific International Ltd. Middx, England) and cell viability determination with Trypan blue vital dye (Invitrogen).

#### 2.2 Chondrocytes Culture Expansion in Different Composition of Growth Factors

After isolation, human chondrocytes were divided into 4 groups, each supplemented with different composition of growth factors in Ham’s F12:Dulbecco’s Modified Eagle medium (ratio 1:1; Invitrogen). Group 1: 1ng/mL bFGF + 1ng/mL TGFβ-2. Group 2: 3ng/mL bFGF + 0.2ng/mL TGFβ-2. Group 3: 3ng/mL bFGF + 0.5ng/mL TGFβ-2 and Group 4: 3ng/mL bFGF + 1ng/mL TGFβ-2. The effective range of the bFGF and TGFβ-2 that can increases human chondrocytes proliferation was predetermine in previous study (data not shown). Media in all groups also added with 2% pooled human serum (HS), 1% ITS (10µg/mL insulin + 5.5µg/mL transferrin + 6.7ng/mL sodium selenite), 200mM L-glutamine, 100U/mL of penicillin, 100µg/mL of streptomycin, 0.25µg/mL of amphotericin B (Invitrogen) and 50µg/mL of ascorbic acid (Sigma, St. Louis, MO). The pooled human serum was collected from three consented healthy individual that age from 25 to 35 years old and stored at -20°C until use. Medium without both growth factors supplementation was used as the control group. Chondrocytes were seeded in six-well tissue culture plate (Falcon, Franklin Lakes, NJ) with cell density of 5,000 cells/cm² in the initial passage (P0). All cultures were kept in 5% CO₂ incubator.
(Jouan, Duguay Trouin, SH) at 37°C with medium changed every three days. When the initial culture (P0) reached confluence, it was trypsinized with 0.05% trypsin-EDTA (Invitrogen) and suspended in PBS for growth rate, viability and total cell yield determination. Subsequently, the chondrocytes were sub-cultured with the same cell density and culture condition until it reached passage 3 (P3).

### 2.3 Total RNA Extraction and Quantitative Polymerase Chain Reaction (PCR)

Quantitative gene expression analysis was done using two steps reverse transcriptase-polymerase chain reaction. Total RNA was extracted from the cultured chondrocytes at passage 1 and passage 3 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 total RNA pellet was then washed with 75% ethanol and dried before dissolved in Rnase and Dnase free distilled water (Invitrogen). The purity and quantity of the extracted total RNA was determined by NanoDrop spectrophotometer (NanoDrop Technologies, USA). Total RNA was stored at -80°C immediately after extraction. Complementary DNA was obtained from reverse transcription reaction on 200-500ng of extracted total RNA using superscript III reverse transcriptase (Invitrogen) at 45°C for 30 minutes. Then, 1μl of cDNA was used in each quantitative PCR reaction using specific primers to determine the expression level of collagen type II, collagen type I and aggrecan core protein. All primers were designed using Primer 3 software based on the published GeneBank database sequences. The primers sequence used in this study was listed in Table 1. PCR reaction was carried out using SYBR Green as an indicator in Bio-Rad iCycler instrument. The DNA amplification was carried out in 40 cycles with denaturation step at 94°C for 10 seconds and annealing/extension step at 61°C for 30 seconds. The specificity of the primers and PCR reaction were confirmed with melting curve analysis and further verified by agarose gel electrophoresis. Relative gene

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Gene Bank Accession No.</th>
<th>Primer Sequence</th>
<th>PCR product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH (F)</td>
<td>GAPDH F</td>
<td>5'-tcc ctg agc tga acg gga ag-3'</td>
<td>217</td>
</tr>
<tr>
<td></td>
<td>NM_002046</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH (R)</td>
<td>GAPDH R</td>
<td>5'-ggg gga gga gtc gtc gct gct-3'</td>
<td>217</td>
</tr>
<tr>
<td></td>
<td>NM_002046</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Collagen Type I (F)</td>
<td>Coll F</td>
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<td>222</td>
</tr>
<tr>
<td></td>
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<td></td>
</tr>
<tr>
<td>Collagen Type I (R)</td>
<td>Coll R</td>
<td>5'-tac aggaag cag acaaggca-3'</td>
<td>222</td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Collagen Type II (F)</td>
<td>Col2 F</td>
<td>5'-cta tctggcaagcagctgg ca-3'</td>
<td>209</td>
</tr>
<tr>
<td></td>
<td>NM_001844</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Collagen Type II (R)</td>
<td>Col2 R</td>
<td>5'-atg ggtgcaatgtcaatgag-3'</td>
<td>209</td>
</tr>
<tr>
<td></td>
<td>NM_001844</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aggrecan core protein (F)</td>
<td>ACP F</td>
<td>5'-cac tgtaccgccacttc ca-3'</td>
<td>183</td>
</tr>
<tr>
<td></td>
<td>NM_001135</td>
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<tr>
<td>Aggrecan core protein (R)</td>
<td>ACP R</td>
<td>5'-acc agcgggaatct ccg-3'</td>
<td>183</td>
</tr>
<tr>
<td></td>
<td>NM_001135</td>
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</tbody>
</table>
expression level of each targeted gene was then normalized to GAPDH. Chondrocyte differentiation index was then determined by calculating the ratio of collagen type II against collagen type I expression level.

2.4 Cartilage Regeneration and Implantation

Chondrocytes at passage3 was used for cartilage tissue engineering because it provided the highest total cell yield after culture expansion. The combination of growth factors that gave the highest cell yield was used to culture the chondrocytes until harvest. The harvested chondrocytes were then suspended into a 30% (wt/vol) co-polymer of polyethylene oxide and polypropylene oxide, Pluronic F-127 (BASF, Mount Olive, NJ) at 4°C with cell density of 50 x 10^6 cells/mL. The admixer (100μl) 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 guideline of Univeristi Kebangsaan Malaysia Animal Ethical Committee.

2.5 Evaluation on the Regenerated Cartilage

The implanted tissues were harvested after a period of 8 weeks. The tissue engineered cartilage was divided into two half with one part fixed in 10% phosphate buffered formalin (Fisher Scientific, Fair Lawn, NJ) for 24 hours and processed into paraffin embedded block. Paraffin blocks were then sectioned at 4μm thickness and the slides sections were stained with standard histological staining; Hematoxylin & Eosin and Safranin O staining. The other half of the excised tissue was digested with collagenase type II enzyme and the total RNA was extracted for quantitative PCR analysis similar way like the monolayer cells. Native cartilage was used as the control for comparison.

2.6 Statistical Analysis

Data for chondrocytes growth rate, viability, total cell yield and gene expression level were collected from six samples. Values were presented as mean ± standard error of mean (SEM). Student’s t test was used to compare data between groups. Differences at 5% level were considered significant.

| Table 2: Relative gene expression level of collagen type II, collagen type I and aggrecan core protein in tissue engineered cartilage compared to native cartilage |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Tissue          | Collagen Type II| Collagen Type I | CII/C I Index   | Aggrecan core protein |
| Tissue engineered cartilage | 3.462 ± 0.244   | 0.118 ± 0.034   | 29.342 ± 7.269  | 0.275 ± 0.048 |
| Native cartilage | 2.042 ± 0.496   | 0.021 ± 0.003   | 95.428 ± 16.211 | 0.456 ± 0.089   |

3.0 Results

3.1 Chondrocyte Growth Kinetic

Human septum chondrocytes adhere to the surface of tissue culture flasks and start to proliferate on the second day after
Generally in all growth factors composition groups, chondrocytes proliferation is at the fastest rate at passage 1 and the growth rate gradually decrease with the following passages. Chondrocytes at P1 in the control group score 9,630 increase in cells/cm$^2$/day; 1ng/mL bFGF + 1ng/mL TGF-B-2 group score 19,874 increase in cells/cm$^2$/day; 3ng/mL bFGF + 0.2ng/mL TGF-B-2 group score 19,160 increase in cells/cm$^2$/day and 3ng/mL bFGF + 0.5ng/mL TGF-B-2 group score 22,529 increase in cells/cm$^2$/day. With the same bFGF concentration at 3.0ng/mL, human septum chondrocytes growth rate increase dependently on the concentration of TGF-B-2. Combination of 3ng/mL bFGF + 1ng/mL TGF-B-2 show the highest chondrocytes growth rate from P0 to P3 compare to other groups. This growth factors composition score 19,816 increase in cells/cm$^2$/day at P0, 24,293 increases in cells/cm$^2$/day at P1, 19,415 increase in cells/cm$^2$/day at P2 and 15,696 increase in cells/cm$^2$/day at P3. The chondrocytes growth rate in medium supplements with 3ng/mL bFGF + 1ng/mL TGF-B-2 score three folds higher value in every passage compare to the control group that supplement with 2% HS and ITS only.

Cumulative cell doubling from P0 to P3 and the number illustrates the degree of expansion of the culture from the initial culture until passage3. Medium supplemented with 3ng/mL bFGF + 1ng/mL TGF-B-2 promoted significantly higher fold of increase in chondrocytes number compared to the other four groups (2.9 x 10$^5$ increased in cell number; Figure 1). Combination of 3ng/mL bFGF + 1ng/mL TGF-B-2 scored 50 folds higher cell number compared to the control group; 2.2 folds higher cell number compared to 1ng/mL bFGF + 1ng/mL TGF-B-2; 3.2 folds higher cell number compared to 3ng/mL bFGF + 0.2ng/mL TGF-B-2 and 1.3 folds higher cell number compared to 3ng/mL bFGF + 0.5ng/mL TGF-B-2 (Figure 1).

3.2 Quantitative Gene Expression of Cultured Chondrocytes

The expression of collagen type II in cultured chondrocytes demonstrates highest expression with the combination of 1ng/mL bFGF + 1ng/mL TGF-B-2 group at P1 as well as P3.

The cell viability in all test groups from P0 to P3 scores minimum level of 92% after trypsinization (data not shown). No significant difference is noticed after statistical analysis. The total fold increase in chondrocytes number is the
passaged (Figure 2). In the other hand, the expression of collagen type I in all test groups show an increment from P1 to P3 (Figure 2). Combination of 3ng/mL bFGF + 0.2ng/mL TGFB-2 exhibit the highest expression level of collagen type I in P1 as well as P3 compared to other groups. The mRNA expression of collagen type I in 3ng/mL bFGF + 0.2ng/mL TGFB-2 group is significantly higher than others group in P1 and P3 (Figure 2). This has resulted in highest chondrocytes differentiation index (ratio of collagen type II/collagen type I expression) in 1ng/mL bFGF + 1ng/mL TGFB-2 group compared to other groups and the differences is significant (Figure 2).

The expression of aggrecan core protein in cultured chondrocytes demonstrates almost 10 fold reduction in expression level in all test groups when P1 culture chondrocytes is compared to P3 (Figure 2). However, no significant difference is recorded for the expression level of aggrecan core protein among the test groups in the same passage.

3.3 Evaluation on Regenerate Cartilage

Combination of 3ng/mL bFGF + 1ng/mL TGFB-2 managed to produce the highest cell yield in the shortest duration of culture. This combination is selected to expand the chondrocytes number for cartilage construction. This combination also gives the second best chondrocytes differentiation index after the group supplemented with 1ng/mL bFGF + 1ng/mL TGFB-2. After 8 weeks of implantation in nude mice, the harvested tissues were glistening white in color and firm in consistency. Histological evaluation using Safranin O staining shows that the tissue sections are positive for proteoglycan matrix staining which denotes high production of cartilage matrix in the tissues (Figure 3A). Hematoxylin and Eosin staining demonstrated that the tissue engineered cartilage consists of lacunae cells in round to oval shape embed in basophilic matrix (Figure 3B).

Quantitative gene expression analysis shows high expression of collagen type II with value even higher than native cartilage (Table 2). However, due to the higher expression level of collagen type I (almost 5 folds), the tissue engineered cartilage only scores 30% of the differentiation index compared to native cartilage (Table 2).

4.0 Discussion

This current study demonstrated that simultaneous supplementation of bFGF and TGFB-2 promoted great growth rate of human septum chondrocytes.

This result was consistent with previous finding for the mitogenic effects of bFGF and TGFB-2 on adult human articular chondrocytes [9,19]. However, the optimum combination and concentration of the growth factors was not determined in previous reports. Among the four tested combinations of growth factors, 3ng/mL bFGF + 1ng/mL TGFB-2 demonstrated the best combination that was able to give the highest growth rate and total cell yield compared to other groups. This combination worked well with 2% pooled human serum and ITS as has been demonstrated in previous studies [18,20]. This current protocol can be easily translated into clinical setting by using just the minimum amount of 2% pooled human serum or 2% autologous serum. Previous study has also demonstrated similar performance of pooled human serum and autologous serum on human articular chondrocytes [21]. Although quantitative gene expression results showed this combination ranked second in the ability to retain the differentiated phenotype of cultured chondrocytes, but it provided almost double the amount of cells needed for cartilage construction compared to the 1ng/mL bFGF + 1ng/mL TGFB2 group. Higher number of chondrocytes is crucial for making larger cartilage in the clinical treatment for cartilage repair [1].

Higher concentration of TGFB-2 was able to prevent chondrocytes dedifferentiation caused by bFGF. This was
observed when group supplemented with 3ng/mL bFGF combined with 0.2ng/mL TGFβ2 demonstrated lower expression of collagen type II and lower differentiation index compared to group supplemented with 3ng/mL bFGF combined with 1ng/mL TGFβ-2. Previous finding has showed complex regulation of bFGF and TGFβ-2 in cartilage matrix homeostasis and repair. Both inhibitory as well as synergistic interaction can occur depending on time of growth factor application and composition of the combination [22]. Supplementation of TGFβ-2 can also reduce the degradation of collagen type II and expression of gene associated with chondrocyte hypertrophy [23].

Although the best combination of bFGF and TGFβ-2 (3.0ng/mL bFGF + 1.0ng/mL TGFβ-2) did not result in the highest differentiation index of cultured chondrocytes, histological evaluation confirmed these cultured chondrocytes can produce good quality cartilage formation. The reduction on type II collagen gene expression during monolayer culture could just be temporary and the cultured chondrocytes can regain the differentiation phenotype by increasing the collagen type II and aggrecan core protein expression when put into the three-dimensional in vivo environment. This finding is in agreement with Bradham and Horton (1998) where they showed that rabbit and human articular chondrocytes lost type II collagen synthesis at P2 when cultured in 10ng/mL bFGF + 1ng/mL TGFβ medium. However, the cultured chondrocytes were able to re-express type II collagen in the subsequence micromass culture [10]. Later, after 8 weeks of in vivo development; the implanted tissue showed similar properties compared to hyaline cartilage. Bohme et al. (1995) showed another advantage of using both bFGF and TGFβ-2 in combination to suppress the terminal differentiation of chick embryo chondrocytes. By using bFGF and TGFβ-2 in combination, engineered cartilage tissues can be prevented from entering into the hypertrophy stage and turning to bone [24]. It is important to regenerate pure cartilage tissue for repairing cartilage lesion and minimize the possibility of generating bone tissue in order to regain its functions.

5.0 Conclusion

The combination of 3ng/mL bFGF and 1ng/mL TGFβ-2 in medium supplemented with insulin-transferin-selenium and 2% pooled human serum promoted human chondrocytes expansion and enhanced collagen type II and aggrecan core protein expression in engineered cartilage. The engineered cartilage also demonstrated high differentiation index with lacunae cells embedded in proteoglycan-rich matrix. It is the ideal combination for human cartilage tissue engineering in this study.

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