Isolation and in vitro reproduction of periosteal cells

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Mechanical and biochemical isolation and reproduction of periosteal cells, enable us to evaluate these fibroblast like cells. The established method of isolation and reproduction of periosteal cells were presented, and compared to other well known periosteal cell culture studies. The effect of hormones, trace elements, osteogenic mediators, and different pressure and oxygenation on bone growth and periosteal bone healing can be evaluated by structural and electron microscopic means on the isolated and cultured osteogenic cells. [Turk J Med Res 1993; 11 (3): 107-111]

Key Words: Periosteum, Cell culture, Fibroblast like cell

Preparation of histological sections of the bone is not so easy. Our knowledge on bone healing and growth is therefore limited, when compared to soft tissues and other organs. Excluding physeal growth, the primary growth and healing of bone is maintained by the endosteum and periosteum (1). If the isolation and culture of periosteal and endosteal cells were possible, it would enable us to increase our knowledge on bone healing and bone growth.

Recent progresses in mechanical (2) and biochemical cell isolation techniques, permit researchers to perform tissue culture of osteoblast like cells (5-11). Knowledge on osteoblast like cells increased by various experiments performed on these cells (12-18).

Basset and Cohen identified two different layers of the periosteum (19,20). The inner layer, which is called the "cambium" maintains its osteogenic potential through the whole life of the human being. The idea to observe periosteal cells for their osteogenic capability in vivo and in vitro is not so new. In 1928, Fell were able to grow fibroblast like cells, and realised that ossification occurs in in vitro conditions by these cells (19). Short after Fells' experiment, the importance of the periosteum in bone growth was estimated by other researchers (21-24). Caplan and his study group and Poussa et al. identified the details of periosteal cell culture from chicks. They also identified the osteogenic potential of these cells by monoclonal antibodies (21), and demonstrated the bone morphology and remodelling of embryonic chick limbs (23).

Isolation of fibroblast like cells from the periosteum, and culture of these cells are still active subjects of today's' orthopaedic research (3,4).

We present the methodology of isolation of fibroblast like cells from the periosteum by mechanical and biochemical means. The way of isolation of chick, rabbit, and human periosteum is discussed, and compared to other methods of cell isolation and culture techniques.

MATERIALS AND METHODS

Isolation of Periosteal Cells

Chick periosteum: One week old ten Leghorn type chicks were used at the experiments. The chicks were sacrificed by high dose diethyl ether inhalation, and the hind legs were cleaned by high concentration of methyl alcohol to avoid bacterial contamination. The skin and muscles were dissected under aseptic conditions, and the periosteum overlying the tibias were incised by a no. 11 scalpel. A quadrangular periosteal tissue, 0.2x0.7 cm in size, were dissected from the tibias of each chick. The isolated tissues were collected at a petri plate containing 7-10 ml Tyrodes salt solution (Sigma, St Louis, USA). The periosteum were dissected free of surrounding soft tissues and muscle. They were rinsed for five minutes by antibiotic (Streptomycin 100 mg/ml, Penicillin 100 IU/ml) containing phosphate buffered saline (PBS).

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Rabbit periosteum: Five two months old New Zealand white rabbits were anaesthetised by intramuscular ketamine hydrochloride and xylasin hydrocloride (100 mg/ml; 23.32 mg/ml) injection. The hind legs were shaved and sterilised by betadine solution. Both tibias were approached by an anteromedial skin incision. Periosteal tissue, 0.5x1.0 cm in size, was isolated and transferred to Tyrodes solution. The isolated periosteal tissues were rinsed with the same method described for chick periosteum.

Human periosteum: The periosteum was removed from the osteotomy site of a six years old child, to whom a corrective femoral osteotomy was performed because of coxa vara. The tissue was 1.0x1.5 cm in size. Following the removal of soft tissues and muscle overlying the periosteum, the tissue was rinsed with PBS for three times and transferred to an antibiotic containing petri dish. The mentioned periosteum was divided into small pieces, 0.1x0.2 cm in size, with a scalpel. Mechanical grinding or ultra sonofication was not used in order to persevere the viability of the cells.

Cultivation of Periosteal Cells

The cultivation of periosteal cells isolated from chick periosteum was performed with the method described by Swartz (25). Small pieces of periosteum was placed into 50 ml disposable tissue culture flasks (Nunc, Intermed, Denmark). To amenable the attachment of the tissue to the bottom of the flaks, the inner surfaces of the flasks was scattered with 100% fetal bovine serum (FBS). Tissue containing flasks were placed into a incubator at 37°C supplied with 5% carbondioxyde. After 4-6 hours of incubation, 20% FBS containing Eagles minimum essential medium (EMEM), (Gibco, Paisley, Scotland) and antibiotic were added into the flasks. The medium was exchanged every two or three days by 10% FBS containing EMEM medium.

For human periosteum, the pieces were placed into a 0.25% type 2 collagenase (Biochrom KG, Berlin, Germany) containing Falcon tube. This tube was placed into a 37°C water bath and shaked for one hour. At the 30th minute of shaking, the elusion was transferred to a vortex machine, and the elusion was vortexed for five to seven seconds. At the end of shaking and vortexing, the tissue suspension was placed into a santrifuge, and isolated cells were precipitated to the bottom of the tube at 1500 rpm for 10 minutes. Following the removal of the supernatant, 100% FBS was added into the tube for the inhibition of the activity of collagenase. The cells were suspended by EMEM, and transferred to 50 ml tissue culture flasks. These cells were followed up with the same protocol used for periosteal cells isolated from chicks (Fig 1).

The Fitton-Jackson modification of BGJb medfum (Sigma, St Louis, USA) was used at the follow up of some human periosteal cells. This medium was described by Biggers (26), and was widely used at cell culture studios by Nakahara et al (3,4).

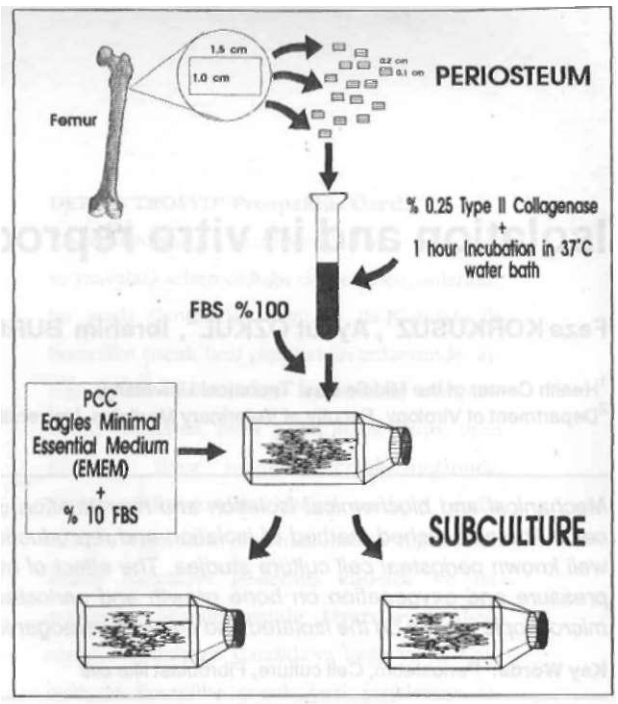

Figure 1. Schematic presentation of periosteal cell isolation and culture.

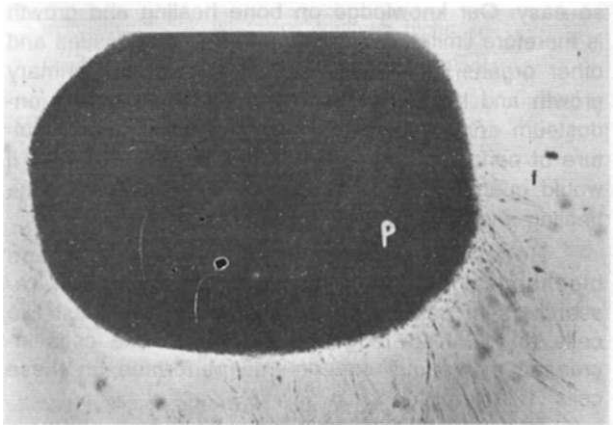

Figure 2. Fibroblast like cell growth (f) from the periphery of chick periosteum (P) cultivated by Swartz method, x 100

The periosteal cells isolated from rabbits were divided into two groups. The same methods used for chick periosteal cells were accomplished at the first group. At the second group, biochemical degradation of cells was utilised by collagenase as described for human periosteum previously.

Subculture: The cells were subcultured when they reached a constant amount in culture. This was called the "confluence" condition, and the medium was removed, and 2 ml of 0.25% trypsin (Gibco Paisley, Scotland) were added into the tissue culture flasks to remove them from the bottom of the flaks. The cells were incubated at 37°C for 10 minutes and dissociation of the cells from the bottom of the flasks were ob-

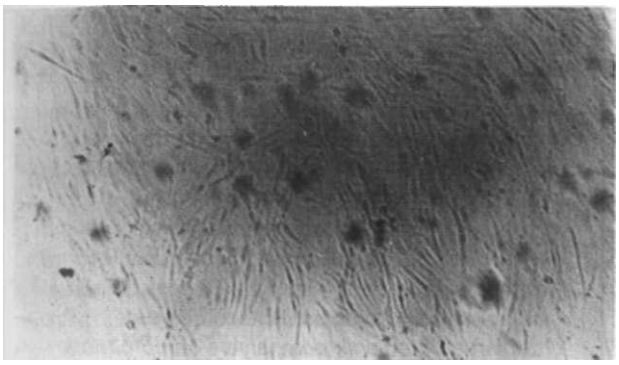

Figure 3. Confluent human periosteal cells in culture, x 200

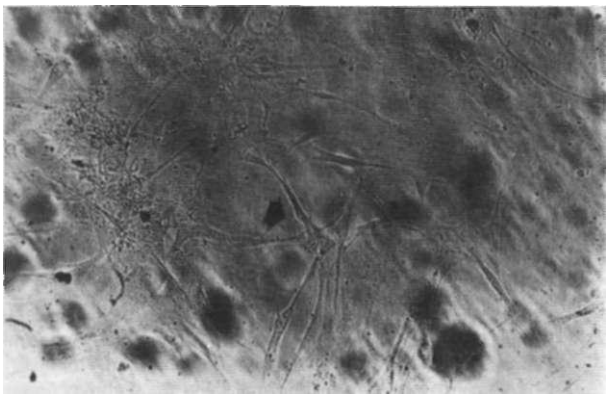

Figure 4. Morphology and integration (arrow) of human fibroblast like cells in culture, x 400

served macroscopically. The effect of trypsin was controlled by adding 100% FBS into the flasks, and the elusion were santrifuged for 10 minutes at 100 rpm. Cell counts were performed and viable cells were diluted to 10^5 cells per ml by EMEM. These cells were then divided into new tissue culture flaks to increase the number of isolated cells in culture.

RESULTS

Periosteal cells grew form the periphery of the periosteal tissue attached to the bottom of the tissue culture flasks (Fig. 2). This was observed between the 15 to 21 days of primary culture of chick periosteum with the Swartz method. Cell aggregates interspersed randomly throughout the tissue culture flask. Reaching confluence, the cells were subcultured to sustain monolayer periosteal cells in culture.

Human and enzymatically treated rabbit periosteal cells reached confluence in a shorter period then chick periosteal cells. Five to seven days later than primary culture, these cells were ready for subculture (Fig. 3). At cultures where BGJb medium was used, the growth of cells lasted longer, and the pH control of the medium was more complex when compared to EMEM.

At cytological examinations, these cells were defined to have similar morphology with fibroblasts. There were spindle-shaped fibroblast like cells as well as large polygonal cells. These cells looked healthy with no signs of degeneration. Spindle like cells were surrounded by a refractive outer boundary. Cells maintained contact with each other through multiple extensions (Fig. 4). Cytoplasmic extensions reveal that these cells have fibroblast like characteristics.

DISCUSSION

The cells of the cambium layer of the periosteum are known to be responsible of periosteal bone growth and healing (19). Periosteum is used as free or vascularised flaps at bone reconstruction for its osteogenic capacity (20,27-32). Although the idea of isolating and culturing periosteal cells by mechanical and biochemical methods is not new, recent research studies determined the standards of methodology (3,4,33). Periosteal cell morphology and their osteogenic potential's were described by this means. Evidence is now available that there is a population of progenitor cells present in periosteum that are capable of differentiating into bone, and cartilage.

The method of periosteal cell culture is described in this current paper. One should take care of several pitfalls in performing periosteal cell culture other than general rules of other tissue cultures. The growth of chick periosteal cells from periosteal tissue by Swartz method takes 2 to 3 weeks. However, treating the tissue with collagenase reduces this time to approximately one week.

Type II collagenase is a specific enzyme to isolate fibroblast like cells, and is known to be the least destructive substance to dissociate periosteal cells from the tissue. 0.5% of collagenase is appropriate for human tissue, where the concentration of 0.25 to 0.125% is better for rabbit and chick periosteum. The effectiveness of collagenase can be increased by vortexing the elusion for 5 to 10 seconds at each 20 to 30 minutes. At lower concentrations the time of treating the tissue with collagenase should be increased to 1.5 to 2 hours.

Although being a specific medium of fibroblast like cells, the BGJb medium was found to be less effective. The cells in the mentioned medium grew slower than suspected, and pH regulation of BGJb was not so easy when compared to EMEM. The BGJb medium was not used at this study for the aforementioned reasons.

For the continuance of human periosteal cells the percentage of FBS in the medium can be as low as 1%. However, 10% of FBS was preferred at our studies FBS in known to stimulate in vitro osteogenesis (26).

The size of periosteal cells were approximately 8-10 um and 15 urn, at chicks and human, respectively. Before subculturing these cells, they can be filtrated

through a 20 urn pore size filter to allow them fall separately, however, this method was not used in our study. Chick periosteal cells and human periosteal cells can be subcultured for 15 to 40-50 times respectively. The osteogenic capacity of these cells decrease, as the number of subcultures increases. We did not exceed 4 to 5 subcultrures in this study to persevere the osteogenic potential of these cells.

The advantages of periosteal cell culture are to increase the number of uniform fibroblast like cells and to evaluate their structural and functional probabilities more easily. Cultured cells can be preserved in liquid nitrogen. These cells can be thawed and used in a future study. An experiment can be truncated at a certain level and the cells can be transferred to another research center to continue the experiment.

Cells in culture present various differences than in vivo cells. They usually present a small part of a tissue, and there exists the lack of factors present at normal tissue circulation. These cells are usually derived from younger individuals, and may present different receptors than suspected in adults. The lack of blood circulation, and environmental factors are other disadvantages of tissue culture.

High mitotic activity of cultured cells disable us to implant these cells back to individuals. Carcinogenic potential, as well as infection, are the drawbacks of retrograde implantation of these cells in human beings. However, active research is performed on these cells to combine them with various carriers, and implant them as tissue substitutes in living organisms. The field of study is encouraging, and may be a promoting area of basic orthopaedic research. These cells can be used at non-unions, and at the treatment of chondral defects.

Endocrinological studies for osteoporosis, and neuroendocrinological bone healing mechanisms can be evaluated through these fibroblast like cells. Various mediators, trace elements, and physical conditions such as pressure, electric stimulation, magnetic fields, and their effects on osseous tissue can be evaluated on cultured cells. However, histological staining of tissue cultured cells, including toluidine blue staining for determining cell viability, alkaline phosphatase activity of periosteal cells, glycosaminoglycan synthesis, growth curves, and ^{3}H-thymidine incorporation studies should be standardised for bone research.

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Periost kökenli hücrelerin izolasyonu
ve in vitro kültür ortamında üretilmeleri

Periost hücrelerinin mekanik ve enzimatik yöntemlerle izolasyonu ve in vitro kültür ortamında üretilmesi, osteojenik potansiyele sahip bu hücrelerin kolay incelenmesi açısından önemlidir. Çalışmamızda uygulanan yöntem tanımlanmış ve yaygın olarak günümüzde kullanılan diğer hücre kültürü yöntemleriyle karşılaştırılmıştır. Periost dokusundan izole edilen fibroblast kökenli hücreler İn vitro ortamlarda osteojenik potansiyele sahip olmalarının yanısıra in vlvo ortamda osteoinduktif özellik taşırlar. Değişik hormonların, eser elementlerin, osteojenik mediatörlerin ve farklı basınç ve oksijenasyonun kemik büyümesi ve periosteal kırık iyileşmesi üzerindeki etkilerinin yapısal ve elektron mikroskopik incelenmesi yönünden periost doku kültürü yönteminin yararlı olacağı düşünülmektedir.

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