Method Description for Obtaining Primary Fibroblast Cultures from Human Tenon's Capsule

İnsan Tenon Kapsülünden Primer Fibroblast Kültürlerinin Üretilmesinde Yöntem Tanımlanması

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ABSTRACT Objective: Human tenon's capsule derived fibroblasts have been widely used in order to constitute in vitro models, especially for studies regarding ophthalmic surgery. Though they are quite difficult to culture primarily, no actual method in detail could be found in literature. In this study, we have aimed to propose an effective procedure including biopsy localizations, reagents and culture conditions. **Material and Methods:** Explants of human tenon's capsule were obtained from 7 patients at the time of pars plana vitrectomy (PPV) surgery. From PPV incision areas 5 tenon's capsule and 2 tenon's capsule + conjunctiva specimens were harvested from 3 and 2 patients respectively, whereas 2 juxtalimbal tenon's capsule specimens were harvested from other 2 patients. The cross-sections were prepared from paraffin blocks for histological analysis. Tenon's tissues were put into dulbecco's minimal essential medium and then placed into a 6 well culture plate containing dulbecco's minimal essential medium +10-20% fetal calf serum. Cells were incubated in a 5% CO_2 humidified incubator. **Results:** From the tenon's capsule samples taken from pars plana vetrectomy incision areas, fibroblasts were detected neither culture nor histological paraffin blocks. Production of confluent fibroblasts was achieved in materials taken from the juxtalimbal area and from tenon's capsule + conjunctiva tissue specimens. It was seen easly when proliferated fibroblasts anchored to plates pre-covered gelatin and serum. Detachment of the confluent cells was obtained via 0.5% trypsin exposure. **Conclusions:** In this study, we have succeeded to produce adequate cells and also standardized ur method of primary fibroblast cultures derived from human tenon's capsule, including tissue biopsy, transport and culture. On the other hand, findings like better production of fibroblasts in juxtalimbal area, may yield the necessity of further studies.

Anahtar Kelimeler: Glaucoma; cell culture techniques; methods; fibroblasts

ÖZET Amaç: Hasta tenon kapsül biyopsilerinden alınan fibroblastlar, başta glokom cerrahisinde antifibrotik ajan kullanımı olmak üzere in vitro modeller oluşturmakta sıkça kullanılmaktadır. Bu hücrelerden primer kültür eldesi zor olmakla birlikte, güncel literatür detaylı bir metot bilgisinden yoksundur. Bu çalışmada alınacak dokuların lokalizasyonu ve kültür koşullarının standardize edilmesi planlandı. **Gereç ve Yöntemler:** PPV uygulanan 7 hastadan, sırasıyla 3 ve 2 hastadan 5 tenon kapsülü ve 2 tenon + konjonktiva doku parçacıkları ile diğer 2 hastadan jukstalimbal bölgeden alınan 2 tenon kapsülü "dulbecco's minimal essential medium" besiyerlerinde, farklı oranlarda FCS ve farklı yapışma faktörleri ile kültür yapıldı. Histolojik analiz için yatay kesitler hazırlandı. "dulbecco's minimal essential medium" + 10-20% FCS içeren multiwellere alınarak %5 CO_2 içeren nemli inkübatöre konuldu. **Bulgular:** Pars plana vetrectomy insizyon bölgelerinden alınan tenon doku örneklerinde, histolojik preparatlarda ve kültürlerde fibroblast gözlenmedi. Aynı bölgelerden alınan tenon + konjonktiva dokularında ise 2-3 gün sonra yoğun metafazda fibroblastlar gözlendi. Jukstalimbal bölgeden alınan 2 materyalde ise ("dulbecco's minimal essential medium" + %15 `fetal calf serum`) yine yoğun fibroblast üretimi başarıldı. Önceden serum ve jelatin kaplanmış "plate"lerde prolifere olan fibroblastlar tabana yapışık olarak kolaylıkla gözlendi. Hücreler %5 tripsin kullanılarak kaldırıldı. **Sonuç:** Bu çalışmada, ihtiyaç duyduğumuz hücrelerin eldesinin yanı sıra, doku alımı, transportu ve primer kültür metodolojisinde bir standardizasyon sağlanabilmiştir. Diğer taraftan, fibroblastların jukstalimbal bölgeden üretiminin daha başarılı olması gibi bazı bulgularla ilgili daha ileri araştırmalara ihtiyaç vardır.

Key Words: Glokom; hücre kültürü teknikleri; metotlar; fibroblastlar

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Effects of many drugs and chemicals which are yet to be applied in vivo, can be investigated in cells produced from the specimens obtained from patient biopsies. In recent years, tenon's capsule derived fibroblasts have been widely used in order to constitute in vitro models to evaluate cellular responses, oxidative stress, toxic effects; especially regarding antifibrotic agents used in glaucoma surgery, in which fibrovascular scar formation is the main cause of failure. So far, many drugs influencing intracellular signaling pathways have been and are tried in order to prevent fibroblast proliferation. Today, the most popular ones are Mitomycin-C, 5-FU. However, either variety of receptor and ion canal subtypes, or state dependent binding of the potential blockers to the unstable sites raises difficulties in producing therapeutics to bind properly to the appropriate site. Furthermore, side effects of these substances restrict their clinical use.[1] This calls for planning in vitro studies in that field, and primary fibroblast cultures derived from human tenon's capsule seem to be the most favorable model for such studies.[2] These cells are known to be quite difficult to produce, however, to our best knowledge, no clear-cut method has been suggested in literature so far. In this pro-study, we have aimed to standardize the procedures of proper tissue harvest and cell culturing, in order to develop a practical method for culturing primary fibroblasts derived from human tenon's capsule.

MATERIAL AND METHODS

BIOPSY SPECIMENS

Explants of human Tenon's capsule were obtained from 7 patients at the time of Pars Plana Vitrectomy (PPV) surgery. During the avivation of conjunctiva and tenon's capsule, specimens were taken from the areas near the scleral incisions, which deprived of conjunctiva or not. None of the donors had had antiglaucomatous or antimetabolite treatment, or ocular surgery before. All patients gave their informed consent before inclusion in the study, which was approved by local ethics committee and conformed the provisions of the Declaration of Helsinki.

Five tenon's capsule and 2 tenon's capsule + conjunctiva specimens from midscleral area, were harvested from 3 and 2 patients respectively, whereas 2 juxtalimbal tenon's capsule specimens were harvested from 2 other patients, all in the same dimensions. The specimens of conjunctival and subconjunctival tissues, some 2 mm deep, 2 mm high, and 5 mm wide (measured under the dissecting microscope via a piece of paper) were harvested under standard regional anesthesia. Care was taken to avoid mechanical damage to tissues.

REAGENTS

Dulbecco's minimal essential medium (DMEM) supplemented with L-glutamine, glucose (1000 mg/L), penicillin (60 IU/mL), streptomycin (60 μg/mL) and fetal calf serum (FCS). The reagents were provided by Sigma and Gibco.

CELL CULTURE

The biopsy specimens were dissected and placed into 6 well culture plates coated with FCS or 1% gelatin and were closed with sterile cover slips. After one hour, 1 mL DMEM or M199 supplemented with L-glutamine glucose (1000 mg/L), penicillin (60 IU/mL), streptomycin (60 μg/mL) and 10%, 15% and 20% FCS were added into culture plates for tissues obtained from each specimen. Cultures were maintained in a humidified 5% CO_2 incubator at 37°C for 3-5 days. Then, suspended fibroblasts were passaged to flasks keeping the attached fibroblasts in place. Culture medium was supplemented twice weekly, keeping the cells under morphological surveillance. Confluent cultures were trypsinized and subcultured, and 95% viability was constantly maintained via trypan blue dye exclusion method. Presence and proliferation of fibroblasts in culture are depicted in inverted microscopes (Figure 1). The proliferative process of each culture displayed individual variations. Patient's cell lines were kept frozen for further studies.

HISTOLOGICAL EXAMINATION

Samples taken for histological analysis were fixed in 10% formaldehyde. The cross-sections were prepared from paraffin blocks. Presence of fibroblasts

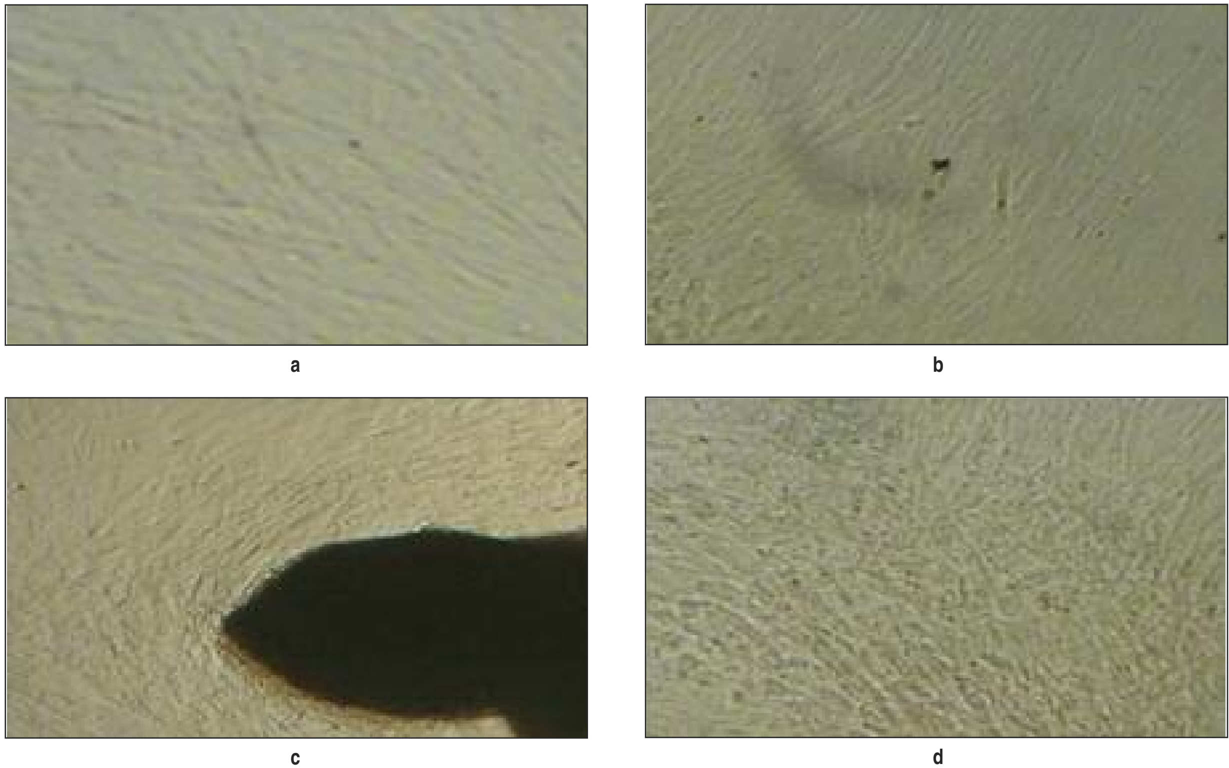

FIGURE 1: (a) Tenon's fibroblasts at the 1st week, (b) 2nd week, (c) 4th week and (d) Confluent tenon's fibroblasts at the 5th week (x40).

was shown with standard techniques including heamatoxylene and eosin staining.[3] Images were taken in light microscopes.

RESULTS

Fibroblast proliferation was absent in cultures prepared from "tenon's capsule-only" tissue specimens. Furthermore, no fibroblast could have been detected histologically with heamatoxylene and eosin staining in these 5 patients. However, abundant fibroblast proliferation was observed in cultures prepared from tenon's capsule + conjunctiva tissue specimens by 3 days, and cells were easily detectable in histological analysis (Figure 2). Confluence was achieved in 6 weeks (Figure 3), followed by trypsinization and liquid nitrogen freezing. Production of confluent fibroblasts was also achieved in 2 materials taken from the juxtalimbal area, with DMEM + 15% FCS. Optimum results have been obtained with the method writen below.

From juxtalimbal areas, 3 x 3 mm of tenon's tissue were harvested.

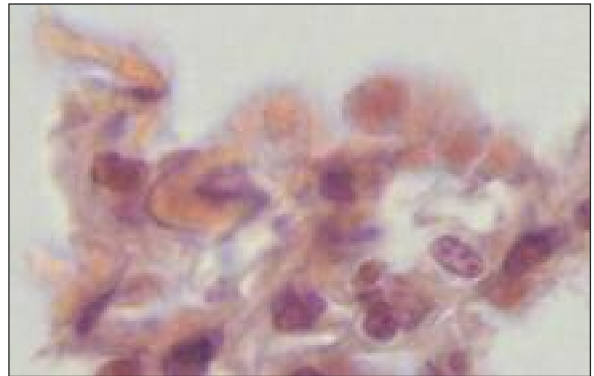

FIGURE 2: Demonstration of fibroblasts in the cross-sections prepeared from paraffin blocks of the juxtalimbal tenon's tissue, with H.E. staining (x90).

Tenon's tissues were put into DMEM and then placed into standard multiwells containing DMEM + 15% FCS in the same day. Bottom surface of culture plates have been washed with FCS and gelatin, sterile coverslips have been placed over the specimens.

Cells were incubated in a 5% CO_2 humidified incubator at 37°C for 3-5 days.

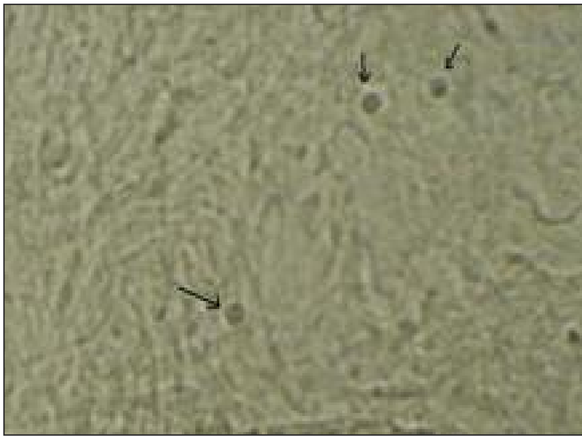

FIGURE 3: Fibroblasts in mitosis phase (arrows) floating in culture supernatant above the attached mature cells (x40).

After seeing the first attached cells, culture medium was supplemented twice weekly.

Detachment of the confluent cells was obtained via 0.5% trypsin exposure 10 min at 37 °C.

Patient's cell lines were kept frozen for further studies.

DISCUSSION

Use of early phase -preferably primary- cell lines is very important for the reliability of cell culture studies. Regarding primary fibroblast cultures derived from human tenon's capsule, literature data is inconsistent and lacks detail. In literature, though the details have not been given about the dimensions and localizations of the tissue removed, Tenon's tissue is reported to be harvested during cataract, glaucoma or strabismus surgeries and immersed in DMEM (3) or Phosphate Buffer Solution (PBS) in sterile conditions and had to be taken into proper culture medium in 12 hours, but no details could be found regarding the optimal localization and dimensions of the tissues to be harvested.[4,5] Aided by correspondences with experienced authors in this field and our personal experiences, we preferred to harvest approximately 3 x 3 mm of juxtalimbal tenon's tissue into DMEM during PPV operation. The biopsies were cut into small pieces and put into culture medium consisted of DMEM with FCS under 37°C temperature and 5% humidified CO_2, consistent with the literature data. [5,6]

Despite the studies suggesting fibrin coated plates, we preferred standard multiwells, like proposed by some others.[4-6] We washed bottom surface of culture plates with FCS and gelatin, placed sterile coverslips over the specimens to anchor it to the plastic well. We used either a small cover slip or fragment a big one and just cover the specimen. After waiting for cells to attach for 1-2 hours, 1 mL of 10% FCS + DMEM have been added. Cells were incubated in a 5% CO_2 humidified incubator. In their study, that was referred many times, Lee et al reported that fibroblasts attached in 4-8 days and culture time was 2 weeks. [5] At the end of the first week, we observed free and attached fibroblasts and replaced half of the medium. Some cultures proliferated better than the others, so we subcultured the fibroblast cultures containing abundant cells in supernatant into additional flasks. We have carried on the culture this way for 1-1.5 months.

Detachment of the confluent cells was obtained via 0.5% trypsin exposure 10 min at 37 °C only, for no significant benefit was observed with additional collagenase II. Although some authors advocate the use of collagenase II, trypsin inhibitor, DNAse in PBS or trypsin/EDTA, there are also studies in which 0.5% trypsin is used alone, apparently with success.[4-6]

CONCLUSIONS

In this study, for our further studies, we have succeeded to produce adequate cells and also standardized our method of primary fibroblast cultures derived from human tenon's capsule, including tissue biopsy, transport and culture. On the other hand, findings of this study may also yield many clinical expansions as well. Therefore, further studies are needed in order to confirm our results and also illuminate the underlying mechanisms.

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