

Development and Characterization of Explant Tissue Culture System as an In Vitro Model for Uterine Leiomyoma (Fibroid) To Screen Natural Compounds

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Abstract: Uterine leiomyomas are the most common benign tumors in women, which arise from smooth muscle cells of the uterine myometrium. The aim of present study was to develop a novel explant tissue culture system as an in vitro model for uterine leiomyoma to facilitate screening of natural compounds. The clinically diagnosed, hysterectomy specimens of uterine myomas were collected and characterized by histochemistry (H & E staining; Masson's Trichome; Periodic Acid Schiff Stain). For in vitro culture, two different isolations methods (mechanical vs. enzymatic) were tested. The cultured cells were characterized by immunocytochemistry using marker antibodies (Ki-67 and Collagen). Our results shown that mechanically (explants culture method) isolated cells possess significantly higher proliferation, sub-culture efficiency and intense staining of Masson's Trichome and Periodic Acid Schiff Stain, Collagen and Ki-67 as compared to enzymatic digestion method. Our results suggest that mechanical isolation (explants culture) method for uterine leiomyoma tissue was better in terms of growth and characterization. In conclusion, leiomyoma explants culture system may offer a suitable alternative model to evaluate anti-tumor properties of natural or synthetic compounds against uterine leiomyoma.

Index Terms: Characterization, Explant tissue culture, In vitro model, Uterine leiomyoma

I. INTRODUCTION

Uterine fibroids or leiomyomas are the most common tumors of the female genital tract, affecting 50% to 70% of females by the age of 50. Even though these tumors are benign, uterine leiomyomas have a significant impact on the reproductive health of women due to their high incidence and lack of proven treatment options other than surgery. It is estimated that up to 77% of all women will develop UL in their lifetime and 15 to 30% of these women suffer from substantial symptoms, including pelvic discomfort, dysmenorrhea, menorrhagia, anemia, urinary incontinence, recurrent pregnancy loss, preterm labor, and in some cases infertility (Catherino et. al. 2013; Bulun, 2013). The prevalence of uterine fibroid in India is upto 20-30% in reproductive females and 10-20% of

infertility is attributed to uterine fibroid (Stewart, 2001; Islam et. al. 2013). Further, according to Ministry of Health and Family Welfare, Govt. of India, it is estimated that at least 15-25 million women will be suffering from uterine fibroids (Khan et. al. 2014). The growing body of literature implicates that unfavorable early life exposure to endocrine disruptors and life style during sensitive window period of development in reprogramming may responsible to induce disease susceptibility later in adult life (Tripathi et. al. 2019; Yang, 2015; Ligon & Morton, 2001).

Despite the significant morbidity associated with leiomyomata, etiology of this disease remains unclear, however it is suggested that genetic factors, cytokines, growth factors, steroid hormones (estrogens and progestogens) and/or their receptors, and excessive production of extracellular matrix plays major role in development of uterine fibroid. Conventional therapies including surgery and medication and chemotherapy are widely used, although in most cases it produces undesirable side effects. Thus, many research groups are evaluated several novel drugs or anticancer compounds using in vitro and in vivo models (Kang et. al. 2009; Morrissey et. al. 2010; Du et. al. 2011). Taking into consideration the importance of fibrosis in leiomyoma pathogenesis, there is a great interest in the development of antifibrotic drugs for this tumor.

Recently, studies have successfully demonstrated that naturally occurring compounds, especially phytochemicals have amazing potential in attenuating tumor progression in breast, lung and ovarian cancer by acting as anti-angiogenic and anti-oxidants factors (Tian et. al. 2017; Wang et. al. 2018). To screen natural compounds against any disease system, a well established, defined and cost effective in vitro cell culture model is required. Such in vitro model would have the additional advantage over in vivo model because it quick, efficient, and easily manipulated. Further, to get the significant effect or data for a particular compound may require large number of animals. These factors make in vivo experiments more time consuming, expensive, and difficult to interpret. Therefore, considering these

limitations and prevalence of uterine fibroid, development of an in vitro cell culture model would be really great. The purpose of this study was to optimize the isolation procedure and development of a suitable in vitro cell culture model from leiomyoma tissues to screen natural therapeutic compounds. This in vitro cell culture models may utilize for screening of potential therapeutic substances to find novel compounds for uterine leiomyoma treatment/ or research.

II. MATERIALS AND METHODS

A. Tissue Collection

Tissue was collected from patients undergoing medically indicated hysterectomy for symptomatic leiomyomas, leiomyoma and myometrial tissue. Preoperative evaluation was performed by gynecologists using available clinical and radiologic data at Institute of Medical Sciences, Banaras Hindu University. Total ten hysterectomy specimens of uterine myomas (3.0-10.8 cm in diameter) were included in this study. The study was performed after approval by a local ethics committee in accordance with the specification stipulated by the institution human ethical committee and signed written informed consent of the patient.

B. Histopathological analysis

1) Hematoxylin and eosin (H&E) staining

To histopathological analysis, the collected leiomyoma and myometrial tissues were fixed in 10% neutral buffered-formalin and then embedded in paraffin using the conventional histological technique. Tissue sections of 5 μ m were prepared on a microtome and mounted on glass slides. Afterwards, the slides were deparaffinized and stained with hematoxylin and eosin (H&E) as per the laboratory established protocol (Pandey et al. 2019). Then permanent sections were prepared with coverslips and synthetic resin. Morphological parameters analyzed in leiomyoma and control (myometrium) explants included collagen deposition, necrosis, viable/damaged tumor cells. Representative photographs of all treatments were showed in results section.

2) Masson's Trichome staining

For Masson's Trichome staining, the sections of both types of tissues were first stained in Weigert's iron hematoxylin working solution for 10 minutes and then after washing, stained in Biebrich scarlet-acid fuchsin solution for 10-15 minutes. Further, sections were stained with in phosphomolybdic-phosphotungstic acid solution for 10-15 minutes. Tissue sections were transferred directly (without rinse) to aniline blue solution and stain for 5-10 minutes. Finally, sections were rinsed briefly in distilled water and then differentiated in 1% acetic acid solution for 2-5 minutes, and after dehydration, sections were mounted with resinous mounting medium and observed under Bright field microscope.

3) Periodic acid schiff (PAS) staining

For PAS staining, tissue sections were deparaffinized and rehydrated through 100% alcohol, 95% alcohol 70% alcohol having time interval for 3 minute, rinsed in distilled water. After that sections were immersed in 0.5% periodic acid solution for 5 minutes and rinsed in distilled water. Further, sections were placed in Schiff reagent for 20 minutes at room temperature and then washed in running tap water for 10 minutes. Finally,

sections were counterstained with hematoxylin for 3 minutes and after dehydration, sections were mounted with resinous mounting medium and observed under Bright field microscope.

All stained sections were observed by a pathologist using a Carl Zeiss Axiostar Plus Bright field microscope. Morphological parameters analyzed in leiomyoma and control (myometrium) explants included collagen deposition, necrosis, viable/damaged tumor cells. Representative photographs of all treatments were showed in results section.

C. Primary cell culture

1) Enzymatic digestion method

Tissue for cell-culture purposes was placed in phosphate-buffered saline (PBS) containing 1 \times antibiotic and antimycotic solution (Invitrogen, Carlsbad, CA) on ice under sterile conditions and transported from operating room to the laboratory. In laboratory, tissues were washed twice in cold 1 \times PBS containing antibiotic and antimycotic solution (Invitrogen; Cat No.#15240062). The tissue were minced into 1–5-mm pieces in a sterile culture dish and transferred into 50-mL conical tubes containing Dulbecco's minimum essential medium/F12 (DMEM/F12; Invitrogen; Cat No. #12634028), supplemented with or without 80 units/mL collagenase type-1 (Sigma: Cat No. #SCR103) and 0.1 mg/mL of DNase-1 (Sigma: Cat No. #5025). All conical tubes were kept at 37°C in a water bath with gentle agitation for 3 hours. At the end of the digestion time, the undigested tissue was filtered using nylon mesh (100 μ m diameter) and the cells were centrifuged at 500 rpm for 10 minutes. The pellet was rinsed twice with DMEM/F12 media, counted, and dispersed in working culture medium composed of DMEM/F12 with 10% fetal bovine serum (FBS), 1 \times antibiotic and antimycotic solution. The cell count and viability were analyzed with the Trypan blue dye (0.4%, Invitrogen; Cat No. T10282) exclusion method.

2) Mechanical or explants culture method

After washing in PBS, tissue was minced into 1–5-mm pieces in a sterile culture dish containing DMEM/F12 supplemented with 1 \times antibiotic and antimycotic solution. Further explants were directly placed into the wells of a 24-well plate, each of which contained 300 μ L of working culture medium composed of DMEM/F12 with 10% fetal bovine serum (FBS), 1 \times antibiotic and antimycotic solution. Fresh medium was added in each well every fourth day, and cells were allowed to grow until they were ~70% confluent, at which time the cells were trypsinized with 0.25 % trypsin-EDTA solution (Invitrogen; Cat No. #25200114) and counted before being plated to undergo a second passage. Once the cells were 70%–80% confluent, they were trypsinized and counted and rest of the cells were cryopreserved. Total ten primary cell cultures that included four myometrium and six leiomyoma cultures were established from five different patients. Plates were pre-incubated for 1 h at 37°C, 5% CO₂/95% air, and agitation at 25 rpm. The interval between resection of the tumor and the incubation of the explants was no more than 2 h. The entire process was performed under aseptic conditions and as per the established laboratory protocol (Tripathi et al. 2019; Pandey et al. 2019).

3) Expression of proliferative markers by immunofluorescence

To study the expression of collagen and Ki-67, myometrium and leiomyoma cells were cultured on glass chamber slides (Nalge

Nunc International, Rochester, NY). After 70% of confluency, cells were fixed with cold methanol and permeabilized with 0.2% Triton X-100 (Sigma-Aldrich, St. Louis, MO). The nonspecific sites were blocked using 1% BSA and 10% normal goat serum, and the cells were incubated with Collagen (COL4A2; Santacruz biotechnology Inc) and cell proliferation marker Ki-67 (Santacruz biotechnology Inc.) antibodies at 1:100 dilution overnight at 4°C. Further, after washing, cells were incubated with FITC-conjugated secondary antibodies (Santacruz; biotechnology Inc) for 1hr at 37 °C. Images were detected and acquired using fluorescence microscope (EVOS 4300 colour imaging system, Life Technologies).

III. STATISTICAL ANALYSES

The statistical analyses were performed by the SPSS for windows version 11.0 software. The differences between groups were analyzed with independent samples T-test. P-value < 0.05 was considered significant.

IV. RESULTS

A. Development of culture method (Explant vs enzymatic)

The H & E staining of uterine leiomyoma explants (tumor tissue) were clearly showed deposition of extracellular matrix (ECM) and more condensed nucleuses as compared to normal myometrium (Fig. 1). Further, Masson's Trichome staining showed intense deposition of collagen fiber (blue color) in the case of uterine leiomyoma as compared to myometrium (Fig. 1). The PAS staining showed significantly more intense deposition of fibrous connective tissue in uterine leiomyoma as compared to myometrium (Fig. 1). These results suggest that collected diagnosed uterine leiomyoma tissue possess tumor and neoplastic characters as compared their counterpart myometrium.

We observed that compared to enzymatic, in explants culture, the proliferation rate of explants derived cells was greater than enzymatic during all the incubation times. It is observed that explants tissue derived cells are morphologically healthier and showing typical fibroblastic nature as compared to enzymatic digested tissue derived cells (Fig. 2). This result suggests that the explants tissue derived cells are more viable, active and possesses higher proliferative capacity as compared to enzymatically digested tissue.

To further evaluate proliferative capacity in long term, both type tissue derived cells were sub-cultured. Our results showed that explants tissue derive cells have more potency to proliferate and maintained their variability and proliferative potency up to 5-6 passages while, enzymatically digested cells were lost their proliferative potency after 2-3 passages (Fig. 2). The explants tissue derive cells were maintained their proliferative potential in vitro up to 28-days while enzymatically digested cells lost their proliferative potential within 10-12 days.

B. Expression of markers proteins in cells

Further, we interested to look more closely at the protein expression patterns of collagen fiber and proliferative marker Ki-67 in both type of cells. Immunore activity for each of these markers demonstrated more collagen deposition and higher

expression of Ki-67 in explants tissue derive cells (Fig. 2E & F) as compared to enzymatically digested cells (Fig. 2e & f).

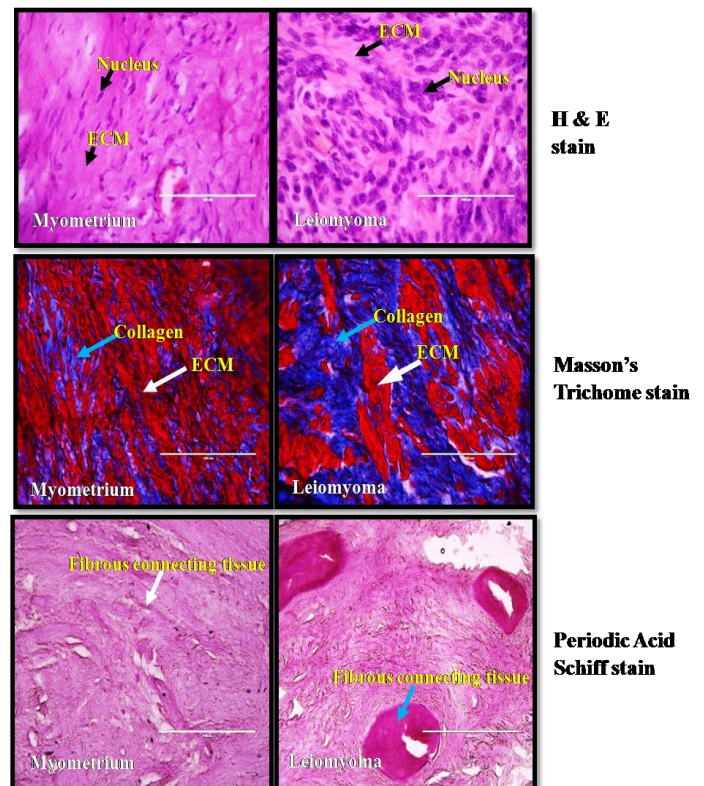


Fig.1. Histological findings in uterine fibroid tissue. Myometrium tissue (control) sections are showing normal morphology with less deposition of ECM and nucleus (arrow head) as compared to their counterpart uterine fibroid tissue showing more condensed nucleus (H&E stain), deposition of collagen (Masson's trachoma stain) and mass of fibrous connective tissue. (Scale Bar = 200 µm).

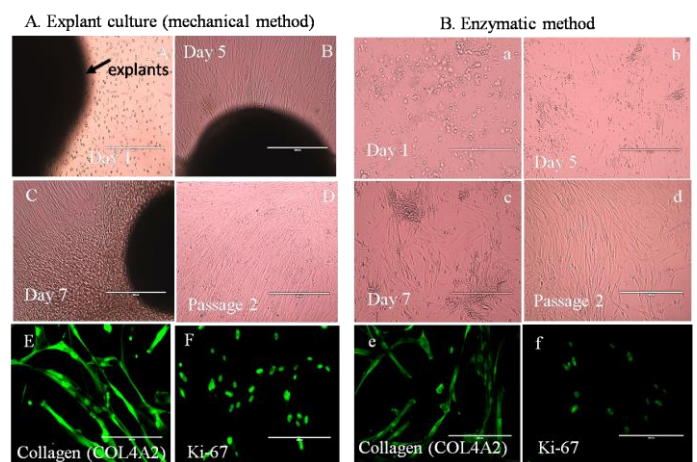


Fig. 2. Figure showing two different in vitro culture methods and characterization of uterine fibroid tissue, (A) mechanically chopped explants tissue derived cells exhibiting typical fibroblastoid phenotype in primary culture (A-C), in vitro expansion of cells (passage 2) showing 90% of confluency (D). (B) In vitro culture of enzymatically digested fibroid tissue

showing slow growth (a,b,c) and 60% confluency (d). The mechanical explants tissue derived cells showing higher expression of proliferative markers (Collagen and Ki-67; higher green fluorescence intensity; E & F) as compared to enzymatically digested tissue derive cells (e & f). Three independent experiments were conducted to confirm the results. (Scale Bar = 200 μ m).

V. DISCUSSION

Present study was conducted to develop a novel tissue culture system that may be utilized for screening of several therapeutic compound and drugs against uterine fibroids. Leiomyoma, or fibroids, are basically benign tumours of the myometrium, and one of the commonest tumours in reproductive aged women (Stewart, 2001; Baird & Newbold, 2005). Leiomyoma poses a threat to females of reproductive age since there are no effective and safe medications available for treating this disease. Surgical procedures including hysterectomy or myomectomy are only the primary option for treatment (Reichardt, 2012). It is defined by the excessive deposition of extracellular matrix (ECM). It is believed that deregulation of steroids hormone, ECM and its associated genes are primarily responsible to cause uterine fibroids. Further, a series of events/factors such as, inflammation, oxidative stress, hypoxia, cytokines and chemokines, proinflammatory and profibrotic growth factors, angiogenic growth factors as well as mechanical stress have also been considered to play important role in the process of fibrosis to drive leiomyoma development and growth (Wegienka, 2012; Fletcher et. al. 2013; Hou et. al. 2014; Islam et. al. 2014; Wang et. al. 2012). Taking into consideration the importance of fibrosis in leiomyoma pathogenesis, there is a great interest in the development of antifibrotic drugs for this tumor. However, till date there is limited studies are conducted to screen the phytochemicals against the UL due to unavailability of established primary cell culture system.

We have developed and characterized a novel technique to culture uterine leiomyoma explants that demonstrates a high degree of tissue viability for at least 28 days ex vivo. We found that the serum supplemented DMEM fortified with antibiotics was able to maintain the viability of leiomyoma explants with regard to every parameter that we have evaluated. Our cultured cells showed higher expression of proliferative marker gene Ki-67 which suggest that cells are more proliferative. Higher expression of collagen further strengthen our results that cultured cells are by nature originated from fibroid tissue and possess unique characters of uterine fibroid. This finding is important, because explants possess unique potential to generate the newer cells for further expansion and processing.

The in vitro model of organotypic uterine leiomyoma explant culture system appears to be an advantageous system in which clinician or researcher can screen the new drug/compound or may be used for differentiation into other cell types. The ex vivo explants culture model may be particularly relevant to screen new compounds for development of new drugs against uterine fibroid or leiomyoma. In this study, total ten uterine fibroid explants were used from five patients and expanded, maintained under in vitro culture condition for at least 28 days, thereby reducing the number of human samples/animals needed for each experiment. Further, the entire procedure of isolation and culture

ten explants requires, at most, 2 hours. A minimal effort of exchanging half of the medium for fresh medium every second day is necessary to maintain the cultures. In this system, conditions can be controlled much more precisely than other in vivo models. Our uterine explant culture model may be useful for screening a large number of compounds for beneficial effects on cellular migration and differentiation, assaying the optimum dosages to achieve the desired effect. This approach may also facilitate reduction and refinement of the number of in vivo procedures which are required to screen therapeutic drugs.

In conclusion, our results showed that organotypic cultures of uterine leiomyoma explants may offer an alternative model for the ex-vivo evaluation of novel compounds with potential antitumor properties, assessing the synergic effect with known antitumor compounds. This model may also open perspectives to study the biological effects of conventional and innovative treatment strategies in uterine leiomyoma or fibroid research and to analyze different mechanisms of carcinogenesis in other human tumors.

CONFLICTS OF INTEREST

The authors have declared that no conflict of interest exists.

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