In Vitro Extracellular Matrix Model to Evaluate Stroma Cell Response to Transvaginal Mesh

Ming-Ping Wu,1,2 Kuan-Hui Huang,3 Cheng-Yu Long,4 Chau-Chen Yang,2 and Yat-Ching Tong5*
1Division of Urogynecology and Pelvic Floor Reconstruction, Department of Obstetrics and Gynecology, Chi Mei Foundation Hospital, Tainan, Taiwan
2Center of General Education, Chia Nan University of Pharmacy and Science, Tainan, Taiwan
3Department of Obstetrics and Gynecology, Chang Gung Memorial Hospital, Kaohsiung, Taiwan
4Department of Obstetrics and Gynecology, Kaohsiung Municipal Hsiao-Kang Hospital, Kaohsiung, Taiwan
5Department of Urology, College of Medicine and Hospital, National Cheng Kung University, Tainan, Taiwan

Aims: The use of surgical mesh for female pelvic floor reconstruction has increased in recent years. However, there is paucity of information about the biological responses of host stroma cells to different meshes. This study was aimed to establish an in vitro experimental model to study the micro-environment of extracellular matrix (ECM) with embedded mesh and the stroma cell behaviors to different synthetic meshes. Methods: Matrigel multi-cellular co-culture system with embedded mesh was used to evaluate the interaction of stroma cells and synthetic mesh in a simulated ECM environment. Human umbilical vein endothelial cells (HUVEC) and NIH3T3 fibroblasts were inoculated in the system. The established multi-cellular Matrigel co-culture system was used to detect stroma cell recruitment and tube formation ability for different synthetic meshes. Results: HUVEC and NIH3T3 cells were recruited into the mesh interstices and organized into tube-like structures in type I mesh material from Perigee, Marlex and Prolift 24 hr after cell inoculation. On the contrary, there was little recruitment of HUVEC and NIH3T3 cells into the type III mesh of intra-vaginal sling (IVS). Conclusions: The Matrigel multi-cellular co-culture system with embedded mesh offers a useful in vitro model to study the biological behaviors of stroma cells in response to different types of synthetic meshes. The system can help to select ideal mesh candidates before actual implantation into the human body. Neurourol. Urodynam. © 2013 Wiley Periodicals, Inc.

Key words: extracellular matrix; Matrigel; pelvic organ prolapse; stress incontinence; transvaginal mesh

INTRODUCTION

Pelvic organ prolapse (POP) is a highly prevalent female pelvic floor disorder. It was reported that 41% of women aged 50–79 years had some degree of POP, including cystocele (34%), rectocele (19%), and uterine prolapse (14%).1 POP with or without stress urinary incontinence (SUI) can have a significant detrimental impact in the patient’s quality of life. Pelvic connective tissue resilience decreases with vaginal delivery and menopause, thus the prevalence of POP increases with aging.2 The lifetime risk of women receiving surgical treatment for POP was reported up to 11.1%; but high recurrence and re-operation rate of 29.2% was also noted.3 Risk factors for POP recurrence include poor pelvic tissue condition, impaired wound healing, chronic diseases associated with high intra-abdominal pressure (obstructive pulmonary disease, obesity, constipation, etc.), and age over 60 years.4 Unfortunately, the largest proportion of POP surgeries has been performed in women aged 60–79 years.5,6 Biologically, defective connective tissue condition both in the vaginal wall itself and its supporting structures are major contributing factors for surgical failure.7 Patients with POP recurrence risk factors may be benefited from the use of mesh implants during pelvic floor surgery.8 Both biological and synthetic meshes have been reported.9–12 In recent years, there has been an increasing popularity for the use of transvaginal mesh (TVM) with procedural kits which include disposable insertion needles, retrieval devices, and large piece of synthetic mesh.11 Among these, the Prolift system (Gynecare, Somerville, NJ) and Apogee-Perigee system (American Medical Systems, Minnetonka, MN) have been two of the most frequently used products. Although some authors have reported good results with these commercial kits,12–14 cases of defective vaginal wound healing were noted. In 2008, the Food and Drug Administration (FDA) issued a public health notification to warn about a number of serious problems that had been associated with TVM procedures. According to reports received by the FDA from nine different manufacturers, over 1,000 people had suffered severe complications from the surgical mesh, including infection, pain, urinary problems and organ perforations. Some women had also reported recurrences of the prolapse or incontinence, and the need for additional surgeries to remove mesh that had eroded into the vagina. In July 2011, the FDA updated the notification and reiterated the concern again. Thus, the establishment of in vitro models to study how meshes are integrated with the host tissue before putting them into a human body is important to minimize post-operative complications associated with TVM surgeries.

Myofibroblasts, also called activated fibroblasts, play an important role in extracellular matrix (ECM) remodeling during...
healing processes. Fibroblasts are the major cellular component of ECM. Compared to normal fibroblasts, myofibroblasts are often characterized by acquisition of high alpha-smooth muscle actin (α-SMA) expression. Phenotypic switching of quiescent fibroblasts into activated fibroblasts is the most prominent ECM stroma reaction during carcinogenesis or wound healing. Mesh incorporation into the host tissue is dependent on the in-growth of activated fibroblasts and other stroma cells. This study was aimed to establish an in vitro experimental model simulating the micro-environment of ECM with embedded mesh and the biological responses of stroma cells toward some commonly used synthetic meshes.

MATERIALS AND METHODS

Cell Cultures

The mouse NIH3T3 cells and normal cervical fibroblast cells were used to represent activated and quiescent fibroblasts respectively. The cells were prepared in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Biological Industries Co., Haemek, Israel). Primary cultures of human umbilical vein endothelial cells (HUVECs) were isolated from fresh human umbilical cord veins and maintained in EGM2 BulletKit (Cambrex, East Rutherford, NJ) containing 2% fetal bovine serum (FBS), basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), insulin-like growth factor-1 (IGF-1), epithelial growth factor (EGF), hydrocortisone and heparin. In some situation, HUVECs were cultured in M199 basal medium containing penicillin/streptomycin and supplemented with 20% FBS. HUVECs were attached on 2% gelatin-coated dish and detached by incubating with 0.5% trypsin/5.37 mM EDTA for 1 min at room temperature. Only passages four to six of the HUVECs were used for experiments in this study. All cells were incubated at 37°C in a humidified incubator with 5% CO₂.

Western Immunoblotting for α-SMA Protein Expression

To characterize the NIH3T3 cell as a suitable representation of myofibroblasts during the ECM remodeling, α-SMA protein expression of NIH3T3 cells was compared with that in human normal fibroblasts (NF). Equal amounts of cell protein lysates were fractionated by SDS–PAGE at 80–100 V for 2.5–3 hr. The proteins were transferred to PVDF membrane (Amershan Corp., Arlington Heights, IL). The blots were incubated with anti-human α-SMA 1A4 antibody (Abcam, Cambridge, MA) or anti-matrix metalloproteases 2 and 9 (MMP-2 and -9; Calbiochem, Darmstadt, Germany; 1:500). Blots were then processed by chemi-luminescent substrate detection system (Amersham).

MMPs Zymography

Activities of MMP-2 and MMP-9, the key regulators for matrix degradation, were tested in NIH3T3 cells. Conditioned medium from the cell culture in FBS-free medium for 3 days was cleared of cells and debris by centrifugation at 3,000g for 10 min and concentrated 10-fold using Amicon Microcon (YM-10). The gelatinolytic activities of MMP-2 and MMP-9 in the conditioned medium were measured by gelatin zymography as previously described. Conditioned medium was mixed with zymography sample buffer and resolved in 10% SDS–PAGE containing 1 mg/ml of gelatin under non-reducing conditions. Following electrophoresis at 4°C, gels were washed twice for 30 min with re-mentation buffer at room temperature, and then incubated overnight in incubation buffer (50 mM Tris–HCl pH 7.5, 200 mM NaCl, 10 mM CaCl₂, 1 mM MnCl₂) at 37°C. Characterization of MMP2/9 activity was determined by inhibition with MMP-2/MMP-9 inhibitor I (Calbiochem; 0–20 μg/ml). After incubation, gels were stained for 1 hr (0.25% Coomassie brilliant blue R-250 in 45% methanol, 10% acetic acid), and de-stained in the same buffer without Coomassie. White bands against a blue background were observed following de-staining which indicated gelatinolytic activity of the expressed MMPs.

Matrigel co-culture system with embedded mesh. To detect the incorporation of stroma cells in different meshes, the multi-cellular Matrigel co-culture system. Meshes (size 1.0 cm × 1.0 cm) from different commercial kits: Prolift (Gynecare); Apogee and Perigee (American Medical Systems); IVS (Tyco Healthcare, Mansfield, MS); Marlex Mesh (Bard, Murray Hill, NJ, USA) were soaked in FBS overnight. Matrigel (BD Biosciences, Bedford, MA) 80 μl was added to each well of a 96-well plate and allowed to polymerize. Then the mesh was placed in the Matrigel. A total of 2 × 10⁶ cells (NIH3T3, HUVEC or equally mixed NIH3T3 and HUVEC cells) in 1 ml aliquot were evenly dispersed in the medium. Following 1–24 hr incubation, microscopic images for the progress of cell recruitment and tube-formation were taken. Matrigel (8 mg/ml) was used to check tube-formation, while Matrigel (1 mg/ml) was used to check cell recruitment. To better visualized migration of different cell types, PKH 26 (Green Fluorescent Cell Linker Kit, Sigma, St Louis, MO) was used to label NIH3T3 cells (green); while PKH 67 (Red Fluorescent Cell Linker Kit, Sigma, St Louis, MO) was used to label HUVEC (red).

The numbers of cells migrated into mesh interstices were counted under a light microscope at 200× and the average cell count from three random microscopic fields was calculated. For quantitative comparisons of stroma cell migration into different types of mesh, the values were presented as: [cell count with mesh/cell count in control medium without mesh × 100%].

Statistical Analysis

All values were reported as mean ± SD (standard deviation). The data were analyzed using one-way analysis of variance. Post hoc pairwise comparisons were performed using Scheffe’s multiple comparisons. The significance level was set at P < 0.05.

RESULTS

Characterization of NIH3T3 Cells

Western blots showed α-SMA protein over-expression when compared with NF (Fig. 1A). Gelatin zymography demonstrated increased MMP-2 activity in NIH3T3 cells which was inhibited by the addition of MMPI in a dose-dependent manner (Fig. 1B). The MMP-9 level was negligible in the NIH3T3 cell.

The Establishment of In Vitro-ECM Model

In order to study ECM micro-environment, an in vitro ECM model to evaluate the recruitment of stroma cells by different synthetic mesh was established (Fig. 2A). Stroma cells included myofibroblasts (NIH3T3) and endothelial cells (HUVEC). Myofibroblasts, like other invasive cancer cells, have the ability to invade tumor cell clusters. In this environment, NIH3T3 cells and HUVEC, but not NF, demonstrated the ability to grow into the mesh interstices and form web-like architecture.
Cell Migration into Different Meshes

NIH3T3 cells and HUVEC, separately or mixed, were dispersed in the Matrigel co-culture system (8%) with or without embedded mesh to evaluate stroma cell migration and tube-formation ability at 1 and 24 hr. NIH3T3 cells were recruited into the mesh interstices and started to form tube-like structures in type I meshes from Perigee, Marlex and Prolift at 24 hr after cell addition. On the contrary, there was almost no NIH3T3 in-growth into the interstices of the type III mesh from IVS (Fig. 2B). Similar results were also found for the HUVEC cells (Fig. 2C), or co-cultured NIH3T3 and HUVEC cells (Fig. 2D).

Matrigel cultured with dye-labeled stroma cells: NIH3T3 (red) and HUVEC (green), was shown in Figure 3. NIH3T3 were recruited into the mesh interstices in type I meshes at 24 hr. On the contrary, there was almost no NIH3T3 migration into the type III mesh of IVS (Fig. 3B). Similar results were also found in the HUVEC (Fig. 3C), or co-cultured NIH3T3 and HUVEC stroma cells (Fig. 3D).

When the experiment was observed up to 48 hr, the results were similar to those at 24 hr. There were still little HUVEC and NIH3T3 cell recruitment into the type III mesh. On the other hand, the numbers of cells migrated into the type I meshes at 48 hr remained stationary to those at 24 hr, suggesting a plateau phase of cell recruitment had been reached.

Quantitative Comparisons of Cell Recruitment Into Different meshes

Figure 4 gives quantitative comparisons of cell migration into different types of mesh at 24 hr. HUVEC and NIH3T3 cells cultured separately or together, showed greater degree of migration into type I mesh materials than type III mesh from IVS.

DISCUSSION

Biocompatibility of long-term implantable medical devices can be defined as "the ability of the device to perform its intended function, with the desired degree of incorporation in the host, without eliciting any undesirable local or systemic effects in that host".21 Mesh biocompatibility is basically...
biologists as a substrate for cell culture. Matrigel contains an environment found in many tissues and is used by cell cells. This mixture resembles the complex extracellular secreted by Engelbreth-Holm-Swarm (EHS) mouse sarcoma host-mesh interaction. Matrigel is a gelatinous protein mixture controllable and adjustable micro-environment to study the setting. One advantage of this system is that it offers a stroma reaction and mesh biocompatibility in an ex vivo shown that it is a potentially useful means of elucidating with surgical mesh is a relatively novel model. Our results have not new, the design of multi-cellular Matrigel-based co-culture in vivo biocompatibility.

The type of mesh polymer, the material weight, the filament structure and the pore size are important determinants on the physiological wound healing and scar formation, the foreign body reaction at the tissue/mesh interface will persist to a chronic inflammatory process. The time course of the reaction consists of several steps that are protein absorption, cell recruitment; fibrotic encapsulation and ECM formation. These dynamic processes involve a complex cascade of immune modulators and are influenced by the biomaterial composition. The type of mesh polymer, the material weight, the filament structure and the pore size are important determinants on the in vivo biocompatibility.

Although the use of cell culture to test prosthetic material is not new, the design of multi-cellular Matrigel-based co-culture with surgical mesh is a relatively novel model. Our results have shown that it is a potentially useful means of elucidating stroma reaction and mesh biocompatibility in an ex vivo setting. One advantage of this system is that it offers a controllable and adjustable micro-environment to study the host-mesh interaction. Matrigel is a gelatinous protein mixture secreted by Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells. This mixture resembles the complex extracellular environment found in many tissues and is used by cell biologists as a substrate for cell culture. Matrigel contains several basement membrane attachment molecules which apparently obviate the need for added purified attachment factors. Matrigel also appeared to play a trophic role in subsequent development by enabling the serum-free growth of myotubes which suggests that Matrigel mediates the cellular interaction of growth or attachment factors. The establishment of an in vitro Matrigel co-culture system is therefore useful to understand the physiology and pathophysiology underlying mesh success or failure in pelvic floor reconstruction. In this pioneer study, we had focused first on the two most basic cell elements of mesh incorporation, fibroblast and endothelial cells. In the future, addition of macrophage into the culture and assay of immune mediators involved in inflammation, wound healing and mesh rejection can also be performed with the system. Thus different stages and aspects of the ECM response to mesh implant can be evaluated. Moreover, the quantitative nature of the assessments makes comparisons of any new mesh products with standard ones possible.

HUVECs are cells derived from the endothelium of veins in the umbilical cord. They are used as a cellular model for the study of the function and pathology of endothelial cells. In this study, HUVEC recruitment was used as an indicator of angiogenesis. On the other hand, the NIH3T3 cell line was originally established from the primary mouse embryonic fibroblast cells that were cultured by the designated protocol, so-called "3T3 protocol". The primary mouse embryonic fibroblast cells were transferred (the "T") every 3 days (the first "3"), and inoculated at the rigid density of $3 \times 10^5$ cells per 20-cm$^2$ dish (the second "3") continuously. The spontaneously immortalized cells with stable growth rate were established after 20–30 generations in culture, and then named "3T3" cells. Our results showed the acquisition of $\alpha$-SMA over-expression in NIH3T3 cells. The $\alpha$-SMA is commonly used as a marker of myofibroblast formation. Actins are highly conserved proteins that are involved in cell motility, structure and integrity. Alpha acts are a major constituent of the contractile apparatus. The differentiation of fibroblastic cells towards the myofibroblastic phenotype, with acquisition of specialized contractile features, is essential for connective tissue remodeling during wound healing. Yet the myofibroblast still remains one of the most enigmatic of cells, partially owing to its transient appearance in association with connective-tissue injury and to the difficulties in establishing its role in the production of tissue contracture. The appearance of differentiated myofibroblasts may be of multiple origins in different pathological situation.

MMPs are zinc-dependent endopeptidases which play an important role in tissue remodeling with various physiological and pathological processes such as morphogenesis, angiogenesis, tissue repair and metastasis. In this study, gelatin zymography demonstrated increased MMP-2 but not MMP-9 activity in NIH3T3 cells. Evidence revealed that fibroblasts surrounding mesh material displayed strong MMP-2 gene transcription, whereas fibroblasts without close contact to mesh material had low MMP-2 synthesis rates. This zonal and

![Image](image_url)
cell-specific regulation of MMP-2 gene transcription suggests an intimate cellular cross-talk during mesh-induced foreign body reactions. According to Amid’s classification, the type I mesh is totally macroporous with pores >75 μm, the size required for infiltration of macrophages, fibroblasts, blood vessels; the type III mesh is macroporous material with multifilamentous or microporous components. Our findings of impaired web-formation ability and recruitment of stroma cells into type III mesh may account, at least in part, of the limitation usefulness of these mesh. An ideal surgical mesh should be infection-resistant, durable, noncarcinogenic, inexpensive, easy to apply, and cause no antigenic response but withstand remodeling by body tissue. The response of host cells to different mesh can explain the different surgical success rates and complication rates. The presence of synthetic mesh may cause interference of stroma cell recruitment into the healing area. For successful mesh incorporation, two important cellular behaviors are stroma cell recruitment and tube-formation. Our results showed that these two functions are impeded in type III mesh from IVS. The IVS is a tension-free vaginal tape variant that uses a multi-filament polypropylene tape to support the mid urethra for the treatment of female stress urinary incontinence. Cases of defective vaginal wound healing have been described in the literature. On the other hand, the monofilament macroporous type I mesh demonstrated more favorable cellular responses in this study. Although to prove the superiority of type 1 mesh was not the primary objective of this study, the fact that our experimental results agreed with the previous clinical observation may support the applicability of this model.

CONCLUSIONS

The Matrigel multi-cellular co-culture system with embedded mesh offers a model to study the ECM micro-environment and biologic responses of host stroma cells to different types of meshes. The impaired recruitment of stroma cells into the type III mesh may account, at least in part, of the less favorable surgical outcomes of these meshes.

REFERENCES


