The Basement Membrane Component of Biologic Scaffolds Derived from Extracellular Matrix

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ABSTRACT

The extracellular matrix (ECM) has been successfully used as a scaffold for constructive remodeling of multiple tissues in both preclinical studies and in human clinical applications. The basement membrane is a specialized form of the ECM that supports and facilitates the growth of epithelial cell populations. The morphology and the molecular composition of the ECM, including the basement membrane, vary depending upon the organ from which the ECM is harvested and the methods by which it is processed for use as a medical device. Processing steps, such as decellularization, lyophilization, disinfection, and terminal sterilization, may affect the morphology and composition of an ECM scaffold, including, but not limited to, the integrity of a basement membrane complex. The present study evaluated the presence and integrity of a basement membrane complex in processed ECM derived from three different tissues: the urinary bladder, small intestine, and liver. Immunohistochemical determination of the presence and localization of three basement membrane molecules, collagen IV, laminin, and collagen VII, was conducted for each ECM scaffold. Scanning electron microscopy (SEM) was used to further explore the surface ultrastructure of selected ECM scaffolds. The effect of a surface basement membrane presence upon the pattern of in vitro growth of two separate cell types, NIH 3T3 fibroblasts and human microvascular endothelial cells (HMEC), was also evaluated for each ECM scaffold. Results showed that the only intact basement membrane complex was found on the luminal surface of the ECM derived from the urinary bladder and that the basement membrane was an effective barrier to penetration of the scaffold by the seeded cells. We conclude that the urinary bladder ECM but not the small intestine- or liver-derived ECM contains a surface with composition and morphology consistent with that of an intact basement membrane complex, that the basement membrane complex can survive processing, and that the basement membrane structure can modulate in vitro cell growth patterns.

INTRODUCTION

The extracellular matrix (ECM) represents secreted products of the resident cells of each tissue and organ. As such, the ECM is a favored substrate for cell attachment, proliferation, and differentiation.1–3 Individual components of the ECM, such as collagen I, collagen IV, laminin, and fibronectin, have been used as surface coatings for biomaterials to facilitate cell attachment and growth and to promote biocompatibility.4,5 The basement membrane represents a specialized form of ECM that serves as an attachment site for various cell populations to adjacent tissues. The presence of a basement membrane within or on an ECM substrate would likely

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affect the pattern of growth of selected cell populations when the ECM is used as a scaffold for tissue engineering applications. The methods of tissue harvesting, processing, and sterilization all affect ECM ultrastructure, including surface characteristics.

The basement membrane is a form of extracellular matrix that is positioned between parenchymal cells and connective tissue, and its presence defines the spatial relationships among various populations of cells and the space that is occupied by connective and supported tissue types. In addition to its role of defining spatial relationships among various cell and tissue types, it plays a crucial role in the healing and remodeling process following tissue injury. A recent study featured bioengineered skin substitutes and evaluated micro-textured basal lamina analogs for their ability to modulate dermal-epidermal interactions. The basement membrane clearly represents an important consideration in the choice of an ideal bioscaffold for tissue engineering applications.

The objective of the present study was to determine the presence of a basement membrane complex in processed biologic scaffolds composed of porcine extracellular matrix derived from urinary bladder, small intestine, and liver. The effect of a surface basement membrane complex on the in vitro growth of human microvascular endothelial cells (HMEC) and NIH 3T3 fibroblasts was also examined.

**MATERIALS AND METHODS**

**Harvest and preparation of ECM scaffolds**

The ECM materials for this study were harvested and prepared using methods previously described. The urinary bladder, small intestine, and liver were harvested from market weight pigs of approximately 260 pounds immediately after sacrifice. Two different ECM constructs were prepared from urinary bladder and one each from small intestine and liver.

**Preparation of urinary bladder matrix.** Excess adipose tissue and collagenous connective tissue were removed from the outside of the bladder with scissors. The urothelial cells were removed by soaking the bladder tissue in 1.0 N saline. Intraluminal water pressure was used to expand and stretch the bladder to facilitate the removal of the muscle layer and the tunica submucosa. The apex of the bladder was removed using scissors. The bladder was then bisected on one side from the opening to the apical region, forming a rectangular-shaped sheet. The luminal side of the bladder was placed downward and the tunica serosa, tunica muscularis externa, tunica submucosa, and the muscularis mucosa were removed by mechanical delamination. The remaining tissue was then soaked in phosphate-buffered saline (PBS, pH 7.4) and represented urinary bladder matrix (UBM).

**Preparation of urinary bladder submucosa.** A second, separate, and morphologically distinct ECM scaffold was prepared from the porcine urinary bladder by removing different tissue layers than were removed in the preparation of UBM. The initial steps of bladder expansion and dissection to prepare a flattened rectangular sheet were the same as described above. Urinary bladder submucosa (UBS) was then prepared by mechanically removing the urothelial cells with the subjacent basement membrane, tunica propria, and muscularis mucosa from the luminal surface of the urinary bladder followed by mechanical delamination of tunica serosa and tunica muscularis externa from the abluminal surface. The remaining tissue consisted only of the tunica submucosa and was referred to as UBS.

**Preparation of small intestinal submucosa.** The preparation of porcine small intestinal submucosa (SIS) was previously described. The jejunum of the porcine intestine was isolated and the mesentery removed by sharp dissection. The lumen of the isolated segment of jejunum was then flushed with running tap water for 2–5 minutes. The intestine was then slit longitudinally and mechanically delaminated to remove the luminal portions of tunica mucosa, including the majority of the lamina propria and the entirety of the tunica muscularis externa and serosa. The remaining tunica submucosa and the basilar layers of the tunica mucosa, specifically the muscularis mucosa and stratum compactum, represented SIS.

**Preparation of liver stroma.** The four lobes of the porcine liver were separated using a scalpel and then trimmed to a uniform shape. The liver lobes were allowed to freeze completely at −80°C for at least 24 h. The frozen lobes were cut into 5 mm slices using a commercial meat slicer. Because the structure of liver tissue is complex and does not exhibit a layered structure, it was not possible to delaminate the desired tissue layers mechanically as in the intestinal and urinary bladder scaffolds. Instead, the liver slices were chemically treated to remove undesired content. The slices were placed in deionized (DI) water and shaken vigorously for 30 min at room temperature. This process was repeated with clean DI water for a total of three rinses. The slices were then massaged to hasten the lysis of hepatocytes and the removal of cell remnants, and then were placed in a container of 0.02% trypsin/0.05% EDTA (#15090-046, Gibco, Invitrogen, Carlsbad, CA) at 37°C for 1 h. The solution was decanted, the slices rinsed in DI water, and the massaging was repeated. The liver slices were then placed back on the shaker at room temperature for 1 h in 3% Triton X-100 (Spectrum Chemicals, New Brunswick,
NJ). Following the massaging and rinsing of the slices, the water rinsing and massaging process was repeated as necessary to lyse and remove any remaining cellular elements. The slices were then placed in 4% sodium deoxycholic acid (Spectrum) for 1 h on a shaker and then rinsed in water. The remaining decellularized connective tissue matrix was referred to as liver stroma (LS).14

**Disinfection, cell lysis, and terminal sterilization of scaffold materials.** Following removal of the appropriate tissue layers and bulk cellular contents from the three organs as described above, the remaining tissue was treated with a 0.1% peracetic acid/4% ethanol solution for 2 h at room temperature on a shaker.15 Traces of peracetic acid were removed and the pH was returned to approximately 7.4 by rinsing the ECM at room temperature, with shaking, in PBS one time, then in water twice, and then again in PBS one time. Each rinse lasted 15 min. The resulting decellularized and disinfected ECM scaffolds were terminally sterilized using 2.4 Mrad of E-beam irradiation. The methods used to sterilize the scaffolds in this study are approved by the FDA for the sterilization of medical devices.

**Immunohistochemistry**

Immunohistochemistry was performed using the Vectastain avidin-biotin peroxidase method (Elite ABC Kit, # PK-6100, Vector Laboratories, Burlingame, CA). Tissues were frozen in optimal cutting temperature solution (Tissue Tek, OCT, VWR International, West Chester, PA) and sectioned on a cryostat at 8 μm. The cryosections were thawed to room temperature, fixed in acetone for 5 min at room temperature, rinsed in PBS, treated with 0.3% hydrogen peroxide in methanol at room temperature for 30 min to quench endogenous peroxidase activity, rinsed in PBS, and then incubated in 1.5% serum (Vector) for 30 min in a 37°C humidified chamber to block binding of antibodies to nonspecific proteins. Serum for blocking was horse serum or rabbit serum, depending on the host species of the secondary antibody. Following incubation in blocking serum, sections were incubated in primary antibody in a 37°C humidified chamber for 30 min and then rinsed in PBS. Sections were then incubated in the appropriate secondary antibody for 30 min in a humidified 37°C chamber and again rinsed in PBS. Sections were then incubated in Vectastain ABC reagent for 30 min in a humidified 37°C chamber, rinsed 3 times in PBS for a total of five minutes, and incubated in 4% diaminobenzidine substrate solution at room temperature while being viewed on a microscope until slides showed the desired darkness of antigen labeling; finally slides were rinsed in DI water to stop the development of the diaminobenzidine substrate. Each PBS rinse in the protocol was performed in a humidified chamber at 37°C.

**FIG. 1.** Immunoperoxidase staining showed the presence of collagen IV in UBM (A), UBS (B), SIS (C), and LS (D). The intact urinary bladder tissue (E) served as a positive control. (× 40)

**FIG. 2.** Immunoperoxidase staining showed the presence of laminin in UBM (A), UBS (B), SIS (C), and LS (D). The intact urinary bladder tissue (E) served as a positive control. (× 40)
col was for 5 min at room temperature, replacing the PBS 3 times, with occasional agitation.

The primary antibodies used were rabbit anti-human laminin (#L-9393, Sigma, St. Louis, MO) at 1:100, rabbit anti-human collagen IV (# T59106R, BioDesign International, Saco, ME) at 1:200, and mouse anti-human collagen VII (a gift from Dr. Irene Leigh, The Royal Hospital of London), which was used undiluted. All primary antibodies cross-reacted with porcine tissue. The secondary antibodies used were biotinylated goat anti-rabbit IgG (#BA-1000, Vector) at 1:200 and biotinylated horse anti-mouse IgG (#BA-2001, Vector) at 1:200.

For collagen IV staining, formalin fixed paraffin embedded tissue samples were sectioned, dewaxed, and then treated as the OCT sections, beginning with the hydrogen peroxide step.

Scanning Electron Microscopy of ECM scaffolds—embedding and ultrastructural examination

The specimens that were previously fixed in cold 2.5% glutaraldehyde were rinsed in PBS, post-fixed in 1% Osmium Tetroxide (Electron Microscopy Sciences, Hatfield, PA) with 0.1% potassium ferricyanide (Fisher Scientific, Pittsburgh, PA) dehydrated through a graded series of ethanol, from 30 to 100%, and then critical point dried in a (critical point dryer, Emscope CPD, EMScope Lab, Ashford, Great Britain). Following critical point drying, the samples were attached to aluminum SEM specimen mounting stubs (Electron Microscopy Sciences) and then sputter coated with a gold palladium alloy (Sputter Coater 108 Auto, Cressington Scientific Instruments, Cranberry Township, PA). Following processing, samples were analyzed using a JEM 6330F (JEOL, Peabody, MA).

Cell culture

NIH 3T3 fibroblasts were cultivated in Dulbecco’s Modified Eagle Medium (DMEM, #12100-046, Gibco) containing 10% fetal bovine serum (HyClone, Logan, UT). HMECs (a gift from Francisco Candal, M.S., M.T. [ASCP], Center for Disease Control and Prevention, Atlanta, GA) were cultivated in MCDB-131 medium (#M-8537, Sigma, St. Louis, MO) containing 10% fetal bovine serum, 2 mM L-glutamine (#G7513, Sigma), 100 U/mL penicillin/100 μg/mL streptomycin (#P0781, Sigma), and 1 μg/mL hydrocortisone (#H0888, Sigma). Cells were grown at 37°C in 5% CO2/95% air and were harvested for seeding when they were approximately 70-80% confluent.

Cell seeding. All scaffolds were placed in 100 mm cell culture dishes, one sample per dish, and stainless-steel tissue culture rings with inner diameters of 1.5 cm2 were placed on the scaffolds. Pressure was applied to create a seal and to hold the sheets of ECM on the bottom of the Petri dish. The scaffolds were incubated in 1 mL of the appropriate media at 37°C and 5% CO2 for approximately 15 min prior to cell seeding, at which point the media was removed just before adding the cells. Cells were trypsinized, resuspended in appropriate growth media, counted, and placed on the ECMs within the tissue culture rings at concentrations of either 1 × 106 or 0.5 × 106 cells per ring. Appropriate growth media specific for the individual cell types was added to the cell culture dishes and dishes were incubated at 37°C and 5% CO2. After 4 h, two-thirds of the media was replaced with fresh media. After 4 h at 37°C and 5% CO2, the stainless steel rings were removed. The cells were grown on the scaffolds for 10 days at 37°C and 5% CO2. The same procedure was used for the culture of both the NIH 3T3 and HMEC cells.

Histologic analysis of cells on ECM scaffolds. After the 10-day growth period the ECM scaffolds were placed in 10% neutral buffered formalin (Surgipath Medical Industries, Richmond, IL), embedded in paraffin, and then sectioned and stained with H&E or Masson’s trichrome stains.

RESULTS

Immunohistochemistry

Positive staining for collagen IV (Fig. 1) was observed in the vasculature of all of the scaffold materials exam-
ined in this study. For the UBM scaffold material, collagen IV staining was also localized at the surface of the tissue in a contiguous pattern consistent with the presence of a basement membrane complex. There was dense positive staining for collagen IV also noted near the surface of SIS consistent with the stratum compactum layer of this ECM. Positive staining for laminin (Fig. 2) was localized to the vasculature of all scaffold materials examined and in a contiguous pattern at the surface of UBM material. UBM was the only ECM that stained positive for collagen VII (Fig. 3). Positive staining for collagen VII in UBM was localized with the collagen IV and laminin on the luminal surface of the tissue, consistent with the presence of an intact basement membrane complex.

**FIG. 4.** SEM showing the presence of a smooth surface ultrastructure, consistent with a basement membrane (arrow) and underlying connective tissue of the tunica propria (arrowhead) in UBM (A). SIS (B) and LS (C) do not contain a smooth surface ultrastructure. (× 400)

**FIG. 5.** H&E staining of cell-seeded EBM scaffolds. NIH 3T3 cells were not able to invade below the luminal surface of UBM (A), but were able to invade the abluminal side of UBM (B). NIH 3T3 had an invasive pattern of growth when seeded on SIS (C) and LS (D). Arrow indicates cells that have invaded the scaffolds. (× 40).
nearly 90% of the dry weight of most ECM. Many noglycans, and growth factors, with collagen comprising ECM include collagen, fibronectin, laminin, glycosaminoglycans, and growth factors. The components of the ECM, such as collagen, have been found to be highly conserved across species, thus mitigating adverse immune responses.

Basement membrane ECM and non-basement membrane ECM, usually of xenogeneic porcine, bovine, or allogeneic human origin, have been used as scaffolds for numerous tissue engineering applications. UBMB, a basement membrane containing ECM, has been used to successfully facilitate esophageal reconstruction in vivo. The basement membrane complex is a thin sheet-like structural component of ECM that provides a stable substratum for the growth of various epithelial cell populations in vivo. The basement membrane complex helps to regulate cell growth, differentiation, and migration during tissue development and reconstruction and is known to contain laminins, collagens IV and VII, nidogens, perlecan, agrin, and other macromolecules. The presence of an intact basement membrane complex in a biologic scaffold used for tissue engineering applications can facilitate and direct the growth of selected cell populations.

The results of the present study would suggest that if invasive cell growth into a biologic scaffold is desirable, then an ECM scaffold such as UBS, LS, or SIS should be used or the abluminal surface of UBM should be positioned in situ to contact tissue. Alternatively, if noninvasive growth is more desirable, such as with epithelial cells like keratinocytes, urothelium, or respiratory tract epithelium, then scaffolds with a surface basement membrane complex may be more desirable.

It has been shown that culturing cells on a surface containing a preserved basement membrane complex induces the differentiation of epidermal cells and promotes the growth of an epithelium with epidermal-like properties. It has also been shown that the presence of an epithelial cell population promotes proliferation, differentiation, migration, and organization of an underlying mesenchymal component during tissue remodeling. It is therefore logical that the use of scaffold materials that facilitate rapid epithelialization during remodeling will improve the chances of a favorable outcome. For example, squamous epithelial cell populations in skin provide a protective covering for the underlying tissue while providing signals that stimulate nerve cell growth and fibroblast migration and minimize fibroblast contraction of wounds. Endothelial cell coverage of vascular grafts tends to inhibit intimal hyperplasia during healing following vascular injury.

The organs from which the ECM scaffolds used in this study were produced were chosen for their diverse architectures and unique compositions. They represent organs that interact with various cell types, including epithelial and parenchymal cell populations. As tissue and organ engineering efforts become more sophisticated, it will be important to optimize cell and scaffold compatibility. The present study shows the similarities and differences in the surface characteristics of ECM scaffolds derived from different tissue sources and how those differences can modulate cell growth patterns.
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