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ORIGINAL ARTICLE

Activation of human mononuclear cells by porcine biologic meshes in vitro

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Abstract
Introduction While porcine-based biologic meshes are increasingly used for hernia repair, little data exist on tissue responses to such products. Host foreign body reaction, local inflammation, and wound healing are principally controlled by monocytes/macrophages (M/MØs). Exaggerated activation of M/MØs may deleteriously influence mesh integration and remodeling. We hypothesized that common porcine meshes induce the differential activation of M/MØs in vitro.
Materials and methods Samples of four acellular porcine-derived meshes, CollaMend™ (CM; C.R. Bard/Davol), Permacol™ (PC; TSL/Covidien), Strattice™ (ST; LifeCell), and Surgisis® (SS; Cook Biotech), were exposed to mononuclear cells derived from the peripheral blood of six healthy subjects. Following a 7-day incubation period, supernatants were assayed for interleukin-1beta (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8), and vascular endothelial growth factor (VEGF) using a multiplex bead-based immunoassay system. The four groups were compared using analysis of variance (ANOVA) and Student's *t*-test.
Results Each mesh type induced differential mononuclear cell activation in vitro. The mean IL-1β expressions for CM (7,195 pg/ml) and PC (4,215 pg/ml) were significantly higher compared to ST and SS (123 and 998 pg/ml, respectively; $P < 0.05$). Similar trends were also seen for IL-6 (range 445–70,729 pg/ml), IL-8 (range 11,640–1,045,938 pg/ml), and VEGF (range 686–7,133 pg/ml).
Conclusion For the first time, we demonstrated that porcine meshes induce M/MØ activation in vitro. CM and PC (chemically crosslinked dermis) induced significantly higher cytokine expression compared to ST (non-crosslinked dermis) and SS (small intestine submucosa). These differences are likely related to proprietary processing methods and/or the extent of collagen crosslinking. Further understanding of immunologic effects of porcine-derived biologic meshes will not only allow for a comparison between existing products, but it may also lead to mesh modifications and improvement of their clinical performance.

Keywords Biologic mesh · Porcine · Dermis · Small intestine submucosa · Peripheral blood mononuclear cells (PBMCs) · Monocytes · Cytokine · Interleukin-1beta (IL-1β) · Interleukin-6 (IL-6) · Interleukin-8 (IL-8) · Vascular endothelial cell growth factor (VEGF) · In vitro

Introduction

Successful repair for many, if not most, herniorrhaphies requires the use of prosthetic implants for reinforcement. For much of the last 50 years, prosthetic implants (meshes) have been constructed of synthetic materials such as

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polyethylene, polypropylene, polyester, expanded polytetrafluoroethylene, or combinations of various materials [1–3]. While synthetic meshes are the most common mesh type used, they are contraindicated in infected or potentially contaminated fields [2, 4–6]. As a result, biologic meshes (BMs) have been developed to provide adequate tissue reinforcement when synthetic materials are contraindicated. While BMs are beneficial for hernia repair in infected or potentially infected surgical fields, failure rates have been shown to be approximately 30% following implantation [7–10].

Following the implantation of biomaterials, the body initiates powerful inflammatory and reparative processes. These tissue responses can either aid in the integration of the implanted BM via normal wound healing mechanisms or they can induce a disproportionate inflammatory response. Such exaggerated inflammatory responses may result in excessive fibrosis, with resultant scarring and encapsulation or rapid mesh degradation, leading to graft weakening and hernia repair failure [11]. The balance between appropriate wound healing and detrimental sequelae is largely controlled by cytokines, growth factors, and other chemical signaling molecules, with monocytes/macrophages (M/MØs) playing pivotal roles [12–15]. Key M/MØ-derived cytokines and growth factors, including interleukin-1beta (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8), and vascular endothelial growth factor (VEGF), play important roles in wound healing and mesh integration in vivo and serve as useful markers of M/MØ activation in vitro [15–18]. Although we have already demonstrated the differential activation of M/MØs in response to human dermis-derived BMs [19, 20], host reactions to xenogenic implants remain unknown. In this study, we aimed to characterize the immunogenic potentials of porcine-derived biomaterials.

Materials and methods

Surgical meshes and mesh preparation

Four different porcine-derived biologic meshes were utilized in this study: CollaMend™ (CM; C.R. Bard/Davol Inc., Cranston, RI, USA), Permacol™ (PC; Covidien, Norwalk, CT, USA), Strattice™ (ST; LifeCell Corp., Branchburg, NJ, USA), and Surgisis® (SS; Cook Biotech Inc., West Lafayette, IN, USA) (see Table 3). CM and PC are chemically crosslinked porcine dermal matrices, ST is a non-crosslinked porcine dermal matrix, and SS is derived from porcine small intestine submucosa. Using sterile technique, 5-mm circular mesh samples were prepared using a sterilized paper drill and immediately placed in sterile 96-well plates as described below.

Blood product isolation

Human peripheral blood mononuclear cells (PBMCs) were isolated from blood and used as an enriched source of M/MØs. After obtaining institutional review board approval and signed informed consent, PBMCs were isolated and prepared from six healthy volunteers. Approximately 60 ml of peripherally drawn blood was anticoagulated with 200 µl of heparin sodium. PBMCs were obtained by standard differential centrifugation of anticoagulated whole blood utilizing Histopaque®-1077 (Sigma–Aldrich, St. Louis, MO, USA) as a density gradient agent. After removal of the PBMC layer, the PBMCs were washed three times with sterile normal saline (0.9%). The cell number for each PBMC preparation was determined using standard microscopic quantitation using an AO Bright-Line hemocytometer (Hausser Scientific, Horsham, PA, USA). The resulting cells were resuspended in approximately 10–30 ml RPMI 1640 tissue culture medium in order to achieve a final concentration of 8.0×10^6 cells/ml of solution. Subsequently, 125 µl of this solution was added to the in vitro cell activation platform as described below.

In vitro cell activation platform

Standard sterile 96-well tissue culture-treated polystyrene (TCPS) plates (Corning®, Corning, NY, USA) were used for our in vitro cell activation platform. Each well received its components (mesh ± cells from one volunteer and tissue culture media) in a sterile fashion in a laminar flow hood. Stock tissue culture media (TCM) was composed of RPMI 1640 medium (Sigma–Aldrich, St. Louis, MO, USA), 10% Fetal Bovine Serum (Gemini Bio-Products, West Sacramento, CA, USA), 1% Penicillin–Streptomycin, and 0.2% Amphotericin B (Invitrogen Corp., Carlsbad, CA, USA).

Four 5-mm circular pieces of each mesh type were placed in their designated wells ($n = 24$ per mesh product). Next, 125 µl of leukocyte–TCM solution was placed into the designated wells (concentration of 1.0×10^6 cells per well, after dilution with TCM based off the initial cell count). Control wells did not receive any mesh pieces, and likewise received 125 µl of leukocyte–TCM solution. The resulting tissue culture plates were left undisturbed for 20 min at 37°C to allow the cells to settle on the mesh or well bottom, thus, ensuring initial adhesion without disruption. Next, an additional 125 µl of TCM was then placed inside the wells to reach a total of 250 µl of solution in each well. While the negative control well received an additional 125 µl of TCM, the positive control well received 125 µl of a TCM–lipopolysaccharide (LPS) solution (concentration: 100 ng LPS/ml TCM). The resulting 96-well plates were incubated at 37°C with 5% CO_2 for 7 days.

On experimental day 7, the resulting culture supernatants were transferred to a new 96-well plate and centrifuged at 3,000 RPM for 3 min in order to create a PBMC pellet at the bottom of each V-shaped well. This step helped limit cellular contamination of the supernatants. The resulting samples were then transferred out of the centrifuged plate, separated into multiple aliquots, and preserved at −70°C for ease of further testing.

Cytokine expression

Cytokine concentrations of IL-1β, IL-6, IL-8, and VEGF in the various culture supernatants were determined by utilizing a bead-based multiplex immunoassay system (Bio-Plex, Bio-Rad, Hercules, CA, USA). Because of the highly elevated levels of IL-8 in pilot experiments, samples undergoing IL-8 analysis were diluted 1:50 in TCM, whereas samples undergoing IL-1β, IL-6, and VEGF analysis were diluted 1:5 in TCM.

All standards, cytokine beads, detection antibodies, and reagents utilized in this study were manufactured by Bio-Rad. In brief, premixed standards were reconstituted with TCM. Ninety-six-well filter plates (MultiScreen HTS, Millipore Corp., Billerica, MA, USA) were prewetted with Assay Buffer A (assay buffer) and vacuum-filtered. Anti-cytokine bead solution (IL-1β, IL-6, IL-8, and VEGF) was prepared with assay buffer, added to each well, then vacuum-filter-washed twice with Wash Buffer A (wash buffer). Standards and culture supernatant samples were added to corresponding wells and allowed to incubate on a plate shaker in darkness for 30 min. Following this and subsequent incubations, the plate was vacuum-filtered followed by three vacuum-filter washings with wash buffer. Detection antibody solution (IL-1β, IL-6, IL-8, and VEGF) was prepared with Detection Antibody Diluent, added to each well, and incubated on a plate shaker in darkness for 30 min. Streptavidin–phycoerythrin solution was prepared with assay buffer, added to each well, and incubated on a plate shaker in darkness for 10 min. After final washings, the contents of each well were resuspended with assay buffer. Each plate was run through the Bio-Plex array reader, where peak fluorescence was measured, along with the corresponding specific cytokine bead (IL-1β, IL-6, IL-8, or VEGF). Finally, cytokine levels were quantitated for each culture supernatant sample using Bio-Plex Manager software and expressed as picograms (pg) of cytokine per milliliter (ml) of culture supernatant.

Statistical analysis

IL-1β, IL-6, IL-8, and VEGF levels for each mesh group were compared using analysis of variance (ANOVA) and Student's *t*-test, with $P < 0.05$ being considered as statistically significant.

Results

Cytokine data were obtained from assayed culture supernatants after seven experimental days. As seen in Fig. 1, the cytokine expression varied among the four different porcine mesh products studied. Both CM and PC induced significantly greater quantities of all four cytokines (IL-1β, IL-6, IL-8, and VEGF) compared to SS and ST ($P < 0.05$–0.001). Overall, CM was the greatest cytokine inducer of the four porcine meshes studied, while ST was the lowest (Table 1). While there was no significant difference between CM and PC for IL-1β, IL-8, and VEGF, there was statistical significance between the two for IL-6 (Table 2).

For IL-1β, CM stimulated over 1.5 times the amount of cytokine production compared to PC (7,195 [range 1,977–22,250] versus 4,215 [range 1,055–15,070] pg/ml, respectively), but this was not statistically significant ($P = 0.14$). However, CM induced IL-1β production over seven-fold more than SS (998 [range 66–4,913] pg/ml; $P < 0.001$) and almost 60 times more than the lowest inducer, ST (123 [range 19–433] pg/ml; $P < 0.001$). Similar trends were seen for IL-8 and VEGF (Fig. 1).

With regards to IL-6, however, CM induced a significantly greater amount of cytokine production (70,729 [range 38,293–105,731] pg/ml) compared to PC, SS, and ST (38,777 [range 7,247–69,731]; 13,796 [range 405–63,751]; 445 [range 20–2,340] pg/ml, respectively; $P < 0.001$).

Discussion

For many decades, implanted meshes have been used to provide structural support for the repair of hernias. The wide implementation of mesh repairs has allowed the development of effective tension-free repairs [2, 3, 21–23]. While an essential component of a hernia surgeon's toolbox, the use of synthetic meshes is limited in infected or potentially contaminated surgical fields. With the advent of human and animal-derived tissues for biologic prosthetic meshes, surgeons have the ability to use biomaterials for the reinforcement of repairs in cases where synthetic meshes are contraindicated [2, 4–6].

Biologic meshes perform by serving as tissue reinforcement, followed by gradual resorption, remodeling, and replacement. Thus, besides adding initial strength, one of their primary functions is to act as a natural scaffold through which the body's reparative processes ultimately replace the mesh with native tissue. Proper mesh integration

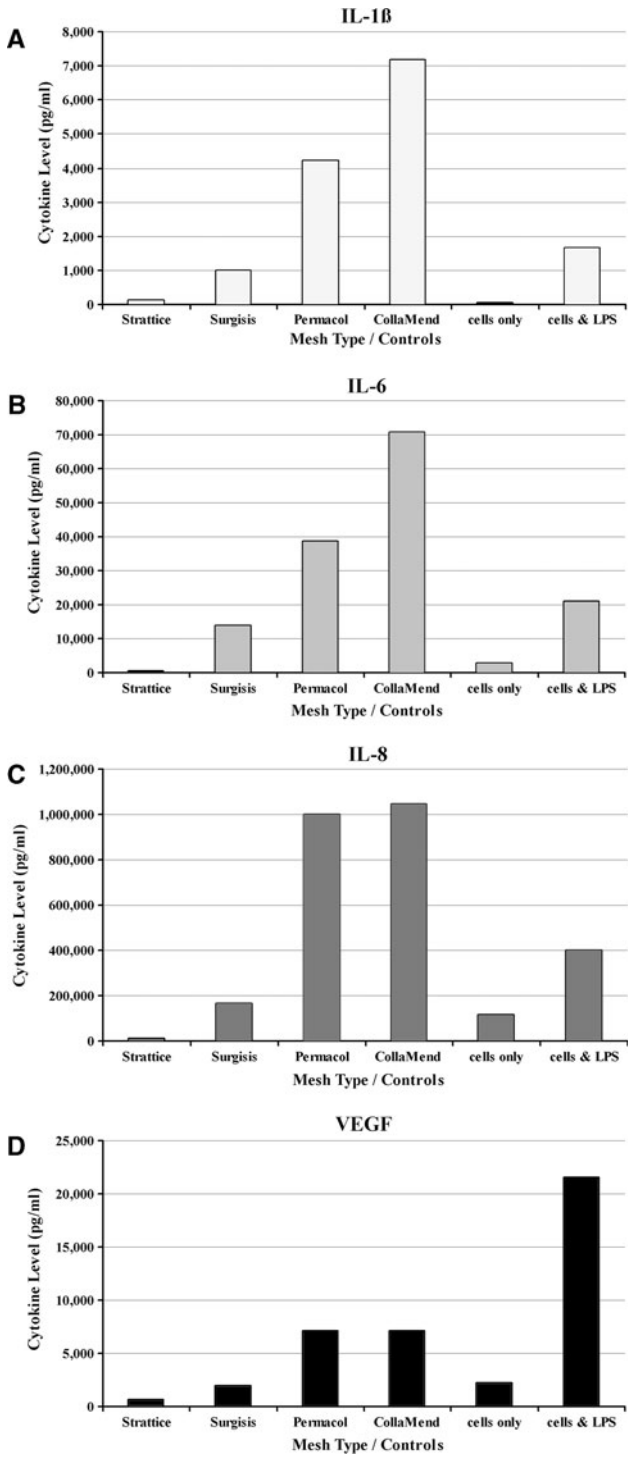

Fig. 1 Mesh-induced cytokine expression from day 7 culture supernatants. The values represent the mean cytokine production for each mesh and are expressed as pg/ml. **a** Interleukin-1beta (IL-1β). **b** Interleukin-6 (IL-6). **c** Interleukin-8 (IL-8). **d** Vascular endothelial growth factor (VEGF). Negative controls (cells only) and positive controls (cells and lipopolysaccharide [LPS]) were not exposed to mesh. Cytokine levels were obtained using Bio-Plex multiplex immunoassay kits. See Table 1 for detailed cytokine expression numbers

depends on cellular and fibrovascular ingrowth, followed by tissue remodeling [4, 24]. These cellular and tissue processes are a result of both acute and chronic inflammation, and are necessary components of proper wound healing. However, excessive inflammation may lead to rapid degradation or encapsulation of the implant [25–27]. At the heart of these inflammatory processes are monocytes/macrophages (M/MØs), which are critical mediators of inflammation, and, thus, of both wound healing and mesh integration. Our previous study has shown the utilization of PBMCs as a valuable in vitro model for the assessment of cellular reactions to implanted human dermis-derived BMs [20]. The current study quantified the levels of four key regulatory cytokines, IL-1β, IL-6, IL-8, and VEGF, in response to various porcine-derived meshes.

For each of the cytokines tested, CollaMend™ consistently induced a higher quantity of M/MØ activation (Fig. 1; Table 1). This was nearly matched by Permacol™ in all but one cytokine (IL-6). These two crosslinked BMs are contrasted by the lowest inducer, Strattice™, a non-crosslinked BM. Such elevated numbers in the CollaMend™ and Permacol™ groups are likely due to their high levels of chemically crosslinked collagen molecules. The chief proposed benefit of collagen crosslinking is to mechanically strengthen the BM and delay degradation of the biologic scaffold, thus, prolonging the lifespan of the tissue reinforcement. Over the course of 30 days to 6 months, non-crosslinked BMs undergo remodeling and are typically fully replaced with native tissue by 4–6 months [27–29]. Intentionally crosslinked dermis products, on the other hand, may take upwards of 12 months or more for complete remodeling [2, 27]. Such products are crosslinked using hexamethylene diisocyanate, carbodiimide, glutaraldehyde, or photo-oxidizing agents in order to add strength and slow degradation times [30, 31]. Interestingly, Gouk et al. suggest that even non-intentionally crosslinked scaffolds may undergo structural changes, including molecular crosslinking, following sterilization via gamma irradiation [32].

However, collagen crosslinking appears to be deleterious from an immunologic standpoint. Prolonged degradation may lead to enhanced immunogenicity, as a competent immune system may perceive the resilient BM as foreign. Over the last several years, two subpopulations of macrophages have been described: M1, which favors a pro-inflammatory state, and M2, which favors wound healing and tissue remodeling [12, 13, 33–36]. Badylak et al. noted that a carbodiimide-crosslinked product was associated with an M1 macrophage-driven response, resulting in chronic inflammation and scar formation versus an M2 macrophage-driven response, resulting in organized tissue

Table 1 Cytokine expression levels from day 7 culture supernatants

Cytokine	Mesh type			
	CollaMend™	Permacol™	Strattice™	Surgisis®
IL-1β	7,195	4,215	123	998
IL-6	70,729	38,777	445	13,796
IL-8	1,045,938	1,000,051	11,640	166,419
VEGF	7,133	7,128	686	1,993

Values are listed as mean cytokine concentrations for each mesh and expressed as picograms of cytokine per milliliter of culture supernatant (pg/ml). Cytokine levels were obtained using Bio-Plex multiplex immunoassay kits

IL interleukin; *LPS* lipopolysaccharide

Table 2 Statistical analysis of mesh-induced cytokine expression from peripheral blood mononuclear cells (PBMCs)

	CollaMend™	*Permacol*™	*Strattice*™	*Surgisis*®
IL-1β				
CollaMend™				
Permacol™	NS			
Strattice™	< 0.001	< 0.001		
Surgisis®	< 0.001	< 0.001	< 0.05	
IL-6				
CollaMend™				
Permacol™	< 0.001			
Strattice™	< 0.001	< 0.001		
Surgisis®	< 0.001	< 0.001	< 0.001	
IL-8				
CollaMend™				
Permacol™	NS			
Strattice™	< 0.001	< 0.001		
Surgisis®	< 0.001	< 0.001	< 0.001	
VEGF				
CollaMend™				
Permacol™	NS			
Strattice™	< 0.001	< 0.001		
Surgisis®	< 0.001	< 0.001	< 0.05	

Data represent *P*-values from two-tailed Student's *t*-test analyses

IL interleukin; *NS* no statistically significant difference

remodeling at 16 weeks in a similar but non-crosslinked product [12]. In addition, incomplete removal of chemical crosslinking agents could result in cytotoxicity from residues leaching from the BM, which may induce an elevated cellular response [30, 31]. While crosslinking extends the life of the collagen scaffold, we found that crosslinked meshes induce greater pro-inflammatory responses from macrophages. Our findings, similar to Schutte et al. [15], are highly suggestive that crosslinked meshes induce higher degrees of chronic inflammation at sites of mesh implantation.

Collagen crosslinking of biomaterials is not the only explanation for differences between the porcine BMs. As we have previously demonstrated [20], a wide range of cytokine induction was seen between three similar human dermis-derived BMs where no intentional crosslinking was implemented by the manufacturers. Additional disparities between mesh products are likely a result of the different methods of processing, decellularization, and sterilization that each manufacturer uses (Table 3). Given the various solvents and methods used by each manufacturer for processing and sterilization, the long-term effects of these proprietary methods have the potential to influence the tissue. It is likely that the processing alters the collagen matrix, inducing lasting biochemical and biomolecular changes [11, 37, 38]. These matrix alterations, in turn, may influence foreign recognition and antigen presentation. The resulting enhancement of the foreign body response and immunogenic potential may, consequently, undercut the mesh's performance in the clinical setting.

While difficult to compare because of dissimilarity of the tissue type, Surgisis® did induce a response greater than the non-crosslinked porcine dermis, Strattice™. Surgisis® differs from the other three BMs in that it is comprised of a laminate of porcine small intestine submucosa instead of being derived from a dermal source. Multiple studies have demonstrated the safety and efficacy of Surgisis® within contaminated as well as clean surgical fields, and it is at least equivalent to other porcine-derived dermal products clinically [39–42] and in animal models [43, 44]. From our data, it appears to have immunogenic potential that supersedes non-crosslinked porcine dermis, but well below that of crosslinked products.

Whether excessive M/MØ activation in vitro corresponds to adverse in vivo performance remains unknown at this time. Although clinical correlations are not well established comparing BMs, in vivo studies by Xu et al. and Connor et al. demonstrate advantageous cellular responses and remodeling to non-crosslinked porcine dermis [45, 46]. BMs that display elevated cytokine production may allow for enhanced mesh integration via fibroblast proliferation and stimulation from high levels of macrophage-produced IL-1β. Conversely, the chronic induction of IL-1β may cause exaggerated matrix metalloproteinases activity, which would lead to accelerated degradation of the mesh [17]. Ultimately, these points may be further counterbalanced by chemically induced collagen crosslinking causing chronic foreign body reaction with resultant fibrosis and encapsulation. Overall, we believe that excessive cytokine production seen in our experiments is likely predictive of an exaggerated immunogenic potential of a crosslinked mesh, with subsequent inferior performance in the clinical setting.

Conclusion

We have demonstrated that profound immunogenic differences exist between porcine biologic mesh products commonly used

Table 3 Characteristics and methods of decellularization, processing, and sterilization for porcine-derived biologic meshes

	CollaMend™	Permacol™	Strattice™	Surgisis®
Source	Porcine dermis	Porcine dermis	Porcine dermis	Porcine small intestine submucosa
Intentional crosslinking	Yes (via EDC)	Yes (via HMDI)	No	No
Decellularization and processing	NaSulfate, lime, NaHypochlorite, water, alcohol, NaCl, HCl, H_2O_2, phosphate buffer, detergent, lyophilization	Proprietary enzymatic process	Non-denaturing detergents, alpha-gal removal	Peracetic acid, hypotonic rinses, multiple sheet layering/compression
Sterilization	Ethylene oxide	Gamma radiation	Low-dose electron-beam radiation	Ethylene oxide

EDC 1-ethyl-(3-dimethylaminopropyl) carbodiimide hydrochloride; *HMDI* hexamethylene diisocyanate; *alpha-gal* alpha-galactosidase

in hernia repairs. Crosslinked porcine dermis meshes are strong inducers of key regulatory cytokines compared to non-crosslinked porcine dermis or porcine small intestine submucosa. While crosslinking is a distinguishing factor between mesh types, the various proprietary decellularization, processing, and sterilization techniques employed by manufacturers may also strongly influence macrophage responses to biologic meshes. We believe that excessive cytokine production seen in our experiments is indicative of higher immunogenic potential of crosslinked biomaterials and is likely associated with a decreased performance of a given mesh in vivo. Further understanding of the immunologic effects of porcine-derived biologic meshes will not only allow for comparison between existing products, but may also lead to mesh modifications and improvement of their clinical performance.

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Conflict of interest statement Y.W.N. has received consulting and/or speaking fees from LifeCell Corp., C.R. Bard Inc., and Covidien. The other co-authors have nothing to disclose.

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