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Effects of sterilization on an extracellular matrix scaffold: Part II. Bioactivity and matrix interaction

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Abstract Small intestinal submucosa (SIS) has been successfully used to treat a variety of damaged or diseased tissues in human patients. As a biologic scaffold, SIS stimulates repair of damaged or diseased tissues and organs with tissue that is similar in structure and function to the material it was meant to replace. To meet clinical safety requirements, biologic materials from animal tissues must undergo processing treatments to minimize host immune response and to eliminate the possibility of disease transmission. The effect of peracetic acid disinfection, lyophilization, and ethylene oxide sterilization on the *in vitro* bioactivity of the processed SIS was therefore examined in murine fibroblasts and pheochromocytoma (PC12) cells. Specifically, the ability of processed SIS to support fibroblast attachment, to stimulate PC12 cell differentiation, and to upregulate fibroblast VEGF secretion was examined. Fibroblasts attach to the sterilized SIS, remain viable, and more than double their secretion of VEGF as a result of interacting with the SIS matrix components. Additionally, PC12 cells exhibit increased neurite outgrowth following stimulation by SIS matrix proteins versus controls. We conclude that a biologic scaffold can be prepared for human use and still retain significant bioactivity.

1 Introduction

Retention of the bioactivity within collagen-based biomaterials is essential if the material is to act as more than just an inert scaffold. Small intestinal submucosa (SIS), a natural biologic scaffold material, has been used in numerous surgical applications [1-4] and has also been reported to stimulate the repair of chronic venous ulcers and other non-healing wounds [5]. In its natural form, SIS consists of several types of collagens [6], with smaller amounts of glycosaminoglycans [7], glycoproteins [8], and growth factors [9,10].

Retention of the non-collagenous matrix components in their natural state is essential to the maintenance of scaffold bioactivity, but biologic scaffolds used to stimulate wound healing undergo a variety of potentially damaging processing treatments designed to minimize host immune response and reduce endotoxin and bioburden levels to insure product safety. These processes often include processing steps that subject the scaffold to acids, enzymes, or other chemical treatments that can denature growth factors and inhibit the ability of structural proteins to interact with the recipient's cells. For example, crosslinking agents such as glutaraldehyde or hexamethylene diisocyanate (HMDI) are often used during the processing of these biologic scaffolds to increase implant strength and reduce their antigenicity, but these compounds also reduce the ability of cells to interact with the treated material [11], to cause cutaneous sensitization [12], and to lead to calcification [13]. Enzymes such as trypsin, amylase, and neuramidase are used to reduce rejection potential of the scaffold, but they remove potentially valuable globular proteins, such as growth factors, that contribute to the bioactivity of the matrix. Oxidizing agents such as hydrogen peroxide or peracetic acid (PAA) are often used as disinfectants during processing, but have the undesirable effects of oxidizing the matrix and reducing the structural integrity of the collagen fibers. Oxidative destruction of glycosaminoglycans fractures them, impairs their ability to interact with growth factors [14], and subsequently increases the susceptibility of growth factors to denaturation.

Even though these processing steps are destructive to scaffold bioactivity, studies have suggested that the composition

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and activity of growth factors bound in the matrix can be retained if individual protein components are sequestered naturally [15] or in combination with other carriers [16-19]. Some growth factors, such as FGF-2, are inherently more stable than others (such as VEGF) and are able to retain some of their activity under acidic and oxidizing conditions even in the absence of stabilizing agents [20, 21].

The purposes of this study were to determine if processed SIS was able: (1) to support fibroblast attachment and proliferation; (2) to stimulate the differentiation of rat PC12 pheochromocytoma cells; and (3) to stimulate fibroblasts to secrete VEGF, a potential mechanism for promoting angiogenesis and wound healing *in vivo*.

2 Materials and methods

2.1 Reagents

PAA was obtained from FMC (Chicago, IL). Human recombinant basic fibroblast growth factor (FGF-2) was purchased from Boehringer Mannheim (Indianapolis, IN) and used at 10 ng/ml. Mouse nerve growth factor (NGF) was from Roche Diagnostics (Indianapolis, IN) and used at 50 ng/ml. Neutralizing anti-human FGF-2 and neutralizing anti-rat NGF antibodies were purchased from R&D Systems. The anti-FGF-2 was used at 40 μg/ml and the anti-NGF was used at 150 ng/ml. Human FGF-2 and mouse VEGF multiplex microsphere analyte sets (Flourokine MAP system) were purchased from R&D Systems and used with a mouse base kit also from R&D Systems. AlamarBlue was from Biosource International (Camarillo, CA). Unless otherwise noted, all other chemicals and cell culture reagents were from Sigma (St. Louis, MO).

2.2 Cells

Swiss 3T3 fibroblasts were purchased from ATCC and cultured as directed in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4 mM Lglutamine and adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 1.0 mM sodium pyruvate, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% bovine calf serum.

Rat Pheochromocytoma (PC12) cells were purchased from ATCC (Manassas, VA) and maintained as directed in RPMI 1640 cell culture medium (Invitrogen Corporation, Carlsbad, CA) supplemented with 5% fetal bovine serum (FBS), 10% horse serum (HS), 100 units/ml penicillin, and 100 μg/ml streptomycin.

NIH 3T3 fibroblasts were purchased from ATCC and cultured as directed in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4 mM Lglutamine and adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 1.0 mM sodium pyruvate, 100 units/ml penicillin, 100 μg/ml streptomycin, and 5% fetal bovine serum. Cells from limited passage numbers were used for all experiments.

2.3 Procurement and Processing of Small Intestine Submucosa (SIS)

Sections of porcine jejunum were subjected to treatment with a dilute concentration of PAA in water for two hours at room temperature, as described elsewhere [22]. Following exposure to the disinfectant, the SIS was prepared as previously described by mechanical delamination [23]. The prepared SIS (SIS_{PAA}) was either stored at 4°C in sterile containers prior to further evaluation or frozen at −80°C and lyophilized overnight (SIS_{LYO}) to produce a dry sheet. Following lyophilization, the SIS_{LYO} was packaged into gas permeable pouches and sterilized with ethylene oxide (EO) gas. EO-sterilized SIS (SIS_{EO}) was stored sterile at room temperature prior to evaluation.

2.4 Cell attachment assay

SIS_{PAA}, SIS_{LYO}, or SIS_{EO} were placed into polypropylene cell culture inserts and evaluated in triplicate for their ability to support the attachment and viability of Swiss 3T3 fibroblasts. Three individual assays were performed. The SIS samples were equilibrated in PBS, pH 7.4 for 30 minutes at 37°C and 5% CO_2. The PBS was aspirated and complete DMEM containing 0.5% bovine serum albumin (attachment medium) was added to the wells. Fibroblasts were harvested from their tissue culture flasks, counted, and suspended in attachment medium at a concentration of 125,000 cells/ml. A total of 50,000 cells were added to each SIS substrate. Samples were allowed to incubate at 37°C and 5% CO_2 for 60 minutes for attachment to occur. After 60 minutes, the SIS substrates were inverted and centrifuged at 250 x *g* for 5 minutes. The attachment medium was carefully aspirated so the cells that had attached to the SIS would remain undisturbed. The SIS substrates were inverted to their original orientation and treated with 10% alamarBlue solution at 37°C and 5% CO_2 for 18 h. Following incubation, 100 μl samples of alamarBlue solution from each of the substrate wells were pipetted into a 96-well plate (Corning Costar, Cambridge, MA).

Because alamarBlue is a metabolic dye that is reduced linearly with cell metabolic activity, a ratio of the reduced form to the oxidized form can be used to measure cell viability. Absorbance was measured and 570 nm (reduced form) and 600 nm (oxidized form). The percent of alamarBlue reduction in each SIS well was measured and compared against control wells in order to correlate to cell number. The results presented represent the percentage of seeded cells that attached to the substrate for 60 minutes and remained metabolically

active during the 18-hour incubation period. Pairwise comparisons were made between groups using a 2-tailed *t*-test.

2.5 PC12 differentiation assay

Conditioned cell culture medium was prepared from SIS_{EO}. Briefly, serum-free RPMI 1640 cell culture medium was incubated with the processed SIS at a concentration of 1 g (wet weight) / 7.5 ml of medium for 48 h at 37°C. The SIS_{EO} was removed and the medium was filtered through a 20 μm filter to remove any particulate matter. The SIS_{EO} conditioned cell culture medium was supplemented with 5% FBS, 10% HS, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Twelve-well culture plates were coated overnight at 37°C with 1 ml of 0.2 mg/ml type I rat tail collagen (BD Biosciences, Bedford, MA). The collagen solution was aspirated and the wells were washed once with phosphate buffered saline (PBS). PC12 cells were harvested, counted using a hemacytometer, and seeded in 1 ml of growth medium at a density of 20,000 cells per well. Growth factors and/or antibodies were then added at the aforementioned concentrations. All conditions were evaluated in triplicate.

The cells were cultured for 48 h at 37°C before being visually examined for differentiation. Cells were considered differentiated if they exhibited at least one neurite-like extension at least twice the diameter of the cell body. Three separate, 20X fields were examined per well; the percentage of cells that met the criteria for differentiation was recorded. Statistical significance was set at $p < 0.05$ and evaluated using a two-tailed *t*-test.

2.6 Fibroblast activity assay

Conditioned cell culture medium was prepared from SIS_{EO}. Briefly, serum-free DMEM cell culture medium was incubated with each ECM at a concentration of 1 g (wet weight) / 7.5 ml of medium for 48 h at 37°C. The ECM was removed and the medium was filtered through a 0.2-μm filter. The conditioned cell culture medium was supplemented with 100 units/ml penicillin, and 100 μg/ml streptomycin prior to use.

NIH 3T3 fibroblasts at 75% confluency were harvested from their tissue culture flasks, counted, and resuspended in complete DMEM at a concentration of 50,000 cells / well in a 24-well plate and allowed to recover overnight. The media was aspirated and replaced with serum-free DMEM for 24 h prior to the assay. Cells were cultured under one of three conditions: 1) serum-free DMEM; 2) conditioned, serum-free, cell culture medium; or 3) conditioned, serum-free, cell culture medium containing anti-FGF-2 at 40 μg/ml to neutralize FGF-2 activity in the wells. Cells were incubated at 37°C and 5% CO_2 for 24 h. During the growth period, 200 μl aliquots of media were sampled at 0, 1, 4, 8, 16, and 24 h. At the end of 24 h, the level of VEGF in the collected cell culture supernatants was measured.

2.7 VEGF quantitation

VEGF and FGF-2 in the cell culture supernatants were measured using multiplex technology. Briefly, VEGF standards diluted in assay buffer and collected samples (200 μl) were added to wells of a pre-wetted 96-well filter plate. The samples were incubated with 50 μl of the antibody-coupled microspheres (anti-VEGF and anti-FGF-2 coupled beads) at 25°C for 3 hr on a plate shaker set to 500 rpm. Wells were washed 3 times with 100 μl/wash of buffer, and 50 μl of freshly diluted secondary/detection antibody was added. The assay plate was incubated at 25°C with constant shaking for an additional 60 min. The wash step was repeated, 50 μl of streptavidin-PE was added to the wells, and the incubation was continued under constant shaking for an additional 30 minutes. The wash was repeated a final time, and the microspheres were resuspended in 100 μl assay buffer for 2 minutes with constant shaking. Fifty microliter samples were then analyzed for median relative fluorescence (RFU) on the Luminex 100 (Bio-Plex Suspension Array System, Bio-Rad Laboratories) according to the manufacturer's instructions, with settings set to detect 50 events/bead and doublet discriminator gates set at 4335 and 7990.

VEGF in the samples was quantitated using a cubic spline curve fit. Pairwise comparisons were made between groups using a 2-tailed *t*-test. Because no standard curve was created for FGF-2, FGF-2 levels in the supernatants could not be calculated, but the median RFU was evaluated for changes in levels over time. Moreover, the absence of fluorescence on the FGF-2 beads in the wells treated with anti-FGF-2 was verified to validate the neutralizing activity of the antigen-antibody complex.

3 Results

3.1 Cell attachment is retained following sterilization

Because rapid interaction between the cells and the ECM is a desirable characteristic of tissue engineered matrices, the effects of lyophilization and sterilization on host cell attachment and short term viability were evaluated. SIS_{PAA}, SIS_{LYO}, or SIS_{EO} were placed into polypropylene cell culture inserts and evaluated for their ability to support the attachment of Swiss 3T3 fibroblasts.

The alamarBlue assay for attachment and viability of fibroblasts indicated that 66% of the seeded cells attached to the SIS_{PAA} during the 1-hour incubation period and remained viable for an additional 18 h. Additionally, SIS_{LYO} supported the viability and attachment of 57% of the seeded cells. SIS_{EO}

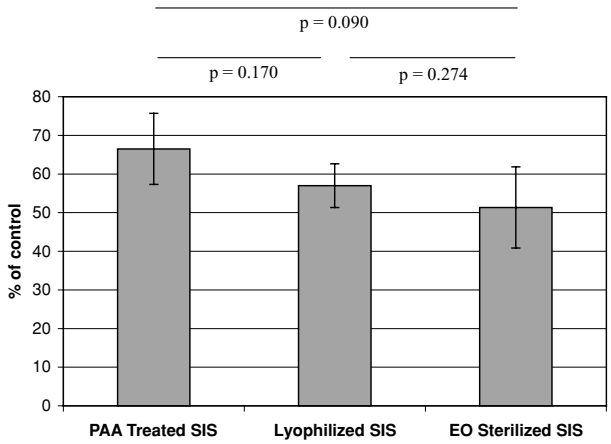

Fig. 1 Cell attachment and viability on SIS at different levels of processing indicates that 3T3 fibroblasts are able to attach and remain viable on lyophilized and EO sterilized SIS. Groups are not statistically different using paired Student's *t*-tests

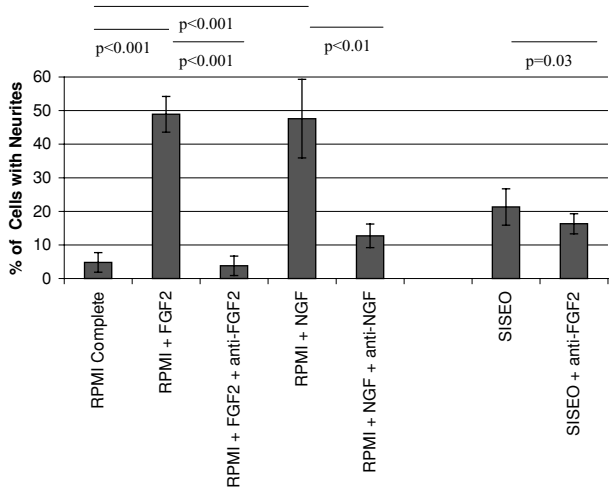

Fig. 2 Addition of 10 ng/ml of FGF-2 or 50 ng/ml NGF into the cell culture medium of PC12 cells results in neurite formation after 48 h. Blocking antibodies abolish this effect. Treatment of PC12 with SIS conditioned cell culture medium similarly stimulates neurite formation, partially through an FGF-2 dependent mechanism. Lines between bars represent significant differences

supported the attachment and viability of this cell line equally as well as the other forms of SIS, with 51% of the seeded cells attaching to the substrate in the first hour after seeding on the matrix (Fig. 1). There was no statistical difference in the percentage of viable cells attached to the SIS matrices after 60 minutes (SIS_{PAA} vs. SIS_{LYO}, $p = 0.170$; SIS_{LYO} vs. SIS_{EO}, $p = 0.274$; SIS_{PAA} vs. SIS_{EO}, $p = 0.090$). Because no statistical difference between groups was indicated, all remaining tests were only performed on terminally-sterilized, clinical grade SIS_{EO}.

3.2 FGF-2 from SIS_{EO} stimulates PC12 differentiation

PC12 cells form neurite-like extensions in the presence of laminin, NGF and/or FGF-2, but fail to differentiate in the absence of these added growth factors [24]. In control wells where cells were seeded in complete RPMI 1640 cell culture medium, no cells formed neurite-like extensions after 48 h. When the RPMI was supplemented with 50 ng/ml NGF or 10 ng/ml FGF-2, many of the cells began to differentiate by forming neurites. Addition of antibodies to the cell culture medium significantly reduced the degree of differentiation observed. Results are displayed in Fig. 2.

Media conditioned with SIS_{EO} was tested for its ability to promote differentiation of PC12 cells. After 48 h in the presence of the SIS_{EO} conditioned media, the PC12 cells were attached to the collagen-coated plate and $21.3 \pm 5.4\%$ of them had begun to form neurite-like extensions. When a neutralizing antibody to FGF-2 was added to the cell culture conditions, differentiation was reduced to $16.6 \pm 3.0\%$ ($p<0.05$). A neutralizing antibody to NGF failed to reduce the number of cells displaying differentiation after 48 h.

3.3 FGF-2 from SIS_{EO} stimulates fibroblast secretion of VEGF

FGF-2 is known to stimulate fibroblast proliferation and secretion of VEGF [25]. To further test whether FGF-2 activity was retained in SIS_{EO}, VEGF secretion by fibroblasts cultured in SIS_{EO} conditioned cell culture medium was measured and compared to wells in which FGF-2 activity was neutralized. Cell culture supernatants from SIS_{EO} conditioned cell culture medium contained significantly greater levels of VEGF at 16 and 24 h than either supernatants obtained from cells cultured in DMEM alone or from cells cultured with SIS_{EO}conditioned medium and anti-FGF-2 (Fig. 3a). Levels of FGF-2 in the supernatants did not change over time (Fig. 3b), showing that the increase in VEGF detected was not secondary to increased endogenous secretion of FGF-2 during the culture time.

4 Discussion

Previous studies have shown that it is possible to retain much of the composition and 3-dimensional architecture of the ECM when it is processed using peracetic acid and ethylene oxide gas [21]. However, retention of identifiable matrix components does not necessarily mean that they are retained in a form that can interact with cells and cause meaningful downstream effects, such as cell differentiation or secretion of growth factors. Retention of the bioactivity inherent within the processed ECM is essential to its ability to incite

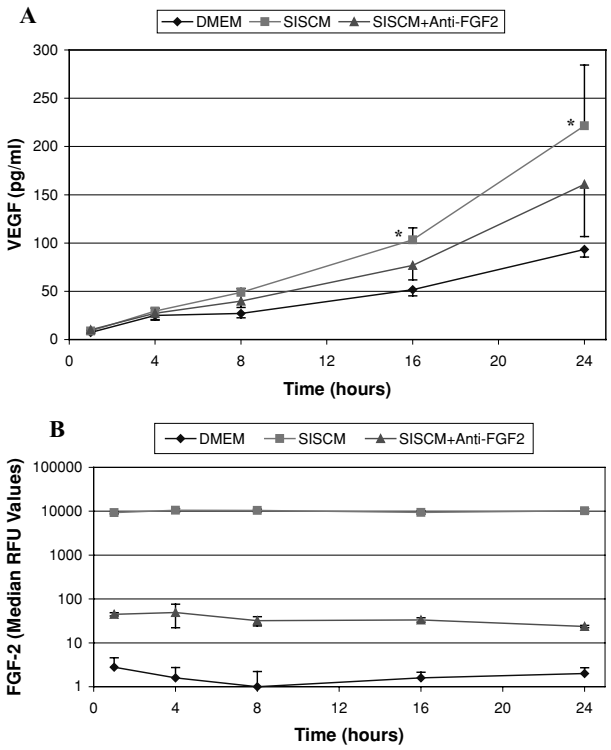

Fig. 3 (A) SIS_{EO} stimulates VEGF secretion by mouse fibroblasts. The addition of a neutralizing antibody significantly reduced VEGF secretion, indicating that SIS_{EO} stimulation of VEGF is mediated through FGF-2. Control cells seeded in unconditioned serum-free media displayed significantly less VEGF secretion; * All three groups significantly different ($p < 0.05$) from each other. (B) FGF-2 levels in the culture media remain steady over time, indicating that VEGF secretion is not caused by endogenous FGF-2 production

meaningful cell and tissue repair and growth when used to stimulate wound healing.

Previously, Hodde et al. [22] reported that endothelial cells retain their ability to interact with SIS that had been oxidized using PAA and terminally sterilized with gamma radiation. The current study supports those findings using a second cell type, but also using a matrix processed through freeze-drying (lyophilization) and terminal sterilization with ethylene oxide gas. Significantly, fibroblast attachment and viability were not altered on SIS_{LYO} when compared to SIS that had not been lyophilized. Because lyophilization has the effect of collapsing the 3-dimensional structure of the matrix [21], these results suggest that complete retention of the three dimensional architecture of the native matrix is not required for SIS—cell interaction and retention of viability. The degree to which the matrix can be collapsed and yet allow cell attachment has not been investigated.

Matrix oxidation by PAA and alkylation by exposure to EO gas are processing steps that ensure the viral safety and sterility of the processed ECM [26–28]. However, these processes have the potential to alter the structure of matrix constituents or render them inactive or unable to interact with each other or with cells [14, 28]. In this study, we have shown that these processes do not alter fibroblast interaction with and viability upon SIS_{EO}. Furthermore, these processes do not alter the bioactivity of FGF-2, a pro-angiogenic growth factor important in wound healing. Specifically, the ability of SIS_{EO} to stimulate the differentiation of PC12 cells was not impaired by lyophilization and sterilization. An activity neutralizing antibody specific for FGF-2 caused a significant decrease in the percentage of differentiated cells in the presence of SIS_{EO}, indicating that some of the neurite formation was due to active FGF-2. We have previously reported that the remaining effect is not due to the presence of NGF in the SIS, but rather is likely due to laminin present within the matrix [29].

The PC12 differentiation assay showed that SIS_{EO} retained the ability to cause a directly observable effect in cells, but we were also interested in the ability of the SIS_{EO} to stimulate cells to secrete growth factors. Therefore, we investigated the ability of SIS_{EO} to stimulate VEGF secretion by fibroblasts, and showed that conditioned cell culture medium made from processed SIS was able to stimulate VEGF secretion. We also showed that this response could be significantly reduced when an activity neutralizing antibody specific for FGF-2 was added to the conditioned cell culture medium, verifying that the upregulation of VEGF by fibroblasts occurs at least partially through an FGF-2 dependent mechanism.

The ability of SIS_{EO} to stimulate cellular production of VEGF is important because VEGF is strongly expressed in the frontline of repopulating epithelial, stromal and endothelial cells following injury, and is critically important in the proliferation and migration of multiple cell types during wound repair and tissue regeneration [30]. In diabetic ulcers, VEGF improves wound healing by locally upregulating other growth factors important for tissue repair [31]. VEGF also mobilizes and recruits bone marrow-derived cells to the local wound environment, where they are able to contribute to blood vessel formation and accelerate diabetic ulcer healing [31]. Importantly, because VEGF is only loosely bound in the ECM and is susceptible to degradation by PAA and EO gas [21], the ability of other, more stable growth factors to stimulate endogenous secretion of VEGF is an important characteristic of an implantable scaffold material. The loss of VEGF during matrix processing is likely not critical to the effective bioactivity of the matrix during wound healing, because we have shown that VEGF is actively secreted by fibroblasts in response to interactions with other components of the processed biomaterial.

In this study, we have shown that SIS retains the ability to support the interaction and growth of fibroblasts. We have also shown that FGF-2 remains in the matrix in a form that is able to stimulate the differentiation of PC12 cells. We have further demonstrated that FGF-2 and other factors present in the SIS_{EO} are sufficient to stimulate fibroblasts to secrete their

own endogenous VEGF, abrogating the need to retain VEGF in the processed matrix. We conclude that processing of SIS using agents that oxidize and alkylate matrix components as required for clinical safety results in a biomaterial that retains its intrinsic bioactivity. This intrinsic bioactivity may help explain the unique healing effects observed when this material is used to treat chronic wounds and repair damaged tissues.

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