Porcine small intestinal submucosa (SIS): a bioscaffold supporting in vitro primary human epidermal cell differentiation and synthesis of basement membrane proteins

Kristina Lindberg *, Stephen F. Badylak

Department of Biomedical Engineering, 1296 A.A. Potter Building, Room 204, Purdue University, West Lafayette, IN 47907-1296, USA

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Abstract

The growth pattern of human epidermal cells, fibroblasts or Swiss mouse 3T3/J2 fibroblasts cultured upon the extracellular matrix (ECM) derived from small intestinal submucosa (SIS) was evaluated. The cell/SIS composites were grown submerged, then maintained in air/liquid interface for 2, 7, 10 or 14 days. The presence of differentiation-related keratins 10, 14 and 16, FN, laminin, collagen type VII and collagen type IV was determined by immunohistochemical methods in SIS alone and in the SIS/cell composite. Only FN could be detected in SIS alone. SIS supported the formation of an epithelial structure with suprabasal expression of K16 and regional suprabasal expression of K10. The epidermal cells were K14 positive and tended to ‘invade’ the SIS to various degrees. Following the growth of epidermal cells and fibroblasts on the SIS substratum, immunolabeling of FN, laminin, collagen type VII and collagen type IV was observed in a cell-associated pattern. The fibroblasts commonly invaded the SIS, when co-cultivated with epidermal cells on the opposite side of the SIS. The ability of SIS to support epidermal cell/fibroblast attachment, migration and/or proliferation and differentiation with deposition of basement membrane (BM) components indicates that the composite model may be useful for studying cell-matrix interactions and for investigation as a dermal substitute. © 2001 Elsevier Science Ltd and ISBI. All rights reserved.

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1. Introduction

Small intestinal submucosa (SIS) is an acellular biomaterial usually derived from the jejunum of pigs [1]. SIS consists predominantly (> 90%) of collagens types I and III similar to the dermal component of skin [2]. Other extracellular matrix (ECM) components of SIS include small amounts of collagens types VI, V and VI, but not collagen type VII (unpublished data). The presence of fibronectin (FN) [3], chondroitin sulfates (A and B), heparin, heparan sulfate, hyaluronic acid [4], and cytokines, such as basic fibroblast growth factor (FGF-2) and transforming growth factor-β (TGFβ) [5] have been documented in the SIS-ECM. These components are known to be important for tissue remodeling and wound healing, especially in full-thickness skin defects [6].

The FN content is 0.08 ± 0.05% of dry weight SIS and is distributed transmurally [3]. This glycoprotein is found in very small amounts in normal skin, but is rapidly synthesized by fibroblasts, endothelial cells and monocytes in soft tissue wound sites, where it acts as a template for collagen matrix assembly [7]. During granulation tissue formation, FN promotes attachment and migration of epidermal cells, fibroblasts, endothelial cells and monocytes. FN also promotes basement membrane (BM) assembly [8]. As wound healing progresses, the production of FN ceases [9].
In animal models SIS has been shown to serve as a resorbable scaffold for in vivo regeneration/remodeling of a variety of specialized structures including the urinary bladder [10–13], dura mater [14,15], anterior cruciate ligament [16], Achilles' tendon [17], arteries and veins [18,19] and abdominal wall [20]. Features common to the SIS-induced remodeling process include a rapid angiogenic response, a mononuclear cell infiltrate during the early post-implantation period and the deposition of a neomatrix that replaces the SIS scaffold. The cause of the host tissue response to xenogeneic SIS is presently unclear, but its three-dimensional structure and composition appear to represent a favorable substrate for wound healing. SIS-ECM has previously been shown to support attachment and proliferation of epidermal cells and fibroblasts [21]. During the past 18 months, SIS has been used successfully in over 4000 human patients for the treatment of burns, chronic non-healing venous stasis and diabetic ulcers, orthopedic soft tissue defects, and as a vaginal sling for the treatment of urinary incontinence (personal communication with Mark Bleyer, Cook Biotech Inc., and Chris Butler, DePuy, Inc.). The remodeling process of SIS-ECM implants in humans is comparable with other mammals.

The presence of dermis or a dermal substitute in split-thickness grafts plays a critical role in the prevention or minimization of contraction [22–24]. Collagen-based materials used as dermal substitutes are favorable substrates for recellularization and revascularization and offer promise for the development of next generation dermal substitutes [25–27].

Tissue cultivation on plastic is the most commonly used method to study cells in vitro. The phenotype of cells grown in monolayer cultures is significantly altered compared with their normal physiological state. Depending on culture conditions, the cells change their biosynthesis and morphology to that of a less differentiated cell type. However, the cells maintain their intrinsic genotype and will differentiate, when placed in a permissive environment, for example, when grafted into an appropriate in vivo location.

In vitro three-dimensional models based on the use of cadaver dermis [28,29] or collagen lattices or gels [30,31] as dermal equivalents, have proven to be of great value for studying cell-matrix and cell-cell interactions. These studies have contributed to the re-evaluation of the ECM as a passive mechanical support system to an integral signaling medium that coordinates not only maintenance of a normal skin structure, but also the events of wound healing.

The purpose of the present study was to evaluate the pattern of cell growth, the degree of cell differentiation, and changes of matrix composition, when epidermal cells and fibroblasts are grown in vitro upon the SIS-ECM.

### 2. Material and methods

#### 2.1. Preparation of SIS

SIS was prepared as previously described [1]. Briefly, the superficial mucosal layers and external muscular layers were removed from the jejunum of pigs within 4 h following euthanasia. The remaining tubular structure consisted of the tunica submucosa and the overlying basilar layers of the tunica mucosa; the most superficial of which is referred to as the stratum compactum (i.e. the luminal surface). The side of the SIS material, from which the muscular layers were removed, is referred to as the abluminal surface. The stratum compactum side of SIS has a smoother surface than the abluminal side. As shown in Fig. 1a and b, the ‘sideness’ of SIS can be easily distinguished by the use of scanning electron microscopy. The material was approximately 80 μm thick and was prepared as a sheet, when the SIS tube was cut in the longitudinal direction. The SIS was disinfected with 0.1% peracetic acid and sterilized with 1.5 mRad gamma irradiation, stored in distilled water at 4°C (hydrated form) or stored in lyophilized form and re-hydrated in phosphate buffered saline for 20 min prior to use. Unless indicated otherwise, all experiments were conducted with the hydrated form of SIS. All experimental conditions described below were tested at least ten times.

The SIS material used in this study was prepared at the Biomedical Engineering Department, Purdue University, or obtained from Cook Biotech Inc., West Lafayette, IN.

#### 2.2. Biopsy specimens

The epidermal cells used in this study were derived from normal skin of a 20-year-old man and from neonatal foreskin. The human fibroblasts used were derived from neonatal foreskin.

#### 2.3. Cultivation of Swiss mouse NIH 3T3/J2 fibroblasts and human fibroblasts

The NIH 3T3 fibroblasts (a gift from Professor Howard Green, Harvard Medical School) and the human fibroblasts were grown in Dulbecco’s minimal essential medium (DMEM) supplemented with 10% bovine calf serum (Hyclone, Logan, UT).

#### 2.4. Cultivation of primary human epidermal cells

The epidermal cells were co-cultivated [32] with lethally irradiated Swiss mouse NIH 3T3/J2 in a 3:1 mixture of DMEM and Ham’s F12 media (Sigma Chemical Co., St. Louis, MO), supplemented with 10% fetal calf serum (Hyclone, Logan, UT), 0.4 μg/ml hy-
dorcortisone (Sigma Chemical Co., St. Louis, MO), 10 ng/ml human epidermal growth factor (Austral Biologicals, San Ramon, CA), $10^{-10}$ M cholera toxin (Sigma Chemical Co., St. Louis, MO), 5 μg/ml Zn-free insulin (a gift from Lilly Research Laboratories, Indianapolis, IN), 24 μg/ml adenine (Sigma Chemical Co., St. Louis, MO) and $2 \times 10^{-9}$ M 3,3',5 triiodo-L-thyronine (Sigma Chemical Co., St. Louis, MO).

Fig. 1. Scanning electron microscope picture of the (a) stratum compactum surface, and (b) abluminal surface of SIS. The stratum compactum surface of SIS is noticeably more smooth compared with the abluminal surface $\times$ 500.
2.5. Preparation of composites consisting of epidermal cells and fibroblasts grown on SIS

Swiss mouse NIH 3T3/J2 were seeded at 3–4 × 10^5 cells/cm^2 on the abluminal surface of SIS. The fibroblasts on SIS were cultivated in DMEM plus 10% CS for 2–3 days prior to adding human epidermal cells to the stratum compactum surface of SIS.

Preconfluent primary epidermal cell cultures in the third or fourth passage were seeded onto the stratum compactum surface of SIS at a density of 3–4 × 10^5 cells per cm^2 SIS. After 3–4 days growth in submerged condition, the composites were lifted to the air/liquid interface and grown for 2, 7, 10 and 14 days.

2.6. Fibroblasts grown on the stratum compactum surface or the abluminal surface of SIS with and without epidermal cells on the opposite surface

Swiss mouse NIH 3T3/J2 fibroblasts or human fibroblasts were seeded at 3–4 × 10^5 cells/cm^2 on the abluminal or the stratum compactum surface of SIS and cultivated in DMEM plus 10% CS for 2–3 days. Human epidermal keratinocytes were then seeded at 3–4 × 10^5 cells/cm^2 SIS on the opposite surface of SIS and grown for 3–4 days submerged in DMEM/F12 medium with supplements. Alternatively, composites containing fibroblasts only were grown for another 3–4 days submerged in DMEM/F12 medium with supplements. The composites were then lifted to the air/liquid interface and maintained for 10 days in DMEM/F12 medium with supplements.

2.7. Epidermal cells grown on the stratum compactum surface of SIS or lyophilized (and rehydrated) SIS

Human epidermal cells were grown on the stratum compactum surface of SIS as described above using SIS that had been stored in hydrated form or in lyophilized form and then re-hydrated prior to use.

2.8. Histological and immunohistochemical evaluation of the composites

At the time points indicated above, the composites were fixed in 10% neutral buffered formalin, paraffin embedded, sectioned and stained with Hematoxylin and Eosin (H and E) or frozen and sectioned for immunoperoxidase staining using the Vectastain avidin-biotin-peroxidase technique (Vector Laboratories, CA, USA) or standard immuno peroxidase staining techniques. The tissue sections were evaluated using light microscopy. The following primary anti-human mouse monoclonal antibodies were used; Anti-cytokeratin 10 (1:75 dilution), anti-cytokeratin 14 (1:20), anti-cytokeratin 16 (1:40) (all obtained from Novocastra Laboratories, UK), anti-collagen type VII (1:100) (Chemicon, CA, USA), LH7.2 (an anti-collagen type VII antibody obtained as a gift from Irene Leigh, London Hospital) and anti-laminin (1:2000) (Sigma, MO, USA). The anti-laminin antibody and the LH7.2 antibodies cross-reacted with porcine tissue. Primary polyclonal rabbit anti-human antibodies to collagen type IV (1:50) (ICN Biomedicals Inc, Chemical Credential, OH, USA) and to FN (1:400) (Sigma, MO, USA) both cross-reacted with porcine tissue. Secondary antibodies used were peroxidase-conjugated anti-mouse IgG (1:1000) and anti-rabbit IgG antibody (1:1000) (Sigma, MO, USA). As negative controls, the tissue sections were reacted with primary or secondary antibodies alone. Frozen sections of human foreskin or porcine skin were used as positive controls.

3. Results

3.1. The pattern of cell growth in epidermal cell/fibroblast SIS composites

By day 2 in air/liquid interface, the epidermal cells seeded onto SIS constituted a two to three layer thick epithelial structure. After a total number of 10–11 days in culture, small epidermal cell clusters were intermittently found beneath the surface of the SIS (Fig. 2a). By days 10 and 14 in air/liquid interface (after a total of 13–18 days in culture), a several cell layer thick stratified squamous epithelial structure had formed (Fig. 2b and c). The ‘invading’ epidermal cells showed a preference for migrating into existing tube-like structures within the SIS (Figs. 2c and 9a).

The 3T3 fibroblasts commonly migrated and/or proliferated into the SIS and could be seen transmurally as well as in close proximity to the keratinocytes (Fig. 2c).

3.2. Fibroblasts grown on the stratum compactum surface or the abluminal surface of SIS with and without epidermal cells on the opposite surface

Human and mouse 3T3 fibroblasts seeded on the abluminal surface of SIS showed the same tendency to...
Fig. 2.
‘invade’ the substratum as fibroblasts that were seeded on the stratum compactum surface (data not illustrated). The presence of epidermal cells on the opposite surface from which the fibroblasts had been seeded, promoted fibroblast invasion compared with when fibroblasts were seeded alone (Figs. 2c and 3). Fibroblasts derived from human donors invaded the matrix to variable extents (Fig. 4). After approximately 2 weeks in culture, sparse numbers of human fibroblasts were seen throughout the SIS matrix. The majority of the human fibroblasts were seen in near proximity of the cell-seeding surface. In contrary, the Swiss mouse 3T3 fibroblasts tended to invade the SIS transmurally and were evenly dispersed throughout the matrix.

3.3. Epidermal cells grown on the stratum compactum surface of lyophilized (and rehydrated) SIS

Epidermal cells seeded on the stratum compactum surface of SIS in the absence of fibroblasts formed a less organized and well differentiated epithelium than in the presence of fibroblasts (human or mouse fibroblasts) (Fig. 5). Epidermal cells seeded on the stratum compactum surface of lyophilized SIS that had been re-hydrated did adhere to the substratum (Fig. 6), but subsequently showed signs of terminal differentiation and failed to form an epithelial structure.

3.4. Histological and immunohistochemical evaluation of the composites

Only FN staining was seen throughout the SIS alone. The amount of FN staining in the SIS (Fig. 7a) increased with time in coculture with epidermal cells and fibroblasts and could be seen in association with the cells (Fig. 7b). In the cell/SIS composites, laminin, collagen type IV and collagen type VII staining was present in conjunction with the epidermal cells along the BM region of the epithelial structure and around cell clusters within the matrix (Fig. 8a–c).

All epidermal cells were K14 positive (Fig. 9a). K16 staining was seen suprabasally (Fig. 9b). In some areas of the composites, the keratinocytes tended to stratify...
Fig. 5. Human epidermal cells grown for 14 days on the stratum compactum surface of SIS. In the absence of fibroblasts the keratinocytes tend to form a less organized epithelial structure than in the presence of fibroblasts (Fig. 2b and c). × 200 H and E staining.

Fig. 6. Human epidermal cells grown for seven days in air/liquid interface on lyophilized and re-hydrated SIS. The epidermal cells consist of a two to three cell layers thick epithelium displaying features of terminal differentiation. × 200 H and E staining.

and differentiate into a more mature epithelial structure with suprabasal expression of K10 (Fig. 9c).

4. Discussion

The present study showed that SIS supports the in vitro adhesion, migration, growth and differentiation of primary human epidermal cells and that the SIS matrix itself is altered by the deposition of new BM proteins from the cultured cells. SIS has several characteristics that make the biomaterial suitable for in vitro as well as in vivo support of epithelial cells.

The presence of FN may play a significant role both for initial attachment of cells to the SIS and for subsequent cell proliferation. FN synthesis by fibroblasts is one of the factors contributing to the so-called ‘feeder layer effect’ (i.e. the ability of fibroblasts to support epithelial cell attachment and growth in vitro). FN-coating and collagen type IV-coating of the tissue culture vessels is commonly used to enhance in vitro keratinocyte attachment, migration and proliferation [33]. Primary epidermal keratinocytes placed in culture rapidly acquire the ability to secrete FN and exhibit FN receptors [8,33]. In epidermal cell/fibroblast/SIS composites, the epidermal cell-associated immunolabelling of FN is presumably a result of de novo synthesis by the keratinocytes. It is possible that the presence of TGF-β in SIS contributed to the increased expression of FN by the keratinocytes [34].

Laminin, which is the most abundant glycoprotein in the BM, was not present in SIS in quantities detectable by the immunohistochemical techniques used in this study. However, as shown in this study (Fig. 8a) and by others [35], laminin is synthesized by cultured keratinocytes. Adhesive glycoproteins, such as FN and laminin are known to link ECM components to one another and to cells. The glycoproteins bind with specific receptors on cell surfaces and on BM components, such as collagen type IV, which accelerates epithelial cell attachment [8]. Furthermore, SIS contains FGF-2, which is known to be angiogenic in vivo and to be mitogenic for cultured keratinocytes, melanocytes, endothelial cells and fibroblasts [36].
Fig. 7. FN staining of SIS matrix alone (Fig. 7a). Small amounts of staining are scattered along the stratum compactum surface (arrows) and throughout the matrix. After 14 days in air/liquid interface, the FN staining of the SIS/cell composite (Fig. 7b) is seen in a cell-associated pattern (arrow head). × 200 immunoperoxidase staining.
Considering the composition of SIS, the biomaterials’ in vitro cell growth promoting properties are not surprising. Interestingly, the lyophilized (and re-hydrated) form of SIS did not support growth and differentiation of the epidermal cells as well as the SIS that had not been lyophilized. It is possible that the three-dimensional structure and/or composition of SIS had been altered by the dehydration process, which in turn may have changed the cell adhesion properties of the matrix. It is conceivable that modifications of the dehydration
Fig. 9. Immunoperoxidase staining of SIS/cell composites using antibodies to keratin 14 (Fig. 9a) labeled all keratinocytes. Note that in one area, the keratinocytes have migrated into a tube-like structure (arrow). Antibodies to keratin 16 showed a suprabasal staining pattern (Fig. 9b). Anti-K10 staining had a regional suprabasal distribution (Fig. 9c) × 200.
process, such as amorphous-phase freeze drying [37], would improve the materials in vitro cell supportive properties.

In addition to the favorable composition of the hydrated SIS/ECM itself for cell growth, the pattern of cell growth appeared to be modulated by interactions between the different cell types that were seeded in the composite. The fibroblasts did not tend to invade the SIS, when grown without epidermal cells (Fig. 3). Although the normal human fibroblasts did not invade the matrix to the extent that the immortal 3T3 cells did (when co-cultivated with epidermal cells), the two cell types' ability to support the formation of an epithelial-like structure was comparable. The lesser organization and differentiation of epidermal cells grown on SIS without fibroblasts (Fig. 5), compared with when the
epidermal cells were grown in the presence of fibroblasts (Fig. 2b and c), was another implication of the impact of cell-matrix interaction in the SIS composite. There is accumulating evidence that interactions between epithelial cells and the underlying tissue play a key role for regeneration and maintenance of site-specific tissue characteristics [38–41]. Clinical studies have shown that the epidermal component of a skin graft is essential for minimization of morbidity and mortality. In addition, a suitable dermal substitute can induce the synthesis of normal dermal components, such as elastic fibers, which provide grafts with mechanical properties that mimic the properties of normal skin [42].

The clinical importance of ECM components for skin wound closure and remodeling is well documented. Dermal ECM (usually derived from cryo- or glycerol preserved cadaver skin) significantly improves clinical results, when used in conjunction with autologous split-thickness skin grafts (STSG) or cultured epidermal autografts (CEA) for permanent repair of full thickness skin defects [41]. Evidence for the positive impact of this ECM/epidermal interaction include the accelerated regeneration of BM components, such as anchoring fibrils (collagen type VII), and structural characteristics of normal skin, such as rete ridges [43–46]. Numerous clinical and animal studies have evaluated the ability of synthetic and biological substrata/dermal analogs to support keratinocyte proliferation for constructing a dermal/epidermal composite. Ideally, this type of construct should provide an ECM scaffold that rapidly incorporates into the wound bed, promotes connective tissue remodeling and delivers epidermal cells to the wound site. The keratinocytes that re-epithelialize a wound produce a large number of cytokines and ECM molecules, which are thought to contribute to the accelerated healing reported in different types of ulcers treated with cultured epidermal keratinocytes [47–49]. Based on the results in this study, and the reports of rapid integration and revascularization of SIS, when the ECM is transplanted to various body sites in animal models and in humans ([10–19] and personal communication with Mark Bleyer, Cook Biotech Inc., and Chris Butler, DePuy, Inc.), the cell/SIS composite may be useful as a carrier of epithelial cells to a wound site. It remains to be determined whether the wound healing properties of composites with recently seeded epidermal cells will be superior to composites containing a more mature epidermal structure. The results of the present study, combined with the results of previous studies that evaluated the utility of collagen-based dermal substitutes, suggest that this class of biomaterials may find utility as either a carrier for keratinocytes or as an alternative treatment modality for patients with partial-thickness and full-thickness wounds [22–24].

In the present study, culture conditions allowed de novo synthesis of BM proteins commonly expressed in monolayer cultures and in three-dimensional in vitro models (laminin and collagen type IV), and the synthesis of the major protein in anchoring fibrils of the BM, collagen type VII.

The cell/SIS composite used in these experiments provides a promising model for studies that could contribute to understanding the mechanisms that regulate cell adhesion, migration and proliferation of primary human epithelial cells and for studying the interaction between ECM components, epithelial cells and mesenchymal derived cells.

References


