Preliminary study of a polycaprolactone membrane utilized as epidermal substrate

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Solvent-cast sheets of polycaprolactone were biaxially stretched to produce 10–15 μm thick films. PCL films were found to have a tensile strength of 55 MPa which is about two and a half times stronger than native skin. One of our previous studies using non-coated PCL membranes showed that only 36% of the membrane surface was covered with keratinocytes after 9 days of culture. The present study examined the effects of coating the surface of PCL membranes with fibrin on the proliferation of keratinocytes. Qualitative analysis revealed that the cells attached and proliferated better on coated PCL films. Keratinocytes exhibited healthy cobblestone morphology and proliferated as continuous monolayers over a period of 16 days. The results indicated that fibrin coated PCL films would support the attachment and proliferation of human keratinocytes and have the potential to be applied as a matrix material for tissue engineering an epidermal equivalent.

1. Introduction

Conventional autologous split thickness skin autografts represent the ''gold standard'' to resurface wounds that do not heal on their own. Unfortunately, donor sites may be limited depending on the extent of lost skin. Allogenic skin grafts may be incorporated into healing wounds initially to bridge the critical time gap in the early phase of burn treatment but they irrevocably cause immunogenic rejection over a long period of time. The application of *in vitro* cultured skin substitutes could solve these problems. Development of *in vitro* cultured skin substitutes has taken place along multiple directions [1]: two of them being the construction of multi-layered epithelial transplants and the construction of composite dermal-epidermal analogs.

The pioneering work of Green to successfully culture and subculture keratinocytes on a feeder layer of lethally irradiated mouse fibroblasts [2] paved the way for clinical applications of cultured epidermal autografts (CEA). Keratinocytes aimed to be transplanted were grown to confluency to form a dense epithelial layer. The cultured epithelium was then detached as a coherent sheet from culture flasks by enzymatic treatment and transferred on a surgically prepared grafting bed to provide permanent coverage of excised third degree burn wounds [3, 4]. The detachment of a coherent epidermal sheet is a difficult and time consuming process. In addition, enzymatic detachment of the epithelial sheet from plastic surfaces causes the sheet to shrink. One of the first improvements was attaching the CEA with surgical clips to a backing of petrolatum gauze. Another problem is the inconsistency of graft take as well as the tendency to form blisters and scars due to exposure to shear forces at anatomical locations such as joints. Hence, the development of a substrate that provides mechanical stability allows applications of epidermal grafts on areas subjected to mechanical stress and might improve the overall outcome of the skin engineering.

Recently, a commercially available hyaluronic acid membrane (Laserskin, Fidia, Italy) is used as a carrier system for cultured keratinocytes [5, 6]. The 200 μm-thick membrane has micro holes of 40–60 μm in diameter that allows vapor permeation and cell migration. Macro holes of 500 μm in diameter are present to prevent extensive wound exudate formation. Campoccia *et al*. had demonstrated that fibroblasts, chondrocytes and bone marrow derived mesenchymal stem cells attached and proliferate on Laserskin [7]. In contrast, the natural polymer membrane must first be seeded with a radiated murine 3T3 feeder layer to allow keratinocyte attachment and proliferation on the substrate.

Clinically, fibrin sealant is a common carrier and matrix vehicle for keratinocytes. Cultured keratinocytes suspensions in fibrin glue are used for the re-epitheliasation of deep partial and full thickness wounds [8, 9].

The use of natural polymers gives rise to problems such as fragility, difficult manageability, substrate contraction and instability. The development of a

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synthetic membrane which has a biomimetic surface could overcome these problems. Our initial study with chemically modified polycaprolactone (PCL) foil as an epidermal matrix showed that keratinocyte proliferation on the non-coated PCL membrane was restricted [10]. In the present study, we examined the biomimetic effect of fibrin on keratinocyte attachment and proliferation when using PCL films as epidermal substrate.

2. Materials and method

2.1. PCL film preparation

PCL pellets (catalog no. 44,074-4) from Aldrich Chemical Company, Inc. (Milwaukee, WI) were used as received for this study. This semi-crystalline polymer has an average M_n of ca. 80 000 (GPC) and a melting point of 60 °C (DSC). Substrates used were fabricated by solution-casting with methylene chloride as a solvent and biaxially-stretched as described previously [11]. All the foils used in this study were perforated by using a robot arm (Sony, Tokyo, Japan) and a 0.54 mm diameter syringe needle at a rate of 50 mm/s. Center to center distance between the perforations in all orthogonal directions were 500 µm.

The perforated foils were cut into 5 mm × 5 mm squares. All the perforated foils were soaked in 5 M sodium hydroxide solution for three hours to make the PCL surface more hydrophilic. The foils were then thoroughly rinsed with deionized water until there was no change in pH of the remaining liquid. The films were sterilized with 70% ethanol and dried in a self sterilizable incubator (Binder, Tuttlingen, Germany) for 4 h.

2.2. Keratinocyte culture and seeding

Keratinocytes were isolated from skin biopsy of a patient who underwent routine plastic surgery and signed a written consent form. The skin was cut into 5 mm × 5 mm pieces. The epidermis was separated from the dermis by placing them in dispase/Hepes solution (5 mg/ml, Roche, Mannheim, Germany) for 16 h at 4 °C. The epidermis was trypsinized in 0.05% trypsin/PBS solution to isolate single keratinocytes. Passage 2 keratinocytes from the same donor were used for the whole study. Keratinocytes were cultured in keratinocyte serum free medium (SFM) from Gibco (San Diego, USA). Two groups were studied: Group 1 consisted of perforated PCL membranes coated with a thin layer of fibrin glue (FC) (Tisseel Kit, Baxter, Vienna, Austria) and group 2 consisted of perforated PCL membranes seeded with a fibrin–keratinocytes suspension (FKS). The two component fibrin glue was used according to manufacturer's instructions. Specimens of group I were dipped first in a thrombin bath and then in a fibrinogen bath. The fibrin glue coating had on average a thickness of 30–50 microns (data not shown) and was allowed for 15 min to completely polymerize before keratinocytes with a density of 100 000 cells cm^2 were seeded. Keratinocytes were left to attach on the stretched PCL films for 2 h in a self sterilizable incubator (Binder, Tuttlingen, Germany) before topping up the individual wells with 1.5 ml of keratinocyte SFM. In group II, keratinocytes (100 000 cells cm^2) were homogenously mixed in fibrin/thrombin suspension and seeded on the perforated foil (FKS). The fibrin glue/cell suspension was allowed for 15 mins to completely polymerize before topping up with the same media volume as in group I. Medium change was done every two days. The duration of the study was up to 16 days.

2.3. Optical, confocal and scanning electron microscopy

Light microscopy of the samples was performed every second day using Olympus IX70.

A cell viability assay using fluorescein diacetate (FDA) (Molecular Probes Inc., Oregon, USA) and propidium iodide (PI) (Molecular Probes Inc., Oregon, USA) was used. Viable cells were stained green by FDA while non-viable cells were stained red by PI. Prior to viewing, the specimens were removed from the culture wells and incubated at 37 °C with 2 µg/ml FDA in PBS for 15 min. After 2 × 5 mins washing with PBS, each specimen was then placed in 0.1 mg/ml propidium iodide solution for 2 min at room temperature. The specimens were then washed 2 × 5 min again in PBS, placed on a

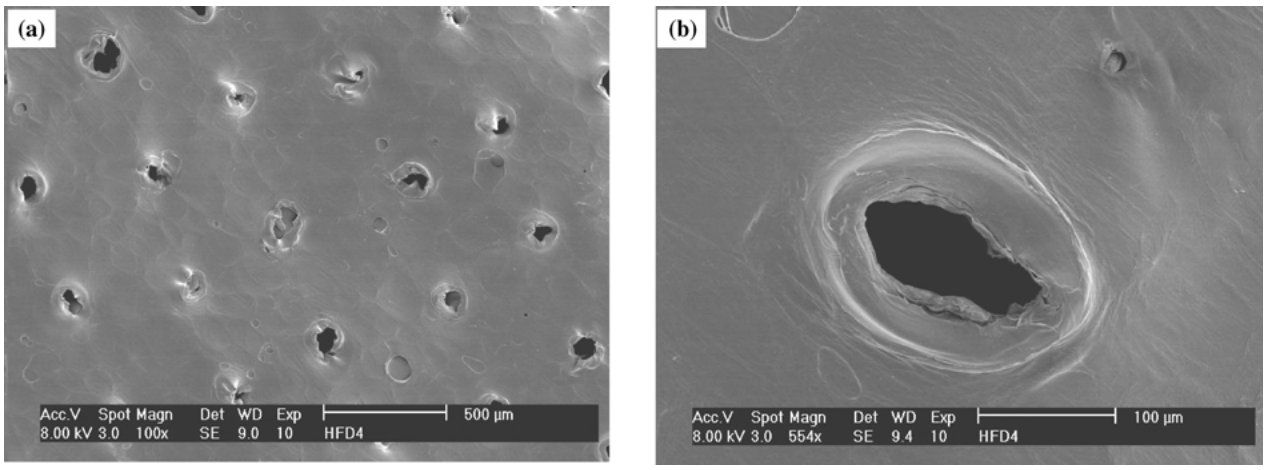

Figure 1 Scanning electron micrographs of perforated PCL membranes after 3 h 5 M sodium hydroxide treatment (a) showing regularly placed holes of approximately 100 µm in diameter. The perforations were not perfectly round (b). Overhanging flaps could be seen surrounding the perforations creating a ''valley-like'' topography around each perforation.

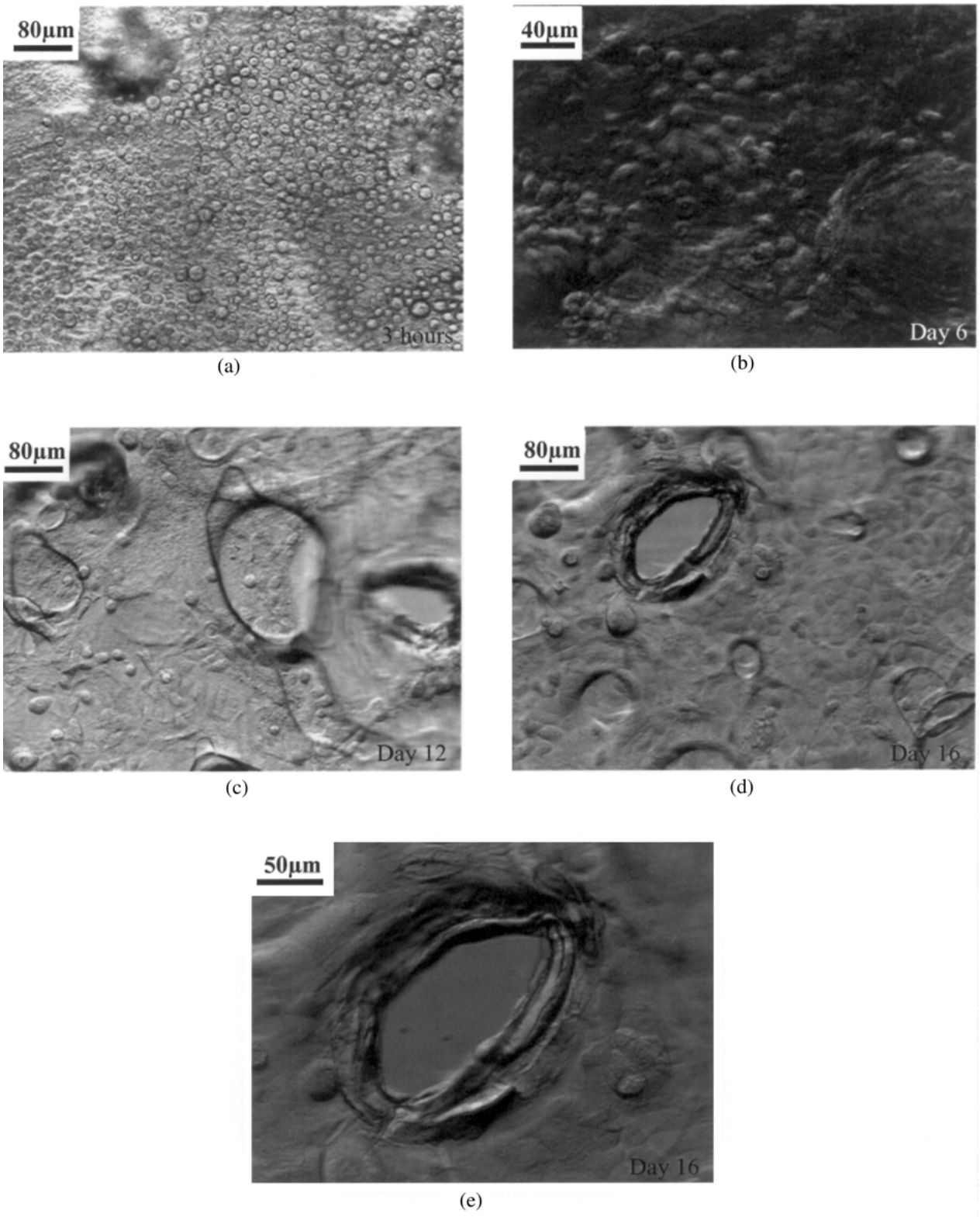

Figure 2 (a)–(d) showed the expanding cell colonies on the fibrin coated PCL films that were seeded with keratinocytes (Hoffman modulation). Fibrin was not present on the films after 12 days. Dense keratinocytes population was observed on the foils at day 16 (e). Keratinocytes were not able to bridge the holes but they orientated themselves around the holes in high cell densities (e). Bars in (b) and (e) represent 40 µm and bars in other figures represent 80 µm.

microscopical cover glass and viewed under an Olympus IX70-HLSH100 Fluoview confocal laser microscope. Images were obtained by superimposing green and red fluorescence.

Scanning electron microscopy (SEM) was used to analyze the HK cultures on the perforated PCL films. Specimens were fixed in 2.5% gluteraldehyde (Merck, Darmstadt, Germany) and dehydrated in a graded ethanol series. Gold coating was done with a Jeol JFC-1200 fine coater at 10 mA for 90 s prior to viewing under a Philips XL 30 FEG scanning electron microscope at an accelerating voltage of 8 kV.

2.4. Histology

The foil/cell constructs were fixed and stained with hematoxylin–eosin for routine histological examination.

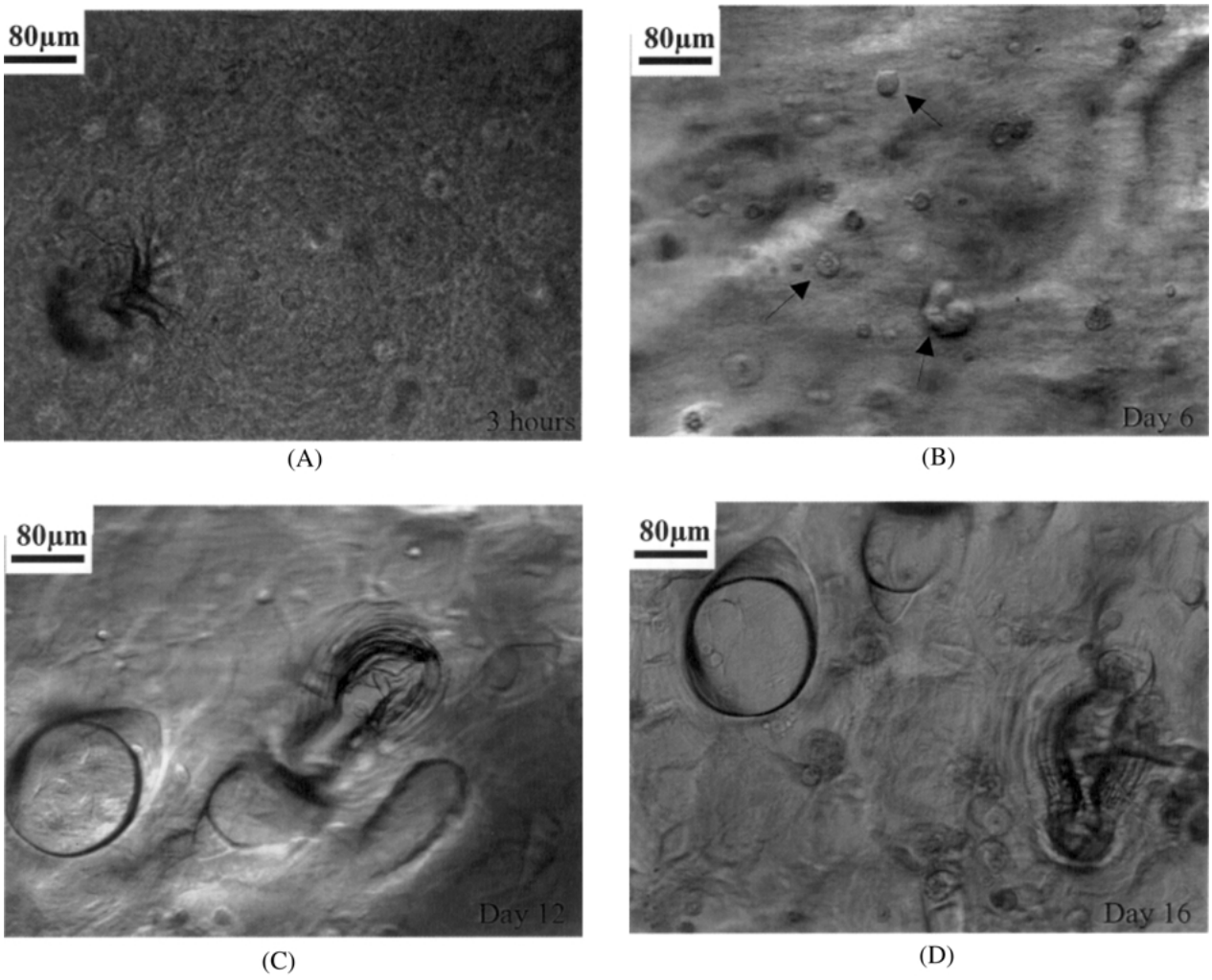

Figure 3 (A)–(D) showed the progressive growth of keratinocytes on the foils that were seeded with fibrin-keratinoctyes suspension (Hoffman modulation). Bars represent 80 µm in the figures. Keratinocytes (arrows) were isolated within the fibrin matrix (B). Fibrin glue was still present at day 12 but keratinocytes could be seen attached to the film (D). Keratinocytes were only sparsely populated on the foil at day 16 (D).

3. Results

Building on the concept of the Laserskin® membrane (Fidia, Padova, Italy), we have introduced microperforations into the ultra thin PCL foils. Microperforations were stamped out using a 3-axis pick and place robot in combination with a syringe needle. The holes, approximately 100 µm in diameter and placed 500 µm apart were not completely round (Fig. 1(a)). In addition, overhanging flaps were observed on the underlying side (Fig. 1(b)). All the foils were treated for 3 h in 5 M sodium hydroxide to increase hydrophilicity. The measured contact angle values decreased from 68° to 45°. In this preliminary study, we qualitatively evaluated the keratinocytes proliferation on the FC membranes as well as that on FKS membranes (Figs. 2 and 3).

Keratinocytes on the FC films appeared to spread out more on the fibrin layer at day 6 (Fig. 2(b)) whereas those on the FKS films were still round after 6 days in culture (Fig. 3(b)). Close cell to cell contacts were established after 6 days on the FC foils (Fig. 2(b)). In contrast, keratinocytes appeared to be still randomly distributed in the fibrin matrix on the FKS foils at day 6 (Fig. 3(b)). Most of the fibrin glue layer on the FC films was degraded after 12 days leaving only a thin layer of fibrin visible on the FC foils. Whereas a significant layer of fibrin still remained on the FKS foils (Figs. 2(c) and 3(c)). Larger areas of the FC films were covered with keratinocytes when compared to the FKS films group. A dense cell layer was observed on the FC films after 16 days in culture (Figs. 2(d), 4(b) and 5(c)).

Close cell to cell contacts as well as cobblestone morphology which are typical of non-differentiated keratinocytes were observed on FC films throughout the entire period of the study (Fig. 4(b)). Hence, no signs of keratinocyte stratification could be seen on both groups. A monolayer of keratinocytes following the topography of the perforated PCL foils covered the FC films (Fig. 5(b)). A well defined extracellular matrix network appeared to be formed on FC foils (Fig. 5(b)). FKS foils were only sparsely populated with keratinocytes (Fig. 3(d)) and the cell size was about 1.5 times bigger than those on FC foils (Figs. 4(e) and 5(c)).

Keratinocytes did proliferate in high concentration around the holes of the FC films even though they were not able to bridge the holes (Figs. 2(e) and 4(c)). Whereas if we compare the growth of fibroblasts (conducted in a separate study), it was observed that fibroblasts had the capability of bridging across the perforations (Fig. 6). Fig. 4(c) showed representative images of how keratinocytes were aligned around one perforation on the FC films. Epidermal cells were aligned longitudinally along the holes, getting elongated as they proliferated around

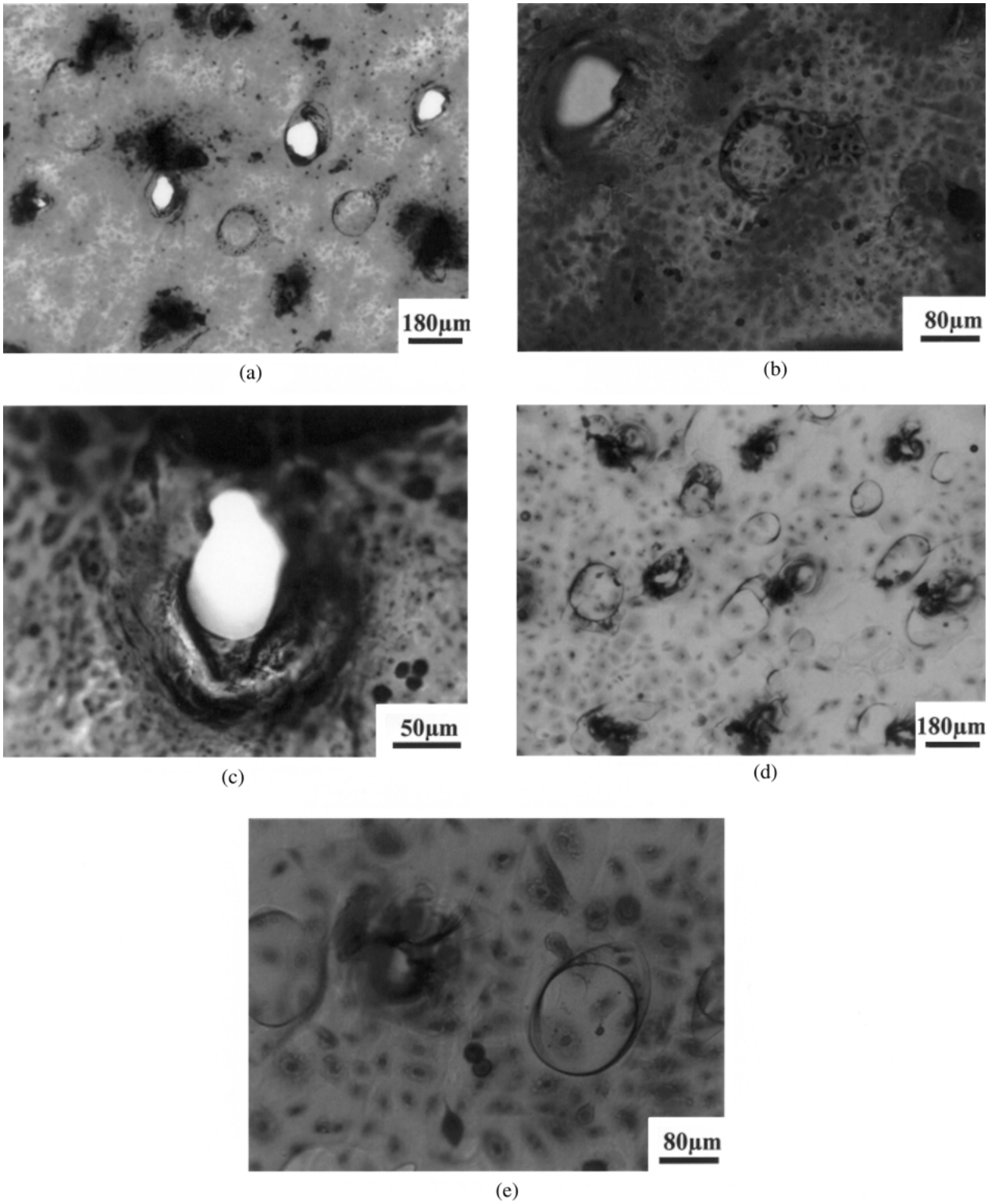

Figure 4 (a)–(c) Keratinoctyes grew in high density on fibrin coated PCL foils (FC) at day 16, (hematoxylin–eosin stain) while only sparse population of keratinocytes was found on foils seeded with fibrin–keratinocytes suspension (FKS) (d)–(e). Bars found in (a) and (d), (b) and (e), (c) are 180, 80 and 50 µm, respectively.

the holes. Keratinocytes were able to adapt to the ''valley-like'' topography around the perforation. Keratinocytes further away from the holes retained their usual cuboidal shapes.

We observed that keratinocytes migrated through the holes and populated the underlying surface of the FC films as keratinocytes grew to confluency on the seeded surface but the underlying surface was covered with keratinocytes in a lower density.

Laser confocal microscopy revealed that larger areas of the FC membranes than the FKS group were stained with viable cells throughout the study (data not shown).

We were able to achieve 100% keratinocytes coverage on the fibrin coated perforated (FC) films compared to those seeded with fibrin–keratinocyte suspension (FKS) (Fig. 7) after 16 days. Hematoxylin-eosin staining showed for both groups intense staining around the perforations (Figs. 7, 4(a) and (d)) which supported the high cell density observation around the holes under light and scanning electron microscope (Figs. 2(d), 5(b) and (d)).

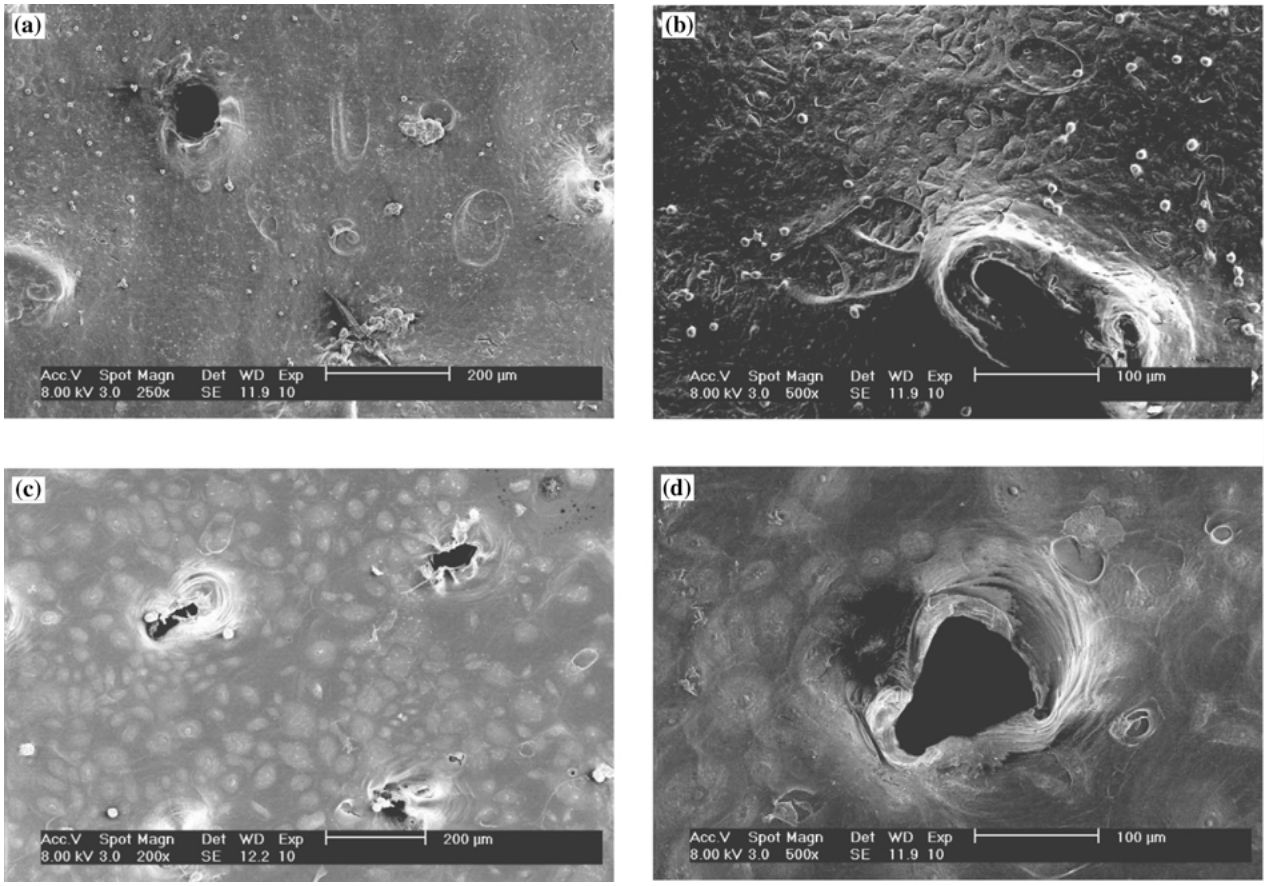

Figure 5 Scanning electron micrographs of fibrin coated films (a), (b) showed a thick layer of keratinocytes on the surface. Whereas on the surface of films seeded with fibrin–keratinocytes suspension (FKS) (c), (d) only a sparse keratinocyte population was observed after 16 days.

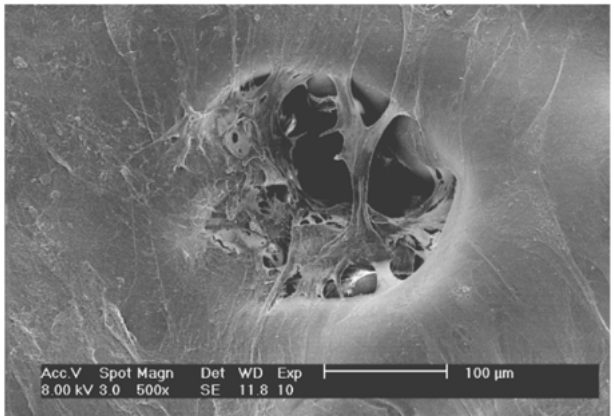

Figure 6 Human dermal fibroblasts are able to bridge a hole on perforated PCL foil (unpublished data).

4. Discussion

Different matrices and cell carriers have been developed to overcome the clinical difficulties of associated with CEA [7, 5, 16, 17]. One of the currently used epidermal substitutes is hyaluronic acid based membrane (Laserskin, Fidia, Pavoda, Italy). Keratinocytes were plated on Laserskin which must be seeded first with a radiated murine 3T3 feeder layer. Keratinocytes were able to proliferate and migrate on both sides of the membranes due to microperforations in the membrane. It was reported that a well differentiated and stratified epithelial was formed on such a substrate. But there is a concern that transplanted murine 3T3 fibroblasts could elicit an immunological rejection response that finally might lead to late graft loss.

Another clinically used keratinocyte carrier is fibrin glue. It has been shown that fibrin, as a naturally occurring polymer leads to hemostasis and provides a provisional matrix for epidermal cell migration during epithelialization [18]. Barrandon *et al*. [19] determined the colony forming ability of keratinocytes cultivated on a fibrin based substrate. Fibrin in this case was applied as a cast thin sheet on which keratinocytes were plated. It was demonstrated that the fibrin substrate prevented the shrinkage of the epithelium formed, a major problem that is inherited by CEA. The authors concluded that the presence of fibrin was found not to inhibit the growth capacity of human keratinocyte colony forming cells as well as their ability to generate human epidermis.

However, poor physical properties such as low mechanical strength and rapid degradation rates are still a clinical concern of fibrin-based cell culturing. Synthetic polymeric membranes with a biomimetic coating could provide the required mechanical stability as well as surface properties which promote keratinocyte attachment and proliferation.

In an earlier study where we used chemically surface modified but non-coated PCL membranes, 10% of the epidermal keratinocytes seeded were observed to attach on the non-coated PCL films 3 h after seeding. Keratinocytes formed dense colonies in the center of the film and they expanded outward towards the edge of the film. Increasing amount of localized stratified clumps of cells and extracellular matrix was observed as cultivation was prolonged over a period of 9 days [10]. This premature differentiation of epidermal keratino-

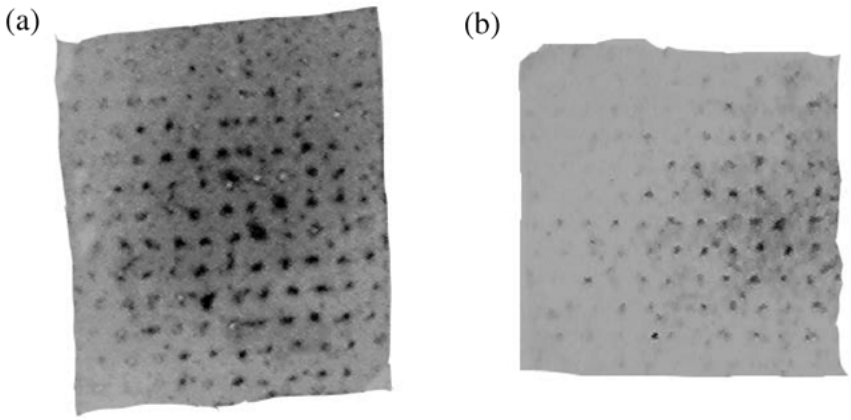

Figure 7 The extent of coverage on the foils was shown by Hematoxylin–eosin stained Keratinocytes/PCL foil constructs at 16 days. PCL foil that was coated with fibrin glue (a) was 100% covered with a dense layer of keratinocytes while PCL foil seeded with fibrin–cell suspension constructs (b) were only sparsely populated by keratinocytes.

cytes could be an indication of stress on the cells as they try to proliferate on the uncoated PCL films. As a result, only 36% coverage was observed on the non-coated PCL films after 9 days in culture.

PCL membranes were assessed in this study to qualitatively examine the ability of the polymeric material to support epidermal growth. Poly(ε-caprolactone), a biodegradable polyester, has shown good biocompatibility, high tensile strength, slow degradation kinetics and good barrier properties.

The fibrin glue coating served as a biomimetic surface for the adherence and proliferation of keratinocytes. Cell to cell contacts could be established very early in the culture on the FC membranes. Although fibrin was degraded through a natural process of enzymatic digestion, keratinocytes did not break away from the FC foils. Keratinocytes had the ability of burrowing through the fibrin layer and adhered directly to the underlying PCL film. This supported the work by V. Ronfard and Y. Barrandon [20] which reported that human keratinocytes are able to migrate through fibrin matrix. Locomoting keratinocytes dig cylindrical and helical tunnels into fibrin matrices while advancing as rounded cells. Mixing the fibrin glue and the cells into a suspension resulted in a keratinocyte encapsulation. Hence, the cells had to three-dimensionally proliferate through a large hydrogel volume in the FKS foil group to establish cell to cell contacts. Hence, on the sixth day the spherical shape of keratinocytes can be still detected (Fig. 3(b)). Keratinocytes suspended in fibrin were consequently isolated from each other on the FKS films and could not build up a network. Thus, when fibrin started to break down after 7 days, keratinocytes suspended further away from the polymeric foil were not able to adhere to the polymeric surface. As a result most unattached keratinocytes were lost in the FKS foil constructs during the media changes. Keratinocytes once they were attached were able to produce collagen, fibronectin and other extracellular matrix proteins and reorganize them as they proliferate and migrate over the PCL membrane surface.

Dense colonies of keratinocytes observed at the location of the perforations even on both groups could be due to the special surface topography and geometry of the holes. The perforations appeared to be points of intense cellular activity as seen from the way keratinocytes deferred from their usual cuboidal morphology into linear, bipolar shape around the holes. Further work will be executed to study the morphological differences observed in more details. The perforations were also points of origin for the migration of keratinocytes from the seeded surface to the underlying surface of the PCL membrane.

Keratinocytes when seeded on fibrin coated perforated membranes were able to proliferate and colonize both sides of the PCL membranes, migrating through holes. Studies are in progress to further evaluate this concept of combining a synthetic polymer membrane with a biomimetic surface towards the development of an epidermal substrate.

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