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Development of a Tissue-Engineered Human Oral Mucosa: From the Bench to the Bed Side

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Key Words

Tissue engineering · Oral mucosa · Keratinocyte · Food and Drug Administration

Abstract

The main objective of this publication is to make the reader aware of the complexity and steps that are necessary to make a Food and Drug Administration (FDA)-approved laboratory produced cell-based device, for use in clinical trials for reconstructive surgery. Most tissue-engineered cell-based devices are considered as 'human somatic cell therapy' and fall under the auspices of the Center of Biologic Evaluation and Research (CBER) and are considered a combination product by the FDA. We have illustrated the algorithm that is necessary to follow an Independent New Drug (IND) application by using our ex vivo produced oral mucosa equivalents (EVPOME), a tissue-engineered oral mucosa, as an example of a cell-based device that needs FDA approval prior to clinical application. By illustrating the experimental approach and presenting resulting data we attempt to explain each step that we address along the way.

Introduction

The nascent field of tissue engineering has generated enthusiasm based on its potential for regeneration and reconstruction of tissues or organs using cells and/or growth factors implanted into or onto suitable carrier scaffolds. Tissue engineering can be defined as 'the recon-

Abbreviations used in this paper

CBER	Center of Biologic Evaluation and Research
CDE	Center for Device Evaluation
COA	Certificate of Analysis
EVPOME	ex vivo produced oral mucosa equivalent
FDA	Food and Drug Administration
GFP	green fluorescence protein
HIPAA	Health Insurance Portability and Accountability Act
IND	Investigational New Drug
IRB	Internal Review Board
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NIH	National Institute of Health
PCNA	proliferating cell nuclear antigen
POK	primary oral keratinocyte
SCID	severe combined immune deficiency
SOP	standard operating procedures

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stitution of tissues and organs, in vitro, for use as model systems in basic and applied research or for use as grafts to replace damaged or diseased body parts or body functions'. Conceptually, tissue engineering differs from the field of cell transplantation in that an organized three-dimensional functional tissue is desired and designed. In the past, reconstructive surgery would focus on the designing of replacement parts for the body. With the debut of the field of tissue engineering this has shifted to the biological components of tissues: the body's cells responsible for tissue building in organisms during development and throughout life and the extracellular matrix, or scaffold, consisting of cell secretions immobilized in spaces contiguous with cells [Bell, 1995]. In addition, the majority of reconstructive procedures utilizes autogenous tissue, which has an advantage of the ability to respond to biological cues, but has the disadvantages of donor site morbidity, and limitation of quantity available. The main objective of this publication is to make the reader aware of the complexity and steps that are necessary to make a laboratory-produced cell-based device, Food and Drug Administration (FDA)-approved, for use in clinical trials for reconstructive surgery. This type of tissue-engineered cell-based device is considered as 'human somatic cell therapy' and falls under the auspices of the Center of Biologic Evaluation and Research (CBER) [Du Moulin and Morohashi, 2000].

An important point to keep in mind is that once tissue/cells has/have been removed from the patient and 'leave' the operating room or surgical suite and is/are brought to the laboratory to be cultured, where the cellular component is amplified and mucosal equivalent fabricated, it becomes biologic and is no longer considered a device. It is referred to as a 'combination product' which is a product that would normally be regulated under different types of regulatory authorities, and frequently by different FDA Centers, such as a device-biologic, into which category a skin or mucosal equivalent would fall (http://www.fda.gov/oc/ombudsman/part15hearing.html). This means that specific guidelines delineated by CBER/FDA must be then followed [Murphy and Epstein, 1998]. In order to precede forward with human clinical trials an Investigational New Drug (IND) application must be applied for. CBER will then require that the protocol for fabrication of the tissue-engineered cell-based device follow specific guidelines. This includes, but is not limited to, the use of serum-free medium, the elimination of any potentially contaminating material such as pituitary extract, exclusion of irradiated xenogeneic 3T3 mouse fibroblast feeder layers, the monitoring of sterility at each step of fabrication, especially at the time of release of the product for patient use, for bacterial, viral, or mycoplasma contamination, a certificate of analysis (COA) to show that all products that come into contact with the cells must be free of contaminants, the necessity of performing pharmacology and toxicology studies to rule out any cell transformation and/or induced cellular inflammatory response, development of a manual of standard operating procedures (SOP) must be filed for fabrication of the cellular devices, obtainment of Internal Review Board (IRB)/Health Insurance Portability and Accountability Act (HIPAA) approval for patient consent and for performing the surgical procedure, the ability to test cell viability of the cell-based device on release for surgical use, the development of a tracking procedure of the tissues/cells at each step of fabrication, the ability to do quality assurance and control, and the ability to follow good manufacturing practice standards [Steel and Roessler, 1999].

Oral mucosal grafting is indicated in the following clinical situations: periodontal surgery, minor and major preprosthetic surgery, dental implant surgery, reconstructive surgery for congenital, developmental or acquired facial deformities and/or defects resulting from trauma and ablative tumor surgery [Hillerup, 1993]. An ideal mucosal graft material should have the following qualities: it should be available in ample supply, maintain a functional epithelial barrier, be able to withstand functional stresses placed upon it, and be adherent to the wound surface and compliant to the undulated and complicated recipient site.

Oral mucosa or skin grafts both require harvesting of tissue from a second surgical site resulting in increased donor site morbidity. Oral mucosa is an excellent intraoral graft material but is limited in supply. In contrast, split-thickness skin grafts are available in ample supply, however contain adnexal structures and express a different pattern of keratinization, resulting interference of function and unpleasantness in the oral cavity. In addition, the wet and microbial environment of the oral cavity complicates reconstruction with skin grafts and their keratinized surface of grafted skin tends to macerate and to become easily infected.

In designing a tissue-engineering human oral mucosal equivalent it should duplicate the tissue's anatomic structures and physiologic functions that they are to emulate. According to Boyce [1996], the following categories should be addressed in the design consideration: (1) restoration of oral mucosa anatomy and physiologic function, (2) genotype of transplanted cells, (3) biocompatibility of

polymeric materials, (4) cost and complexity of fabrication, and (5) storage or banking of components. Oral mucosa or skin substitutes have been designed and manufactured in consideration of those categories but the fulfillment may vary and depend on the end use for each type of substitute/equivalent.

In development of a mucosal equivalent, two basic components exist: the superficial portion or epidermis that contains keratinocytes, and the deeper portion or dermis. Investigators, in the past, have attempted to graft skin and oral mucosal defects with epithelial sheets. These epithelial sheets have been friable and difficult to handle with a low engraftment rate. They also need to be conveyed to the surgical site or wound with a carrier vehicle such as petrolatum gauze that is secured in place with either a stent or metallic surgical clips.

Studies have shown that the concurrent grafting of a dermal component aids in enhancing the quality and time of wound and that the rate of closure of the wound and the increase in percentage of wound repair are enhanced with the presence of a dermis [Marks et al., 1991; Parenteau et al., 1991; Kangesu et al., 1993; Martin et al., 1995]. In addition, the maturation process and biological events of skin regeneration are accelerated with the presence of a dermal substrate [Compton, 1993]. Others have found that autogenous fibroblasts, within the grafted dermal matrix, facilitated the long-term maintenance of the reorganized cultured epidermis by supporting self-renewal of the epithelium in vivo [Inokuchi et al., 1995]. The absence of a grafted dermis resulted in a contracture of cultured keratinocyte autografts on the order of 50% [Clugston et al., 1991].

The development and grafting of a dermis can assist in epithelial graft adherence, minimize wound contraction and assist in epithelial maturation while encouraging the formation of a basement membrane [Gallico and O'Conner, 1995]. In order to resist sheer stresses placed on it, the epithelia should form a continuous basement membrane with a basal lamina and an anchoring zone [Ralston et al., 1999]. Several models that have consisted of keratinocytes combined with a mesenchymal or dermal component have successfully shown enhanced epithelial morphogenesis and an increase in expression of differentiation markers [Lee et al., 2000; Ojeh et al., 2001; Hinterhuber et al., 2002]. This was seen when they were especially grown at an air-liquid interface with fibroblasts grown in a contracted type I collagen, or on lyophilized collagen-glycosaminoglycan membranes cross-linked by chemical agents. However, those models showed an immature basal lamina and anchoring zone in an in vitro environment [Monteiro-Riviere et al., 1997; Nishiyama et al., 2000; Hinterhuber, et al., 2002].

A major drawback of the available cultured composite skin grafts was their poor handling characteristics. It was felt that the dermal component needed improvement in its tensile strength as well as the production of a lamina densa and anchoring fibrils. A dermal equivalent should be best made out of human, rather than animal, collagen. The human collagen (dermis) helped to promote deposition of additional basement membrane constituents, showed a better pattern of keratinocyte differentiation and had less immunogenicity than the animal collagen [Boyce, 1996; Pomahac et al., 1998]. Lastly, the use of a dermal equivalent enhances the handling characteristics of the equivalent.

The ideal mucosal graft should be constructed of autogenous oral keratinocytes, grown in a serum-free or defined medium without a feeder layer. Prior to application into the oral cavity, the oral keratinocytes should be applied to a human dermal matrix that is instructive and communicative with the cultured oral keratinocytes. In addition, the composite graft should possess these characteristics: they should be biodegradable and nontoxic, have physical properties of strength, compliance and density similar to the tissue it replaces, be capable of promoting cell attachment, be recognizable and remodelable by host and/or grafted tissue cells, have low immunogenicity, be suturable, encourage neovascularization, and be a suitable substrate for extracellular matrix enzymes [Bell, 1995].

Present Protocol for Fabrication of ex vivo Produced Oral Mucosa Equivalent

Using CBER/FDA guidelines, we have recently developed a protocol for fabrication of an ex vivo produced human oral mucosa equivalent (EVPOME), comprised of autogenous oral keratinocytes and a cadaver acellular dermis, AlloDerm®, in a serum-free culture system without a feeder layer or the use of pituitary extract, resulting in a completely chemically defined culture system. Keratinocyte proliferation was enhanced and shown to be in a hyperproliferative state when they were cultured in a serum-free medium devoid of essential fatty acids (table 1). Our EVPOME is cultured submerged for 4 days, then raised to an air-liquid interface for additional 7 or 14 days, to enhance epithelia stratification, based on Bell's work on skin equivalents [Bell et al., 1981]. From a 4×4 mm^2 oral mucosa biopsy of the

Table 1. Average population doublings and population doubling times at each of four cell passages in serum-free and serum-containing protocols

Cell passage No.	Population doublings		Population doubling times, days	
	serum-free	serum-containing	serum-free	serum-containing
1	2.04 ± 0.48 (n = 19)	1.83 ± 0.75 (n = 8)	2.30 ± 0.77[a]	3.05 ± 1.54
2	1.83 ± 0.59 (n = 19)	1.90 ± 0.74 (n = 4)	2.29 ± 0.65	2.98 ± 1.15
3	1.73 ± 0.45 (n = 17)	1.83 ± 0.77 (n = 4)	2.69 ± 1.07	4.30 ± 3.59
4	1.23 ± 0.33 (n = 15)	0.80 ± 0.27 (n = 3)	4.02 ± 1.41	6.93 ± 3.76

[a] Comparisons of population doublings and population doubling times between serum-free and serum-containing protocols yielded no statistically significant results for each passage (by t test, $p < 0.05$).

palate, it would take approximately 40 days to fabricate an EVPOME the size of one US dollar bill. This size EVPOME is large enough to reconstruct most oral mucosa defects.

Characterization of EVPOME

Conventional histology and immunohistochemistry were done to characterize the EVPOMEs [Izumi et al., 2000]. We found a continuous oral keratinocyte monolayer on day 4 equivalents. The one to two layers of continuous epithelial cells showed little sign of differentiation (fig. 1a). Day 11 equivalents, cultured for 4 days submerged and 7 days at an air-liquid interface, showed a continuous, thin, stratified layer of 3–6 cells on the dermal equivalent, AlloDerm, with the uppermost aspect of this layer parakeratinized (fig. 1b). After 14 days, at an air-liquid interface, day 18 equivalent, a more fully differentiated, confluent, 5- to 10-cell layer of keratinocytes was evident (fig. 1c). Filaggrin, a differentiation marker of epithelium, first appeared in the middle layer of day 11 EVPOME and was highly expressed in the middle layer of day 18 EVPOME (figure is not shown). In conjunction with the fact that keratin 10/13 expression was restricted to the uppermost layer on day 18 equivalents, epithelial cell layers of the EVPOME were more immature when compared with noncultured keratinized oral mucosa. In contrast, the presence of proliferating cell nuclear antigen (PCNA) and Ki-67-immunopositive cells, in the basal and parabasal layers on day 11 and 18 equivalents, was indicative of a hyperproliferative state similar to that seen with reconstructed skin epithelial layer [Gibbs et al., 1997] (fig. 2, 3). A more active, proliferating basal and parabasal keratinocyte population within the epithelial layer of EVPOMEs was proven by the persistence of the number of Ki-67-positive basal layer cells (fig. 3). p63, a recently identified member of the p53 gene family, was found to be very important in epithelial stratification and craniofacial and limb development, and its protein may have a function in maintaining the proliferative potential of keratinocytes and prevention of terminal differentiation. We found that p63 was predominantly present in basal and lower suprabasal layers in day 4, 11, 18 equivalents as well as in native oral mucosa keratinocytes (fig. 4). These findings support the concept that the epithelial layer of our EVPOMEs may contain a highly proliferative stem cell population.

Electron-microscopic evaluation of the EVPOME showed that the AlloDerm retained an intact basement membrane and anchoring fibrils on the papillary surface [Izumi and Feinberg, 2002]. Day 4 equivalents showed several layers of keratinocytes adherent to one another via desmosomal attachments while specific junctional structures between basal cells and the basement membrane of the AlloDerm were not seen at this time (fig. 5a). Day 11 equivalents had numerous rudimentary hemidesmosome-like structures (basal electron-dense plaques) that were incorporated into the anchoring fibrils of the basement membrane of the AlloDerm. In the cytoplasm, dense plaques were associated with intermediate filaments (keratin) (fig. 5b). This seemed to indicate that the basal cell layer was firmly attached and integrated to the underlying dermal equivalent of the day 11 equivalents suggesting an ability of the epithelial layer to withstand shear stress that might be found after grafting to the oral cavity.

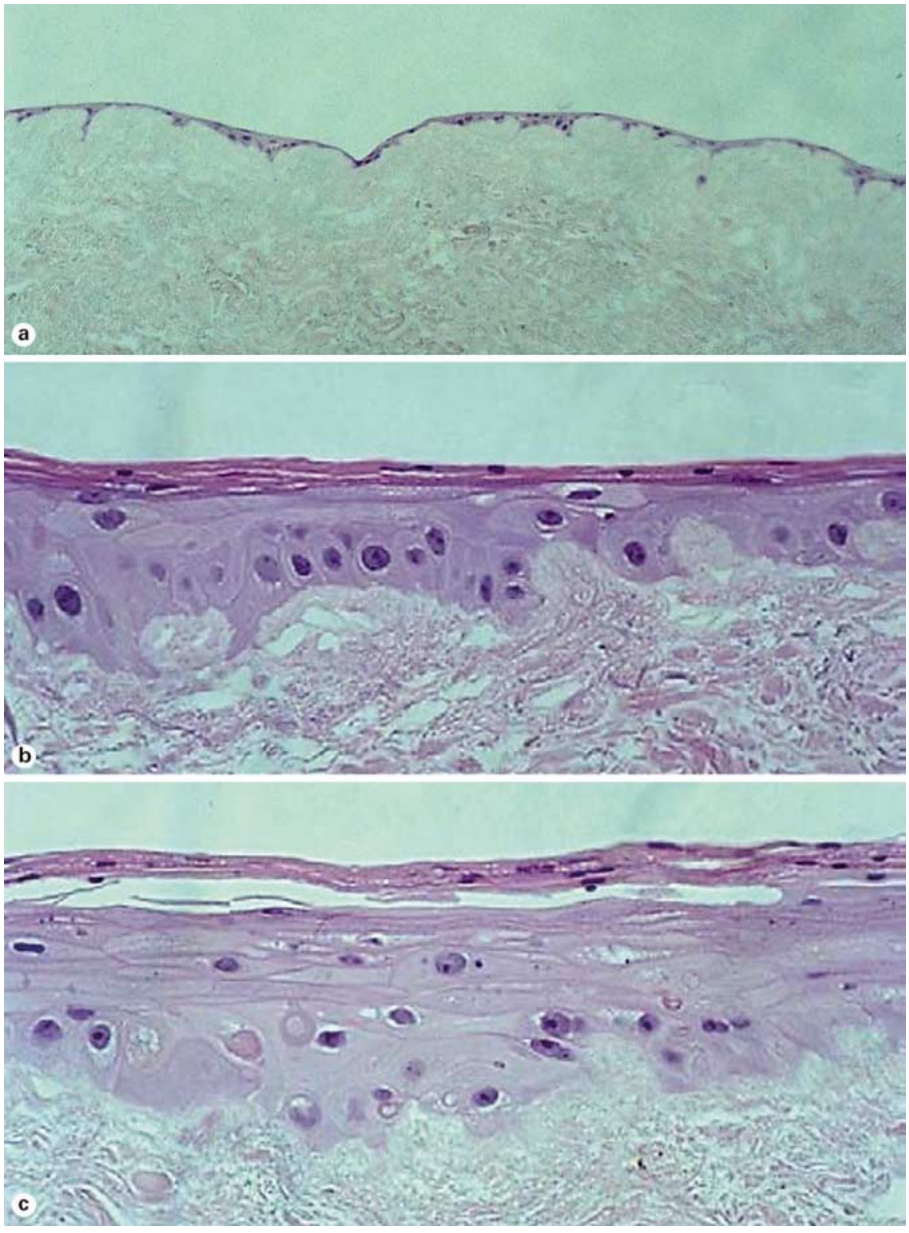

Fig. 1. Histological findings of EVPOME. **a** At day 4, continuous oral mucosa keratinocyte monolayer is developed. ×100. **b** At day 11 the continuous monolayer begins to stratify and show parakeratinization in the uppermost layer when raised to an air-liquid interface. ×250. **c** At day 18, the epithelial layer shows an increase in stratification and differentiation when cultured at an air-liquid interface. ×250.

Determination of Viability of Human Primary Oral Keratinocytes Maintained in Continuous Tissue Culture Using AlloDerm as a Substrate

It was then necessary to show that human primary oral keratinocytes (POK) retain their viability through the course of fabrication of the cell-based device, EVPOME. The FDA requires that you show that viable autogenous cells are grafted back into the donor. In order to quantify the growth and viability of the POK seeded onto AlloDerm, we employed a classic 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) or MTS assay [Mosmann, 1983]. This quantitative colorimetric assay has been developed to determine mammalian cell survival, proliferation and activation. The assay is based on the tetrazolium salt MTT that is a specific substrate for various types of dehydrogenases. The tetrazolium ring can be cleaved only in active mitochondria and the reaction proceeds only in living cells.

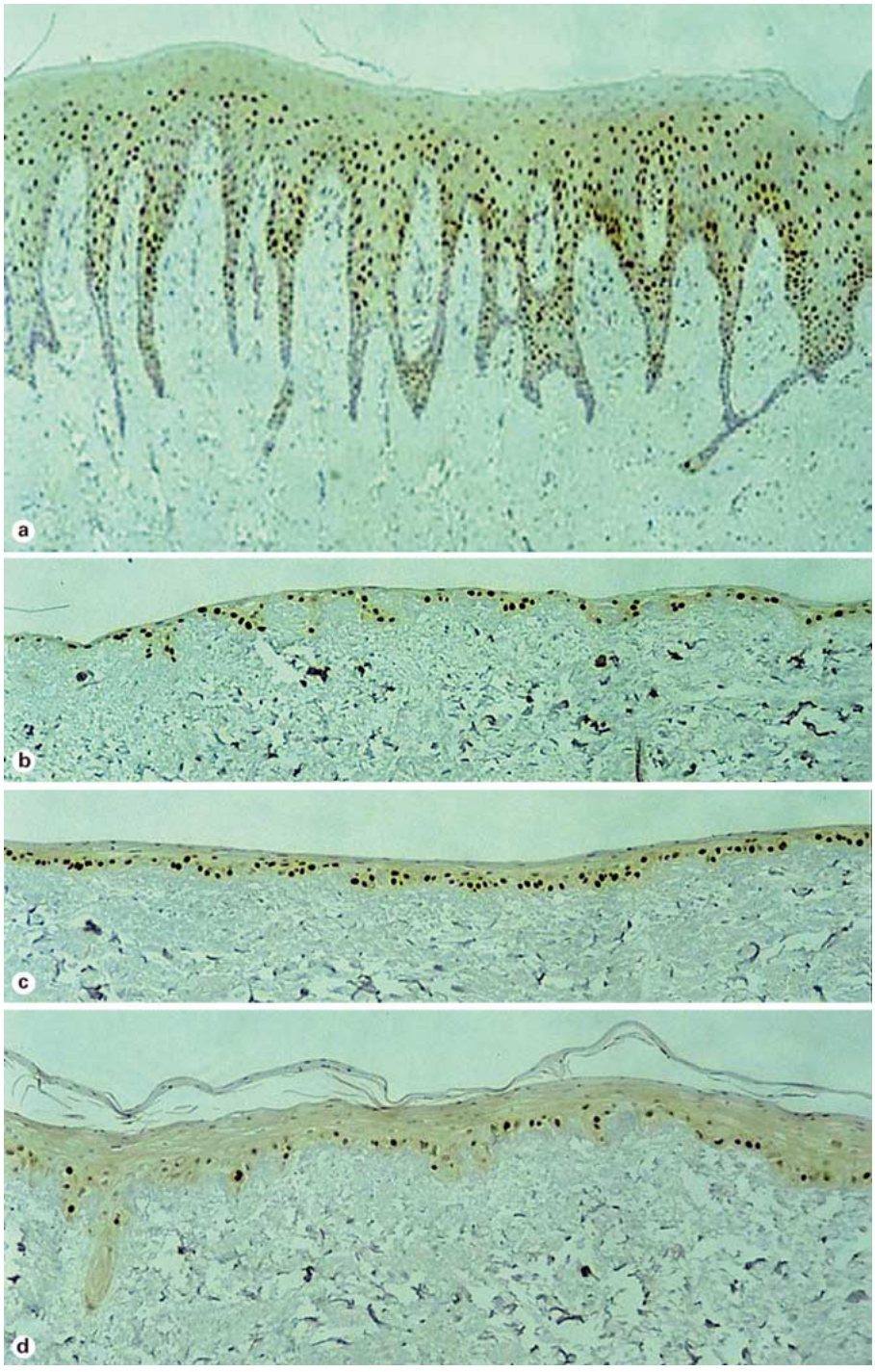

Fig. 2. PCNA expression of uncultured oral mucosa (**a**) and EVPOME at day 4 (**b**), day 11 (**c**), and day 18 (**d**). ×250. PCNA-immunopositive cells are present in basal and suprabasal cell layer in uncultured oral mucosa (**a**). In day 4 (**b**) and day 11 (**c**) equivalents, the basal and lower suprabasal layer cells show PCNA immunoreactivity. In contrast, in day 18 equivalent (**d**), the number of PCNA-immunopositive cells have decreased but are still present in the basal and lower suprabasal cell layer.

We performed MTT assays on POK seeded onto AlloDerm substrate and then maintained them in culture for up to 18 days. The standard MTT assay was modified to fit the condition of the experiments, but modification only involved an increase in the volume of the reagents used during performance of the assay. The concentrations and ratios between solutions used were preserved as in the original method. Because the endpoints of the assays were not simultaneous, we maintained strict conditions regarding the time of the MTT conversion (4 h at 37°C)

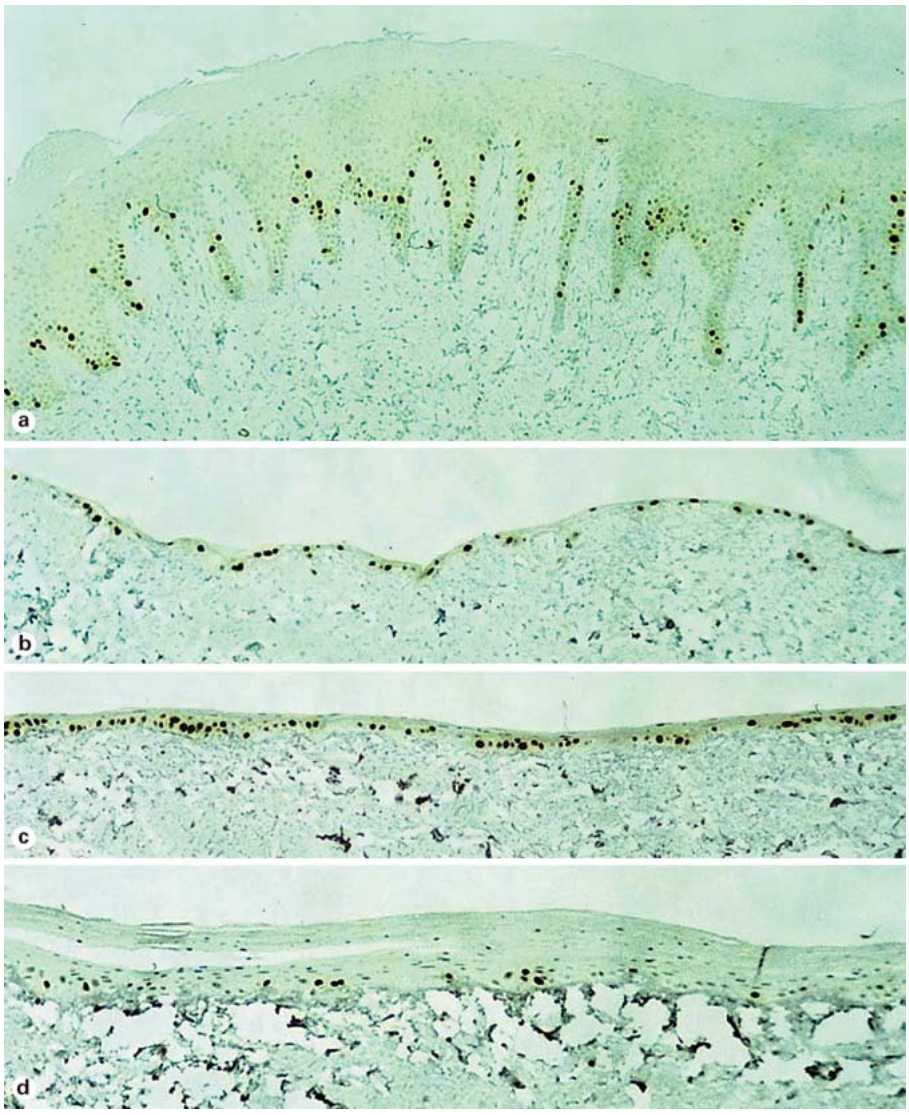

Fig. 3. Ki-67 expression of uncultured oral mucosa (**a**) and EVPOME at day 4 (**b**), day 11 (**c**), and day 18 (**d**). ×250. Ki-67 immunoreactivity is expressed in mostly basal layer cells and the several lowest suprabasal layer cells in uncultured oral mucosa (**a**). In day 4 (**b**) and day 11 (**c**) equivalents, Ki-67-immunopositive cells are located in basal cell layer. In day 18 equivalent (**d**), the number of Ki-67-immunopositive cells are on the slight decrease but still present in the basal cell layer.

and solubilization of the product (12 h at room temperature).

POK were isolated from the gingival tissue of a healthy 30-year-old male patient, expanded in serum-free culture conditions and then seeded as duplicates onto AlloDerm. To address the issues related to sensitivity of the MTT assay, as well as growth properties of POK, cells were seeded at three densities: 0.6×10^5, 1.2×10^5 and 2.4×10^5 cells per dermal equivalent. The 1.2×10^5 density was previously identified as the optimal concentration for the morphologically developed EVPOME [Izumi et al., 1999]. After 4 days in the submerged cultures in serum-free medium (1.2 m*M* Ca^{2+}), the POK-seeded AlloDerm were placed at an air-liquid interface and continued to cultivate up to 3 weeks. Parallel samples of AlloDerm of identical size but without POK were used as negative controls.

AlloDerm seeded with POK and unseeded controls were assayed on days 4, 11 and 18. Samples were transferred to 24-well plates and incubated 4 h (in tissue culture incubator) with 300 µl fresh medium containing 30 µl of 5 mg/ml MTT reagent. The reactions were stopped/solubilized by adding 330 µl to acidified isopropanol (0.04 *N* HCl). Triplicate 100-µl aliquots of the supernatants were placed into a spectrophotometer and the

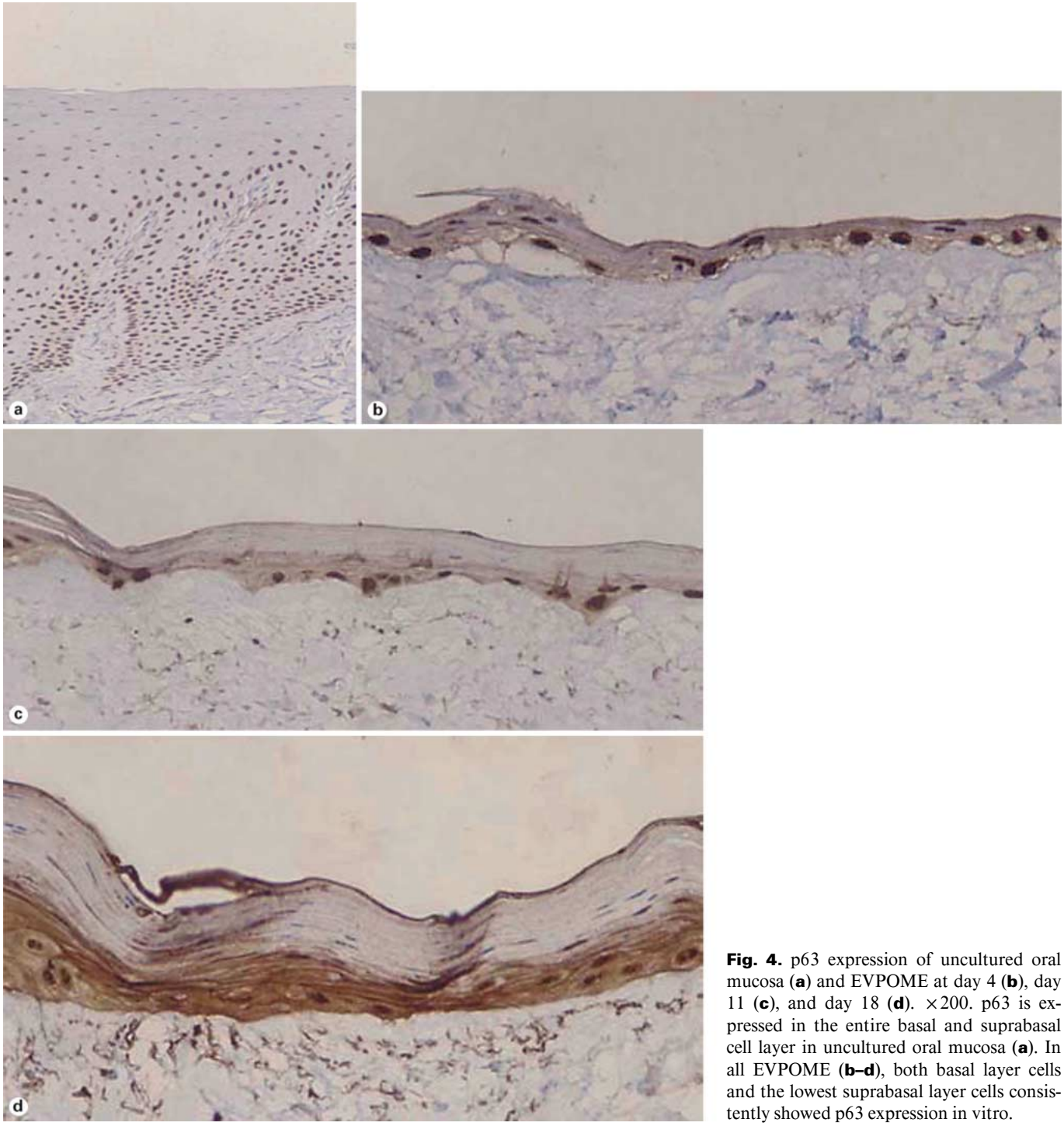

Fig. 4. p63 expression of uncultured oral mucosa (**a**) and EVPOME at day 4 (**b**), day 11 (**c**), and day 18 (**d**). ×200. p63 is expressed in the entire basal and suprabasal cell layer in uncultured oral mucosa (**a**). In all EVPOME (**b–d**), both basal layer cells and the lowest suprabasal layer cells consistently showed p63 expression in vitro.

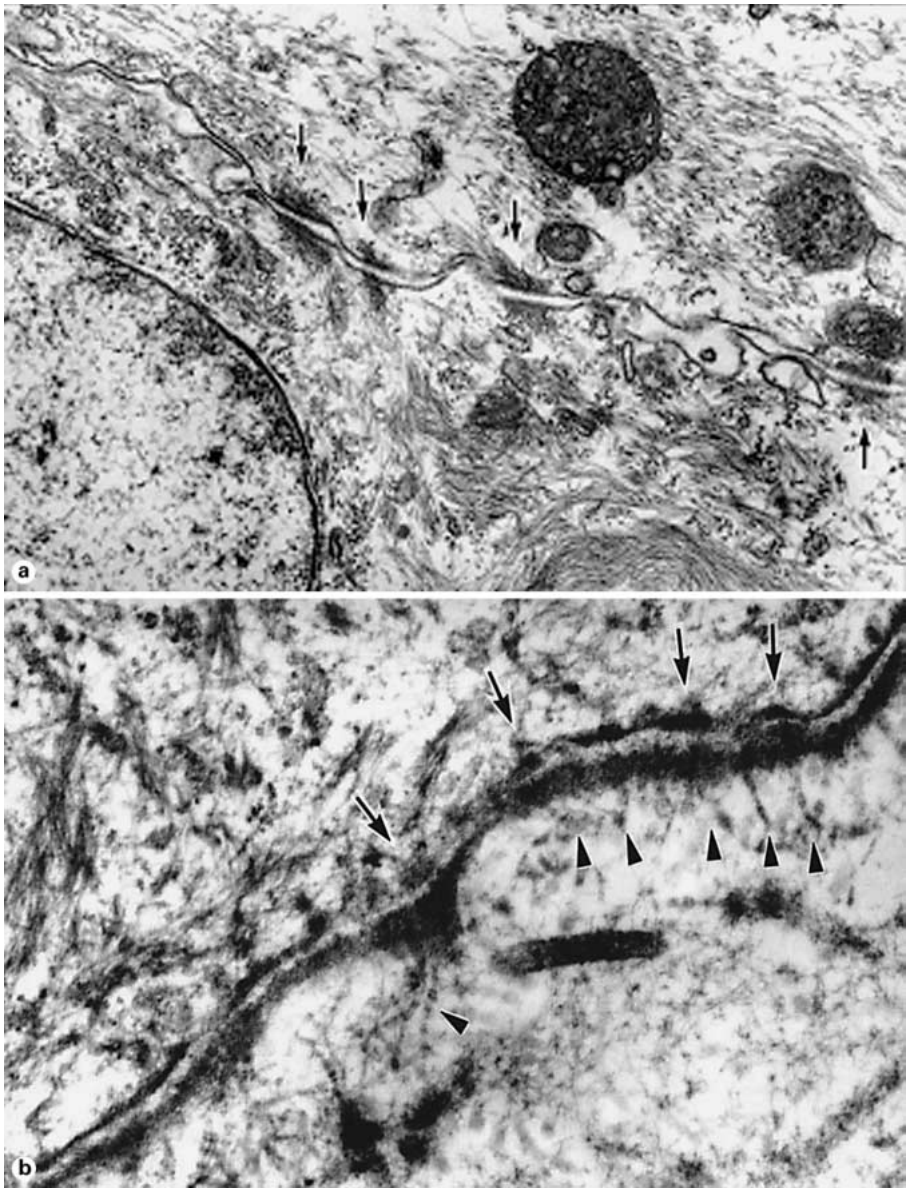

Fig. 5. Electron-microscopic evaluation of the EVPOME at day 4 (**a**). Arrows show the desmosome structure between keratinocyte layers; numerous intermediate filaments (keratin) are seen in their cytoplasm. At day 11 (**b**), arrows show the formation of hemidesmosome-like structures whose dense plaques were associated with intermediate filaments (keratin). Arrowheads show the anchoring fibrils of the basement membrane of the AlloDerm.

$OD_{570/630}$ were determined. Data were recorded as mean values ± standard deviations and analyzed.

Data presented in figure 6a shows that POK cultured on AlloDerm remained viable for at least 14 days of continuous interface culture (day 18). Although increases in the mean values of the MTT assay were statistically significant when day 4 means were compared to day 11 and day 18 means, there was no significant difference noted in the mean MTT values obtained at day 11 when compared to day 18. This data is consistent with morphological data documenting cell differentiation and keratinization of the outer layers of the composite mucosa that occur by days 11–18. Cell viability at day 11 and 18 appeared to be unaffected by seeding density, although the largest number of viable cells correlated directly with the initial seeding density.

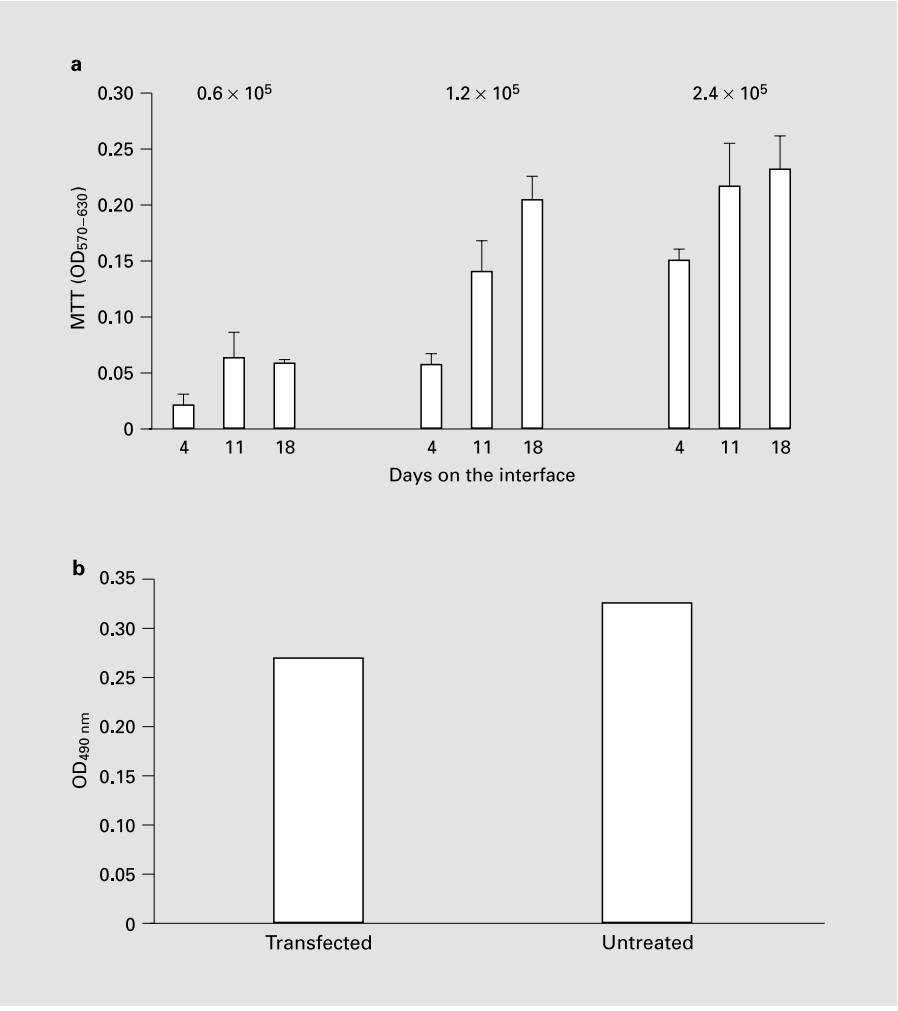

Fig. 6. a Viability of POK cultured on dermal equivalent, AlloDerm (patient STF, male, 30 years old, gingiva). Oral keratinocytes have been viable at least 14 days in an air-liquid interface culture system. Optical density (OD) values are in direct proportion to seeding cell density. **b** Viability of cells on mucosal equivalents (7 days, MTS assay). Viability of oral keratinocytes on mucosa equivalent after 7 days' culturing: MTS mitochondrial respiration assay. $p = 0.11$.

Indirect Assessment of Oral Keratinocyte Viability on EVPOME Using Glucose Metabolism

A study was done to correlate glucose levels in tissue culture medium with that of the MTT/MTS assay to assess oral keratinocyte viability on the EVPOMEs prior to placement in vivo. MTT/MTS assay, although reliable, requires destruction of the cell-based device that is being evaluated. In a clinical trial, it is essential to establish cell viability and metabolic activity of the EVPOME, which will be used to treat a patient, without directly compromising cell integrity of the cell-based device. With this in mind, we utilized glucose metabolism as an indirect indicator of cell viability and metabolic activity.

EVPOMEs were fabricated per our standard protocol [Izumi et al., 2003a]. Day 11 equivalents, which are to be used in the human clinical studies, were cut into three pieces. One piece was used to perform an MTS assay, a second piece was grafted into a subcutaneous pouch on the back of a severe combined immune-deficient (SCID) mouse, and the third piece was placed back in vitro to evaluate glucose utilization during the 7-day culture period to monitor glucose uptake by the oral keratinocyte on the surface of the EVPOME. The MTS assay (fig. 6b) showed viability of the cell on all four of the EVPOMEs evaluated, both green fluorescent protein (GFP)-transfected and untransfected oral keratinocytes.

The EVPOMEs that were grafted into SCID mice were harvested after 14 days and processed for routine histology [Izumi et al., 2003b]. All transplanted EVPOME grafts

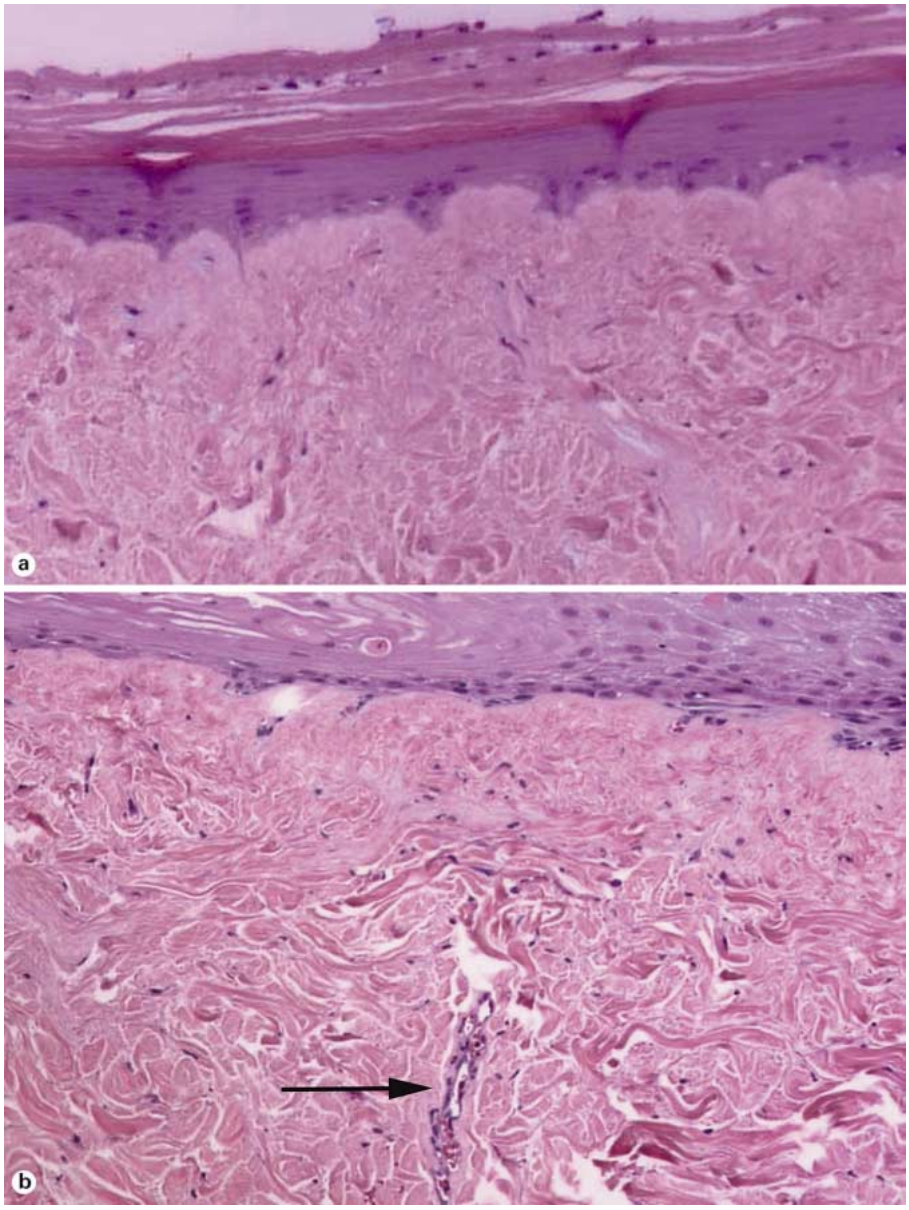

Fig. 7. a EVPOME (day 4) grafted into SCID mouse. Transplanted EVPOME grafts harvested at 10 days postoperatively were shown to be viable in vivo with continued maturation of the epithelial layer. No degenerative changes were seen. **b** EVPOME (day 11) grafted into SCID mouse. Transplanted EVPOME grafts harvested at 10 days postoperatively showed blood vessel formation (arrow) with red blood cells within the lumen, in the underlying dermal component, AlloDerm, as well as evidence of fibroblast infiltration.

were shown to be viable with continued maturation of the epithelial layer (fig. 7a). In addition, the SCID EVPOME grafts showed microvessel formation with lumens containing red blood cells, and infiltration of fibroblasts into the underlying dermal equivalent, AlloDerm (fig. 7b).

Glucose concentration in the tissue culture medium was assessed using a Beckman Glucose Analyzer 2 instrument. The EVPOMEs were cultured for 7 days beginning at the same time period of the MTS assay and grafting of SCID mice. At 3, 5 and 7 days after culturing, medium was decanted and stored frozen at –20°C. After each medium was removed fresh complete medium was replaced in the wells containing the EVPOMEs. Samples thus reflected 72, 48 and 48 h glucose utilization by each of the cultured EVPOMEs. Once all samples were harvested, they were defrosted and a 10-µl sampling of each time period was taken for analyzing on the Beckman Glucose Analyzer 2.

Figure 8a shows an increase overall uptake of glucose by EVPOME over the 7-day culture period starting from zero up to 79 mg/dl at 3 days, 142 mg/dl at 5 days and 189 mg/dl for the 7 days. Figure 8b illustrates the glucose

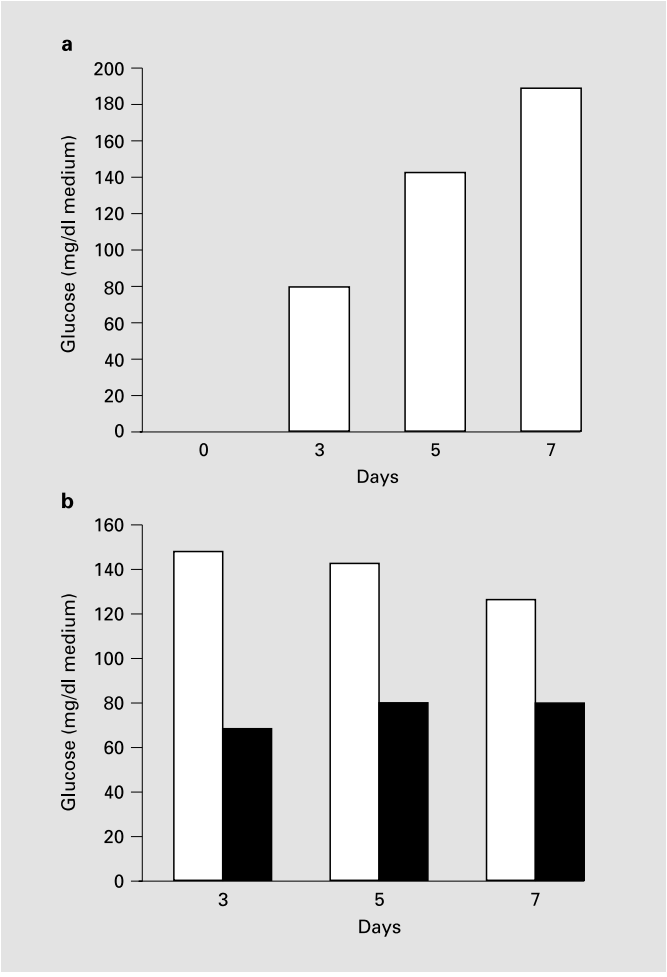

Fig. 8. a Glucose uptake of EVPOME analysis over 7 days' culture. Cumulative uptake of glucose over the 7-day culture period is shown (□). **b** Glucose uptake in spent medium of three culture periods of 72, 48 and 48 h. □ = Glucose levels in medium at time of placement into culture wells containing EVPOMEs; ■ = concentration of glucose in the spent medium 72 h (day 3), 48 h (day 5) and 48 h (day 7) after culturing with EVPOMEs. Fresh medium was placed into wells on day 3, 5 and 7.

present in 'spent medium' of the three culture periods of 72, 48 and 48 h. The time intervals of each of the samples of culture medium of the EVPOMEs showed a drop in the glucose concentration when compared to the background control. This decrease in glucose concentration over the background control indicates that the cells are metabolically active and thus viable.

The data from the MTS assay and SCID mouse in vivo transplantations correlate well with the glucose metabolism of the EVPOMEs in vitro data. It thus appears that monitoring of glucose uptake is an excellent noninvasive method to assess oral keratinocyte viability on the EVPOMEs prior to grafting in vivo for a human clinical trial.

Small Animal Model (SCID Mice)

The FDA requires for filing an IND application to have a small animal model that could be used for pharmacology and toxicology data. In addition, it allowed us to determine the optimal stage of development of the EVPOME for clinical grafting and as well as to assess revascularization of the dermal component of the free mucosal graft.

We grafted EVPOMEs subcutaneously to the backs of SCID mice to assess graft take and development in an in vivo environment [Izumi et al., 2003b]. The SCID mouse is considered an excellent model for xenografts and for assessing neoplastic potential or cell transformation for uncontrolled neoplastic-like growth characteristics of cells. Our data showed complete graft take, with incorporation onto the panniculus carnosus, with continued maturation of the EVPOMEs without any sign or evidence of malignant transformation or unusual growth characteristics that could be evidence of neoplastic growth (fig. 7a). The survival and thickness of overlying epithelium, in vivo, were inversely proportional to the length of time of in vitro culturing of the EVPOME at an air-liquid interface. In contrast, the microvessel density of the dermis varied directly with the degree of epithelial stratification of the EVPOMEs (fig. 9, table 2). The presence and stratification of the epithelium developed in vitro correlated with the revascularization of the underlying dermis in vivo. The EVPOMEs cultured at an air-liquid interface for 7 days had the optimal balance of neoangiogenesis and epithelial differentiation necessary for in vivo grafting [Izumi et al., 2003b].

Large Animal Model (Dog)

The FDA requires a large animal model to evaluate cell-based devices prior to initiating human clinical studies. Our large animal was developed in a dog to evaluate the fate of grafted keratinocytes, and, as a future model, for use in grafting of EVPOMEs with transfected oral keratinocytes.

The procedure of fabrication of a canine EVPOME, as well as the development of a POK culture, are identical to our current protocol for the fabrication of a human

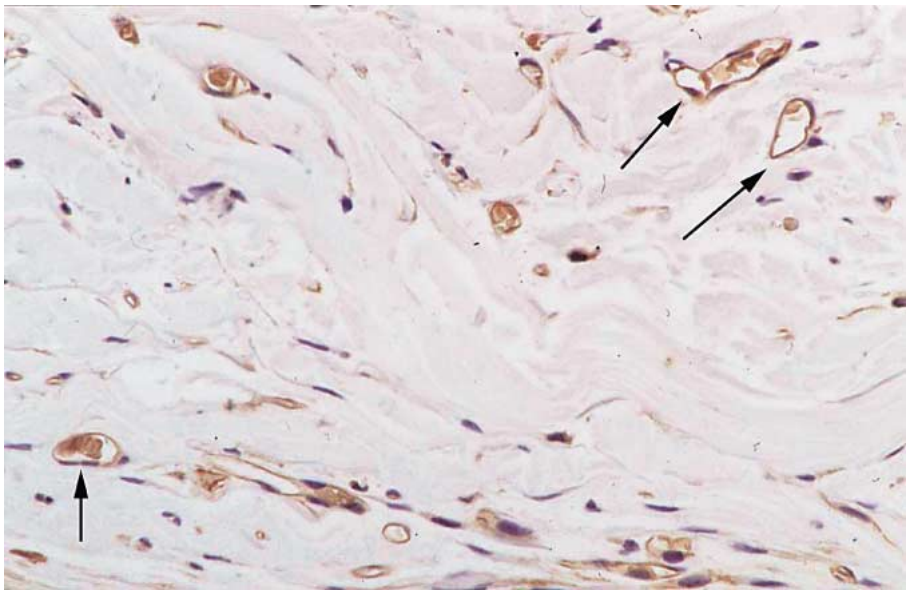

Fig. 9. Microvessel formation is evident within the dermal component of EVPOME transplanted into SCID mice (arrows). Vessel wall or red blood cells within the lumen are immunohistochemically stained with WGA. Magnification ×400.

EVPOME. This is an important point in that the dog model provided us the opportunity to simulate intraoral surgical procedures and to determine the ultimate fate of the cells of the EVPOME grafts in situ. These studies are not possible to carry out adequately in humans.

Histology of in vitro fabricated canine EVPOMEs, cultured submerged for 4 days, showed a continuous keratinocyte monolayer with no evidence of differentiation. The monolayer of epithelium stratified in the day 11 equivalents when raised to an air-liquid interface, as occurred in the human EVPOME. In day 18 equivalents, the epithelial layers continued to stratify and showed increased evidence of differentiation. Parakeratinization was evident in the most superficial layer as was seen in the native buccal mucosa (fig. 10).

Present studies are under way to transfect canine oral mucosa keratinocytes with GFP mediated by a lentivirus vector. GFP-positive cells are then sorted and seeded onto the dermal equivalent, AlloDerm, to fabricate canine EVPOMEs. The GFP-labeled EVPOMEs are grafted to the palate of dogs, protected with a surgical stent, and biopsied at specified times postimplantation.

Table 2. Microvessel count within EVPOME over time after grafting

	Days post-grafting		
	3	10	21
Control[a] (n = 5)	1.2 ± 0.4	2.6 ± 0.4	11.2 ± 3.6
D4E (n = 15)	1.7 ± 0.2	15.5 ± 5.0*	26.0 ± 5.4
D11E (n = 10)	2.4 ± 0.6	69.7 ± 7.5*, +	19.2 ± 1.9
D18E (n = 10)	3.0 ± 0.2*, +	68.6 ± 10.7*, +	28.5 ± 5.1*

Values represent mean ± SEM. Significantly different from control at * $p < 0.05$; significantly different from D4E at + $p < 0.05$. D4E = Day 4 equivalents.

[a] AlloDerm without an epithelial layer.

Human Clinical Trial in Niigata, Japan

The FDA requires, if available, data on the use of the cell-based device in humans. An approved human clinical trial was initiated in the fall of 2000 at the Dental School Hospital of Niigata University, Niigata City, Japan. The clinical protocol required a 5 × 5 mm punch biopsy of keratinized oral mucosa, which is done in an outpatient setting under local anesthesia [Izumi et al., 2003a]. The biopsy was taken prior to the surgical procedure in sufficient time, usually 4 weeks, to assure an adequate size of EVPOME is fabricated for the anticipated surgery. EVPOMEs were prepared in the standard fashion previously described and grafted to patients at day 11 of development, as was determined by the previous SCID mice study. All oral lesions were resected with the use of electrocautery. EVPOME was then grafted and sutured in

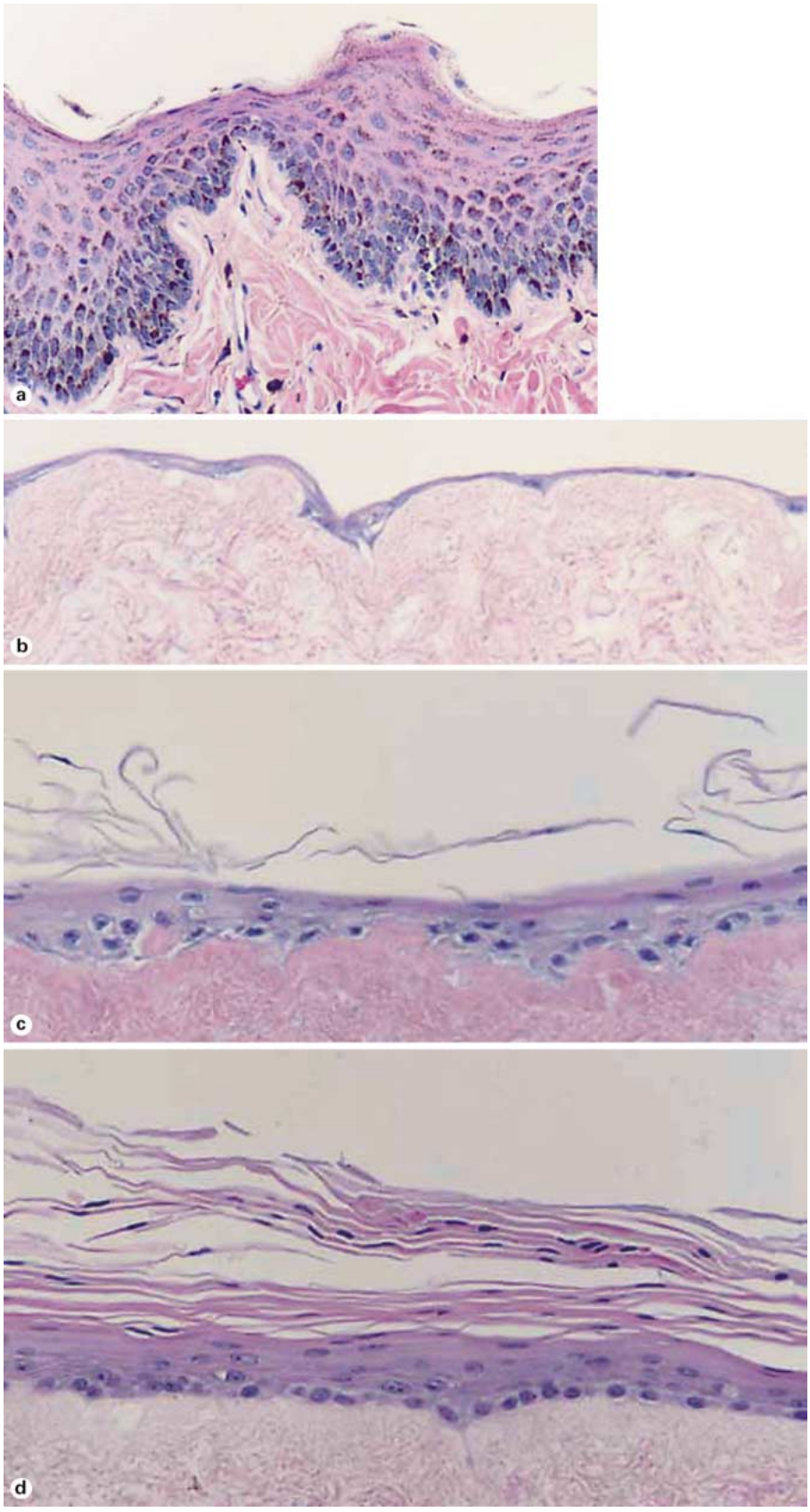

Fig. 10. Histological findings of native canine oral mucosa (**a**) and canine EVPOME of day 4 (**b**), day 11 (**c**) and day 18 (**d**). ×200. **a** Native canine buccal mucosa shows a thick well-differentiated epithelial layer with nonkeratinization. There is prominent pigmentation within the epithelial layer. **b** A continuous keratinocyte monolayer is developed in the day 4 equivalent. **c** The epithelial monolayer has become stratified in day 11 equivalents. **d** In day 18 equivalents, the epithelial layers continued to stratify and showed increased evidence of differentiation with nonkeratinization.

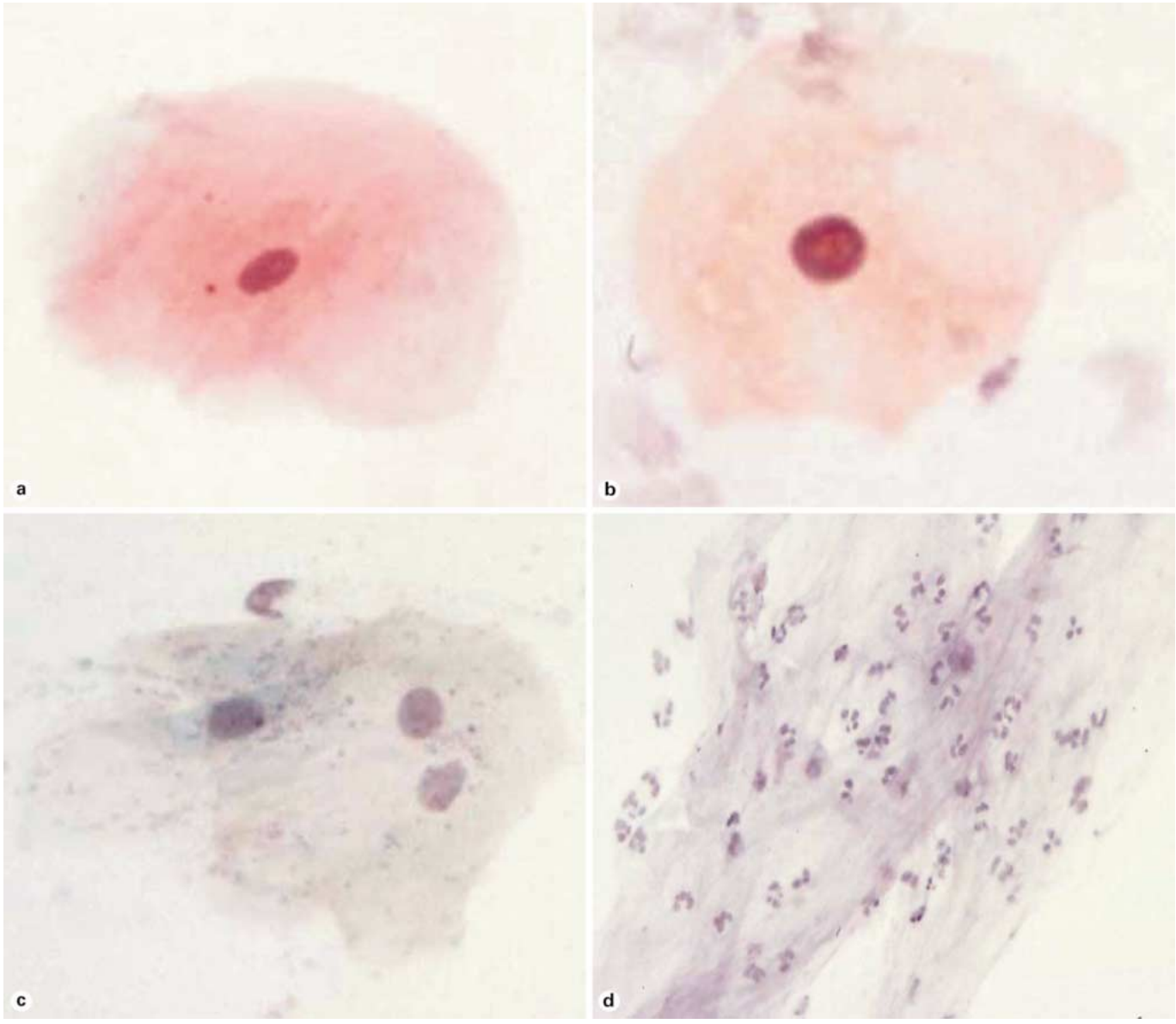

Fig. 11. Cytological smear with Papanicolaou staining performed at day 6 postoperatively. Squamous cells scraped off of the grafts with a cotton swab suggested the persistence of cultured oral keratinocytes on the EVPOME grafts (**a–c**); in contrast, there are only neutrophils and mucus scraped from AlloDerm grafts (**d**) which lacked a grafted keratinocyte layer.

place. A pressure dressing was applied at the time of surgery to prevent hematoma formation beneath the graft, which could interfere with vascularization of the dermal equivalent. As a control, the dermal equivalent, AlloDerm, without autogeneous keratinocytes was transplanted in selected patients. The pressure dressing was removed at 6 days postoperatively and a cytological preparation was made from the surface of the transplanted EVPOME. The presence of small, round-shaped cells suggestive of basal cells was indicative of the presence of grafted oral keratinocytes on the transplanted EVPOME (fig. 11).

Three variables were assessed at the time of removal of the pressure dressing at day 6: postoperative pain, adherence of the EVPOME to the underlying tissue, and the

Table 3. Clinical evaluation of the EVPOME (group 1) and AlloDerm alone (group 2)

	Group 1	Group 2
Postoperative pain	– (15)	– (15)
Adhesiveness	+++ (15)	+++ (13)
	++ (0)	++ (2)
Epithelial coverage	+++ (13)	+++ (0)
	++ (2)	++ (12)
	+ (0)	+ (3)
Required days for epithelialization	27.4 ± 1.2	46.0 ± 2.8

Postoperative pain: + and – units used for Visual Analog Scale (VAS) values ranging as follows: – = 0, + = 1–24, ++ = 25–49, +++ = 50–100. Adhesive: + and – units represent 'pull off strength': – = nonadherent, + = adherent but easily removed, ++ = adherent difficult to remove, +++ = adherent unable to remove. Epithelial coverage: + and – unit for % of epithelial coverage of grafted area at 4 weeks postgrafting: – = 0–50%, + = 51–75%, ++ = 76–99%, +++ = 100%. Required days for epithelialization: Values were expressed as the mean ± SEM. p value of < 0.05 indicates statistical significance.

degree of epithelial surface coverage of the transplanted graft, which was done at 4 weeks posttransplantation. Pain was assessed using a visual analogue scale, attachment by a subjective 'pulling' of the graft, and percent epithelial coverage was calculated by the formula of A – B/A (%). 'A' was the wound area transplanted with EVPOME or AlloDerm and 'B' was the area with exposed AlloDerm (lack of epithelial coverage). Areas of the grafts that had epithelial coverage appeared red, while white or opaque regions at the grafted site were seen with AlloDerm which lacked an epithelial layer (– = 0–50%, + = 51–75%, ++ = 76–99%, +++ = 100%) (table 3).

The Internal Review Board in Niigata University Dental Hospital approved only one time period for a biopsy of the grafts. This was done either at 2 or 4 weeks postgrafting. On histological examination at 4 weeks after surgery, EVPOME grafts showed a more mature, differentiated, overlying epithelium in contrast to AlloDerm alone which lacked the uniformity and epithelia regularity. An inflammatory response was noted within the dermal layer of the AlloDerm controls when compared to the EVPOME grafts (fig. 12). There was no clinical evidence of a foreign body response to either the EVPOME or AlloDerm grafts which all healed uneventfully [Izumi et al., 2003a].

Future Studies

Future studies entail shortening the time for fabrication of the EVPOMEs necessary for reconstructive surgery, as well as isolating a long-live transplanted cell that would be able to continue secretion of a transfected recombinant product, such as a cytokine or growth factor, over a sustained period of time. This will require the identification and isolation of a putative stem cell. Preliminary immunohistochemical studies have shown the presence of the nuclear transcription factor, p63, in both our human and dog EVPOMEs as well as native human and canine oral mucosa (fig. 4, 13). This is significant in that both the human and dog EVPOMEs are produced by identical methodology, thus reinforcing the validity of the use of our dog model as a precursor to human in vivo investigations.

Conclusions

There is presently a desire on part of the National Institute of Health (NIH) to increase clinical research and enhance the translation of basic science advances into the arena of patient care. Presently, the ability to successfully go from 'bench to bedside' is fraught with a litany of administrative guidelines. Initially, tissue engineering constructs were labeled as devices and came under the auspices of the Center of Device Evaluation (CDE). At present, cell-based devices, the group into which most tissue-engineered constructs fall, have been labeled as a 'combinational product', device-biologic, and fall under the perusal of CBER.

One of the first steps in this process is obtaining an approved IND application from the FDA. This requires a strict and detailed process of cell culturing techniques to assure that the cellular component of the tissue-engineered device does not come into contact with any material during the fabrication process that might in some way cross-contaminate the product and put the patient at risk. This necessitates growing cells in a defined chemical medium free of serum, pituitary extract, or irradiated feeder cells from a cell line. The cellular devices must be constructed under good manufacturing principles consistent with US government guidelines and each step of fabrication monitored for sterility.

The determination of dose and potency of a biological component is a unique problem in the use of cell-based devices that are not seen in pharmacological drug studies. There are known variations in each patient so it is not

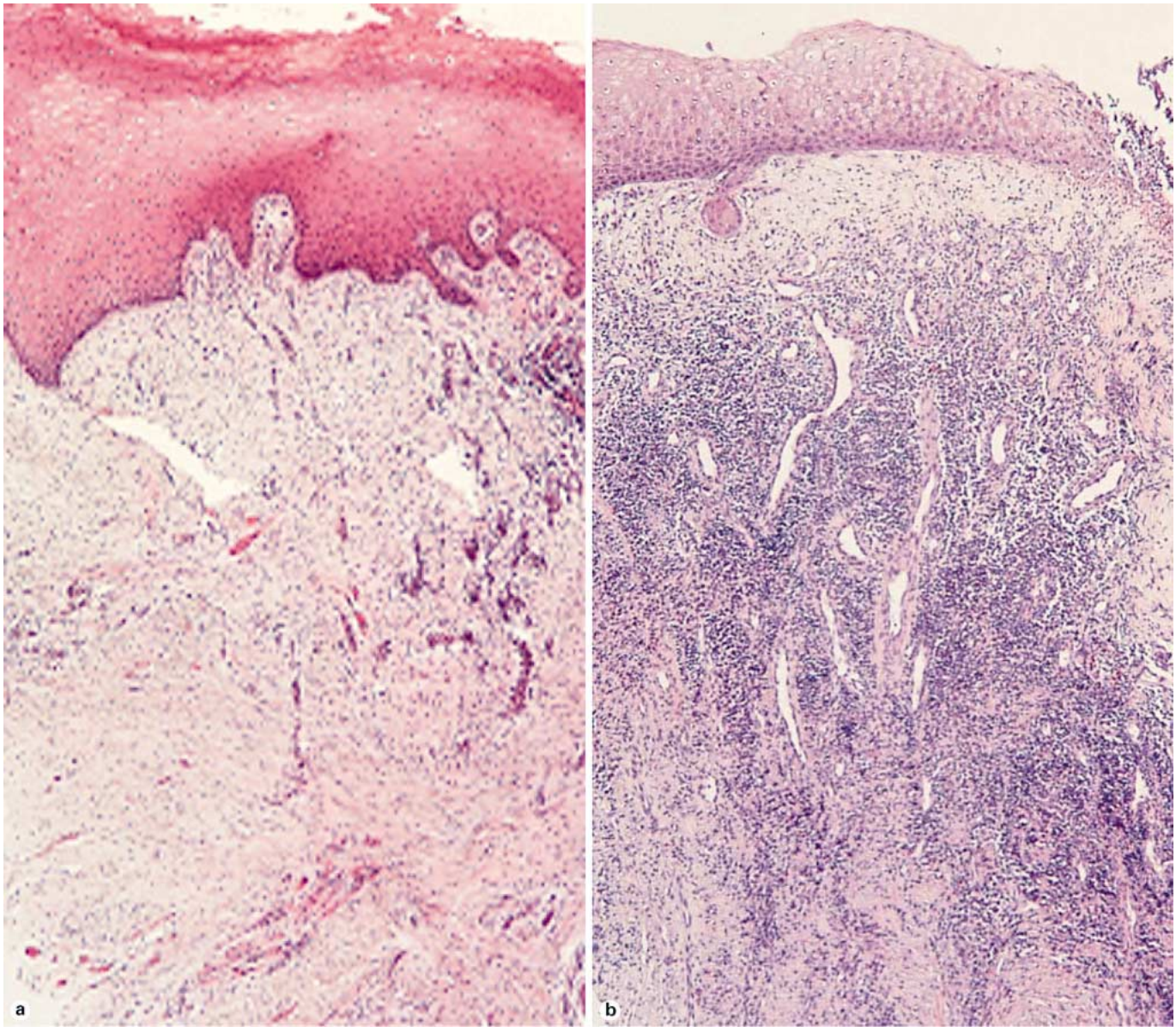

Fig. 12. Histology of a biopsy taken at day 28 after grafting of EVPOME (**a**) and AlloDerm without an epithelial layer (**b**). **a** In the EVPOME grafts, a thick differentiated epithelial layer in association with a decreased inflammatory cell infiltration is present within the dermal component. **b** However, there is disarray of the epithelial layer in the AlloDerm grafts. An intense inflammatory response and numerous diluted blood vessels are still persistent in the AlloDerm only grafts.

unexpected to see that each individual primary cell culture used to fabricate the EVPOMEs has varied levels of metabolic and proliferative activity. We have seen this in cell doubling times and secretion of vascular endothelial growth factor (data not shown). To a certain extent, potency and dose are directly correlated to cell viability. Thus, the ability to ascertain cell viability on the transplanted cell-based device becomes critical. This needs to be done in a manner that does not compromise its metabolic activity or the ability of the cellular component to divide. This necessitates the development of a noninvasive methodology to assess viability of the cellular compo-

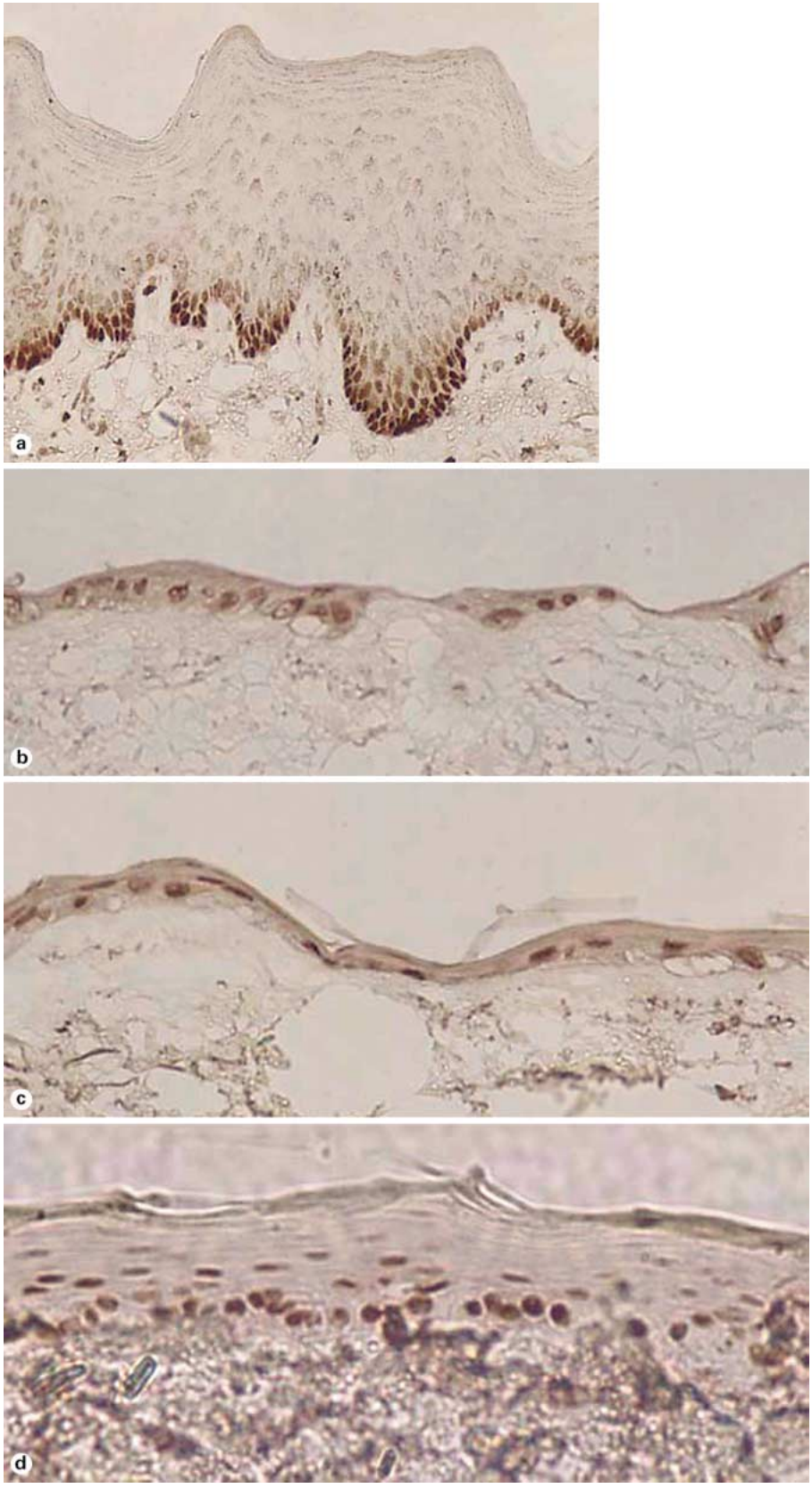

Fig. 13. Immunohistochemical staining for p63 of native oral canine mucosa (**a**) and canine EVPOME of day 4 (**b**), day 11 (**c**) and day 18 (**d**). ×200. p63 expression is restricted in the deep rete ridge basal layer and the lowest suprabasal layer cells in native canine oral mucosa (**a**). In canine EVPOME, basal layer cells and the lowest suprabasal layer cells continue to express p63 over days in culture (**b–d**).

nent of EVPOME, for example, the use of our glucose metabolism protocol. Studies are also planned to assess cell activity using microarray technology to determine the active gene products that might be correlated with cell viability and metabolic activity. This information becomes critical if one is also to assess quality control of the cell-based device at the time of completion of fabrication and after it has been transported to distant clinical sites. This point is critical if multicenter studies are contemplated, a necessity if eventual FDA approval for clinical use is the goal of the investigational studies.

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