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Cultured Mucosal Substitutes; from lab bench towards bedside?

Gekweekte orale slijmvliessubstituten, van laboratorium richting ziekenbed?

Proefschrift

ter verkrijging van de graad van doctor aan de Erasmus Universiteit Rotterdam
op gezag van de
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Chapter 1

Introduction and Outline

Introduction and Outline

Oral cancer is the sixth most common cancer in the world. It accounts for approximately 4% of all cancers and 2% of all cancer deaths worldwide. Malignant tumors of the oral cavity account for approximately 30% of all head and neck cancers. With an incidence rate of invasive lip and oral cavity tumors in The Netherlands rising to 6.6%, oral cancer is a common malignancy that often requires resection of the tumor followed by reconstruction (1,2).

In oral reconstruction, availability of mucosa is extremely restricted. Large intraoral defects are ideally reconstructed using like-with-like principles, with non-keratinizing, thin, pliable and well-vascularized tissue without significant wound contraction. Unfortunately mucosa is scarce and can only be reconstructed using skin using free or pedicled skin or musculocutaneous flaps from arm or leg. These techniques are well-established and successful. However, they have drawbacks such as donor site defects, bulkiness, sweating and hair bearing of the flaps (3-5). Therefore, a demand exists for further refinement using thin, non-keratinizing mucosa.

In order to develop thin mucosal flaps, tissue engineering strategies were explored for expanding mucosa *in vitro*. Techniques are based on the Rheinwald and Green technique for culturing keratinocyte sheaths of keratinizing keratinocytes from skin on a feeder layer of lethally irradiated fibroblasts. This technique grows $1m^2$ of keratinocyte sheath from $1cm^2$ biopsied skin within 3 weeks (6) and is used to produce keratinizing skin substitutes for treatment of e.g. large burns. Similar to burns tissue is scarce in mucosal reconstruction. Using tissue engineering strategies, it proved possible to take a small biopsy of mucosa, isolate and culture keratinocytes and fibroblasts *in vitro*, seed them onto a dermal carrier and produce a Cultured Mucosal Substitute (CMS) to reconstruct the defect. The protocol takes approximately 4 weeks to produce a CMS from biopsy to a graft with a multi-layered, differentiated epidermis and has been tested clinically.

The first clinical trials used collagen gels and membranes as dermal carriers and reported significant contraction of the CMS as a challenging problem (7-9). Other problems include infection, variable graft take (10) and high production costs. A number of problems can possibly be reduced by further *in vitro* and *in vivo* optimisation of protocols.

In design and optimisation of protocols of CMS, a number of *in vitro* conditions have to be met for successful transplantation (table 1) . The choice of the dermal carrier is one of the key decisions to take in design of a CMS. Protocols should minimise culturing times using

optimised isolation protocols and culture media. Furthermore, culturing protocol must be robust with minimum variability in outcome and infection rates. After transplantation, goals are achievement of maximum take and survival. The constructs must be easy to handle and easy to suture in place by the surgeon. After grafting, hypoxia occurs as CMS is a-vascular. The first days after transplantation of a CMS cells are supported by plasmic imbibition and diffusion of nutrients and oxygen to the cells. During these first days after transplantation grafts are under hypoxic conditions until vasculature invaded the CMS from the wound bed by means of angiogenesis. The process of angiogenesis should be optimized. Finally, contraction of the CMS should be minimized and, as a vast majority of patients receive post-operative radiotherapy, it should have reduced radiosensitivity.

***Table 1**: Determinants of successful culturing and clinical application of CMS*

		Factors for success
In vitro	Isolation protocol	- Straight-forward surgical protocol - Communication between laboratory and surgeon - Planning of isolation before reconstructive procedure - Bacterial and fungal contamination rates - Fibroblast contamination - Cells/cm^2 biopsy - % stem cells/cm^2 biopsy
	Costs	- Culture time - Isolation protocol - Technical difficulty protocol - % of successfully produced CMS - Infection rates
In Vivo	Surgery	- Communication between laboratory and surgeon - Logistics, e.g. transportation to OR - Mechanical properties, handling of CMS for surgeon - Resistance of CMS to handling by the surgeon - Straight-forward surgical protocol - Straight-forward wound protocol for surgical and nursing staff
	Graft take	- Angiogenesis - Resistance to hypoxia - Resistance to shearing forces - Susceptibility to Infection
	Long term	- Wound contraction - Resistance to radiation - Impairment of intra-oral functions such as speech

As mentioned, the choice of a dermal carrier is important for successful culturing and grafting. Dermal carriers reduce wound contraction and blistering compared to the use of

keratinocyte sheets alone. Various types of carriers are available and, such as collagen gels and acellularized, immunoligically inert, cadaver skin. To date, clinically used CMS were prepared using collagen gels. Clinical application of collagen gels, however is limited due to a number of problems. A collagen gel is mechanically weak; it cannot be sutured in place, in particular in the oral cavity where the tongue as well as the cheek is constantly moving. Secondly, a collagen gel contracts more than other dermal scaffolds, and is therefore less feasible for transplantation. On the other hand, cadaver skin that is acellularized and therefore immunologically inert, is commercially available as Alloderm® and clinically approved. Acellularisation is achieved by removal of the epidermis and subsequent radiation or soap treatment. Importantly, the basement membrane proteins remain present at the basal membrane site of the dermo-epidermal junction (DEJ), as well as in the remnants of the vascular basal membranes. This facilitates *in vitro* keratinocyte attachment and migration e.g. in wound margins (11,12). Furthermore, the DEJ is responsible for resistance to shear forces that could separate the epidermis from the dermis, resulting in blister formation. Also, endothelial cells invade the dermis using basement membrane proteins at the remnants of capillaries as guides (13).

Traditionally, acellularized cadaver skin is used for CMS in cultures without the supplementation of fibroblasts to the dermis. However, fibroblasts have various beneficial effects on the quality of CMS both *in vitro* as well as *in vivo*. Abdoel el Ghalbzouri, (LUMC Leiden, the Netherlands) developed a technique to centrifuge fibroblasts into a-cellular dermis (14). A clear improvement of epidermal morphology and a better formation of the DEJ as a result of the incorporation of fibroblasts in skin substitutes were seen using keratinizing skin keratinocytes. Moreover, fibroblasts made the addition of growth factors, such as Keratinocyte Growth Factor (KGF) and Epidermal Growth Factor (EGF) unnecessary (14,15). In addition, *in vivo* studies have shown that fibroblasts enhance angiogenesis (16). Clinical reports in patients with extensive skin loss, such as in burns, report reduced wound contraction and enhanced wound healing upon the addition of fibroblasts to a dermal matrix (16-18). These results suggest that incorporation of fibroblasts in non-keratinizing mucosal substitutes might lead to important improvement of the quality and clinical performance of CMS.

For clinical performance the behavior of CMS in hypoxia and its resistance to radiotherapy need to be studied. It has been demonstrated that epidermal hypoxia occurs frequently under physiological conditions (19). For example, wound margins become hypoxic immediately after wounding due to a reduced dermal blood supply by intravascular cloth

formation (20). Upon transplantation the a-vascular CMS becomes hypoxic as oxygen has to diffuse from woundbed to the cells. Semi- occlusive dressings render oxygen levels to a minimum in anticipation of angiogenesis to provide new vasculature with oxygen and nutrients.

Hypoxia has a number of well-documented effects on cells and tissues. It enhances the secretion of survival enhancing factors such as Hypoxia Inducible Factor 1 (HIF-1), which in turn induces the secretion of angiogenic factors, such as VEGF i-NOS and PDGF-B(21) (22). Furthermore, hypoxia induces metabolic changes enhancing production of glycolytic enzymes favouring anaerobic glycolysis and cell survival (23,24). The effect of hypoxia *in vitro* on cell morphology, proliferation, survival and growth factor secretion is not known.

Resistance or response of CMS to radiation therapy is another important clinically relevant issue as the vast majority of patients receive post-operative radiotherapy. In order to radiate this tissue, as a side effect, healthy mucosa is also irradiated. A major side effect of radiation therapy is oral mucositis. Clinical symptoms may vary from a mild erythema to severe, extremely painful ulcers in the mouth resulting in malnutrition and placement of a feeding tube (25). Incidences of 60% in patients receiving radiotherapy have been reported. For quite a few of these patients it requires breaks in therapy for periods up to several days or weeks and pain relief using morphine (26). This allows the defects to heal, while on the other hand oncological therapy is compromised and tumor cells can repopulate (27). Also, long term effects of radiation therapy, e.g. osteoradionecrosis, mucosal atrophy are related to the severity of the acute radiation response (28,29)

The aim of this thesis was to develop, improve and test a Cultured Mucosal Substitute as a step towards clinical application, to take a step from lab bench towards bedside.

Goals were to:

- Study the performance of current reconstructive techniques to identify possible issues that can be improved and challenges that the CMS will meet upon transplantation.
- Choose an optimal isolation protocol.
- Choose an optimal design of the CMS by choosing a dermal carrier and decide on the cell types included.
- Test our constructs behavior *in vitro* to clinically relevant environments and conditions such as hypoxia and gamma irradiation.

Various goals are discussed into detail in subsequent chapters of this thesis; **Chapter 2** reports functional and quality of life outcomes of the use of Free Radial Forearm Flaps, a commonly used flap in reconstruction of oral defects in the Erasmus Medical Centre in Rotterdam. The advantages and shortcomings of these reconstructions with skin flaps replacing mucosa are reported.

An overview of techniques and Tissue Engineering strategies are discussed into further detail in **chapter 3** of this thesis.

Chapter 4 describes the comparison of two widely used techniques for keratinocyte isolation using histology and cell size analysis to minimize culture time of keratinocytes and our CMS.

In **chapter 5** the effect *in vitro* of adding fibroblast to the CMS is decribed in order to enhance morhphology and performance while once again reduce production times. Cell morphology, cell differentiation, proliferation and apoptosis were studied.

Chapter 6 describes the effect of hypoxia on epidermal morphology, proliferation and apoptosis, as well as secretion of angiogenic growth factors in CMS.

Chapter 7 describes the effect of radiation on CMS *in vitro*, and the development and validation of a quantitative model for damage to CMS *in vitro*. Gross morphology as well as various markers for DNA damage and DNA repair were used.

Finally, in **chapter 8**, results are summarized and discussed with special focus on future directions of the (clinical) use of CMS in reconstructive procedures.

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Chapter 2

Long-term Functional Outcome and Satisfaction after Radial Forearm Free Flap Reconstructions of Intraoral Malignancy Resections

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Abstract

Purpose: Evaluation of objective and subjective functional follow-up results of intraoral radial forearm free flap reconstructions.

Methods: A total of 149 patients had received radial forearm free flaps between January 1996 and December 2005. Seventy-two patients completed an EORTC H&N35 questionnaire in the follow-up study. Patients were divided according to location of defect (anterior or posterior) or irradiation. Thirty-nine patients with anterior positioned flaps received standardized physical examinations.

Results: Flap survival was 99.3%. Complications were divided in early (< 2 weeks; 23%) and late complications (20%). Most common complications were dehiscence or fistula, responding well to conservative treatment. Analysis of questionnaire subscales showed no statistically significant differences between anterior and posterior defects. Irradiation showed significant impairment for the ability to smell and taste. No important donor site impairment was found.

Conclusion: The radial forearm free flap is an adequate method for reconstructions after resection of intraoral malignancies. Subjective functional outcome seemed to be defined by adjuvant radiotherapy, patient coping, and to a lesser extent flap bulk for anterior defects.

Keywords: radial forearm free flap, head and neck cancer, quality of life, microsurgery

Introduction

Radial forearm free flaps for intraoral reconstructions have been investigated extensively (1). Versatility of the radial forearm free flap in combination with low flap loss and complication rates traditionally have been the most important factors for choosing this flap to restore inner lining if no bulk was needed(2). In contrast, others have emphasized the benefit of radial forearm free flap bulk, which is necessary for swallowing after partial glossectomy or soft palate reconstruction(3).

Over the years, however, the classic radial forearm free flap for intraoral reconstruction has been under scrutiny due to newer flaps (4,5). Concerns with the radial forearm free flap consisted of excessive flap bulkiness due to replacement of very thin oral mucosa by skin and subcutaneous tissue, or insufficient bulk when reconstructing large defects of the area involved with deglutition. To overcome excessive bulkiness, efforts have been made to decrease flap thickness by applying oral mucosa to fascial forearm flaps without the skin component (6). To overcome insufficient bulk, alternate donor sites have been investigated (4,5). Donor site concerns consisted of the need to sacrifice the radial artery, decreased sensation in the course of the superficial radial nerve, decreased overall function of the hand and an unsightly donor site scar. A few studies have addressed these issues, generally showing acceptable cosmesis and normal function, especially after suprafascial radial forearm flap harvest (7,8).

In literature, emphasis exists on technical refinements of reconstructive procedures to optimize functional results; however, little has been published on long-term subjective or objective functional outcome and associated quality of life with respect to individual reconstructive techniques (9,10).

The purpose of the current study was to evaluate perioperative flap complications, as well as oral functional outcomes and quality of life in patients who had radial forearm free flap reconstructions after resection of intraoral malignancies. In this fashion, the validity of its continuing use in head and neck reconstruction was evaluated.

Patients and methods

Patient and Tumor Characteristics

A database search resulted in 149 patients who had received a radial forearm free flap for intraoral reconstruction after resection of an intraoral malignancy between January 1996 and

December 2005. Medical files were used to retrospectively retrieve patient characteristics, medical history, surgical data, and complications. Tumor characteristics are presented in table 1. The group consisted of 86 male and 63 female patients with an average age at time of operation of 58 ± 11 years (± SD; range 22 to 82 years). Risk factors in this group consisted mainly of smoking (59%), alcohol abuse (26%), cardiovascular impairment (28%), and pulmonary impairment (9%). In 52 patients (35%) more than one of these risk factors was present. The entire group of 149 patients was evaluated for perioperative complications.
Follow-up Study Population Characteristics

Since 64 of 149 patients had died, 85 patients were potentially available for inclusion in the follow-up study. Thirteen patients either declined to participate or were no longer traceable. The follow-up study group, therefore, consisted of 72 patients (44 male, 28 female) with a mean age of 57 ± 11 years (± SD; range 33 to 79 yrs). Patients were divided into two groups according to the location of the defect. Group I with a tumor located at the floor of mouth and/or mobile tongue consisted of 39 patients (25 male and 14 female) with a mean age of 57 ± 12 years (± SD; range 33 to 78 yrs). Group II with a tumor location in the posterior oral cavity consisted of 33 patients (19 male and 14 female) with a mean age of 58 ± 9 years (± SD; range 41 to 71 yrs). Risk factors in the follow-up study group consisted of smoking (I, 51%; II, 58%), alcohol abuse (I, 18%; II, 9%), cardiovascular impairment (I, 31%; II, 36%), and pulmonary impairment (I, 8%; II, 12%). In 33% of patients in each group more than one of these risk factors was present. Radiotherapy had been used as adjuvant therapy in 79% of patients included in the study (79% in group I and 79% in group II).

To rule out a possible selection bias, several patient characteristics (age, sex, location of defect, flap size, radiotherapy, and postoperative complications) between 72 responders and 77 non-responders were compared. Chi-square and Fisher's exact tests, Mann-Whitney U tests and Student's t-tests did not reveal statistically significant differences (p-values > .05; data not shown) between responders and non-responders, so we presume that participants are representative of the total group.

Procedure

All patients (n = 85) were invited by a letter explaining the study and asking them to participate after approval of the ethical committee of the local research institution. An EORTC H&N35 questionnaire was sent, which they were requested to complete and return by means of a stamped envelope. The questionnaire was returned by 72 patients (85%).

All patients (n=39) in group I also visited the outpatient clinic. During this visit, a standardized physical examination of the oral cavity was performed. Patients with a radial forearm free flap reconstruction of the posterior part of the oral cavity (group II) were not invited to the outpatient clinic since it was felt that reliable inspection of these flaps would be too invasive and add no further information to that given in the questionnaire.

Table1: Tumour characteristics and staging in study group patients

Tumour characteristics	No. of Patients
Tumour type	
Squamous cell carcinoma	139
Adenoid cystic carcinoma	2
Carcinoma in situ	2
Muco-epidermoïd carcinoma2	
Adenocarcinoma	2
Undifferentiated carcinoma	1
Osteosarcoma	1

Tumour stage (TNM classification)

	0	1	2	3	4	x
T	*na*[a]	4	60	57	20	8
N	64	27	46	3	*na*	8
M	139	0	*na*	*na*	*na*	10

[a]na, not applicable.

Follow-up Study of Functional Outcome and Satisfaction

Differences in functional outcome and satisfaction were studied between group I and II, as well as between irradiated and non-irradiated patients. Subjective functional outcome and patient satisfaction after radial forearm free flap reconstruction in the oral cavity were studied by using the EORTC H&N 35 questionnaire. This questionnaire was specifically designed for patients with a malignancy of the head and neck region (11). It is a validated instrument for measuring functional outcome and satisfaction, which assesses symptoms directly related to site of reconstruction. It also comprises items concerning side effects of treatment, social function, body image, and sexuality. Results are given on a scale from 0 (no limitations) to 100 (severe limitations). A change of 10 units on these response scales have been regarded as clinically important(11). Functional outcome and satisfaction were evaluated in general as well as in relationship to various risk factors.

Standardized physical examination of the oral cavity was performed by an independent investigator to prevent inter-observer bias. Flap appearance and consistency, and tongue mobility were assessed. Sensitivity was tested with static and moving two-point discrimination using a previously developed set of intraoral two-point discriminators (12).

Statistical Analysis

Chi-square tests, Fisher's exact tests, Mann-Whitney U tests, and Student's t-tests were used. Two-tailed p-values ≤ .05 were accepted as statistically significant.

Results

Medical Data

Patient characteristics

Defects that were reconstructed with radial forearm free flaps were located in the anterior floor of the mouth and/or mobile tongue in 90 cases, and in the posterior part of the oral cavity in 52 cases. The remaining 7 defects were located on the inner cheek (Table 2). In 10 cases an osteocutaneous radial forearm free flap was used because of a segmental mandibular defect. The superior thyroid (54%) or facial (25%) arteries were mainly chosen as recipient vessels. Venous anastomoses were mainly made to the internal jugular (67%) or superior thyroid (12%) veins. Total operating theatre time for the combined ablative and reconstructive operation was on average 11.4 hours ± x (± SD; range 6 to 18 hours). Donor site closure was originally by split thickness skin graft exclusively, but changed to full thickness graft V-to-Y transposition (8) in most defects during the second half of the study period because of surgeon preference. Postoperative radiotherapy of 66 or 70 Gy was administered to 108 patients (72%) according to the protocol of the multidisciplinary head and neck team of our hospital.

Post-operative complications

Post-operative complications were divided into early (within two weeks) and late complications. Eighty-nine patients (60%) had an uneventful postoperative course. Critical review showed early complications in 34 patients (23%) and late complications in 30 patients (20%). One patient had insufficient perfusion of the hand requiring intraoperative placement of an interposition vein graft. Twelve patients (8%) required re-operation within 24 hours

because of compromised flap vascularization. In five patients venous thrombosis was found and in four patients both the artery and vein were occluded, which prompted microvascular revision resulting in one partial and one total flap necrosis. Persistent post-operative bleeding and/or hematoma compromising flap vascularization required re-operation in the remaining three cases. Overall flap survival was 99.3%, with one total flap loss. Other early complications were partial wound dehiscence and/or fistula formation (n = 17) and hematoma (n = 3), which responded well to conservative treatment. Late complications consisted of mandibular osteoradionecrosis requiring further reconstructive surgery with fibula free flaps (n = 3) and one neck abscess which had to be drained. One patient required removal of infected internal fixation material. Late complications, responding well to conservative treatment, were dehiscence and/or fistula formation (n = 16). In one case a permanent tracheostomy was necessary.

Table 2: Tumour location in study group patients

Tumour location	No. of Patients
Anterior	
Floor of mouth	41
Mobile tongue	38
Floor of mouth combined with tongue	11
Posterior	
Tonsil	21
Soft palate	9
Retromolar trigonum	8
Posterior alveolus	5
Pharyngeal wall[a]	6
Base of tongue	3
Other	
Inner cheek	7

[a]Combined with one or more of the above mentioned structures.

Subjective Follow-up Results of Functional Outcome and Satisfaction

The average follow-up was 43 ± 27 months (± SD; range 2 to 120 months). Analysis of the EORTC H&N35 questionnaires resulted in the following individual results of oral function.

Speech

No difference in mean speech impairment between groups I (38 ± SD 26) and II (28 ± SD 21) with different defect locations were seen (Mann-Whitney U test; $p>0.05$). Speech scale scores around 30 indicate moderate speech problems.

Mastication and swallowing

No statistically significant differences were found between the mean scores for 'swallowing' in groups I (30 ± SD 32) and II (28 ± SD 23) (Mann-Whitney U test; $p>0.05$). The scores of two other items of importance for mastication 'dry mouth' and 'sticky saliva' also showed no significant differences.

Effect of postoperative radiotherapy

Postoperative radiotherapy was administered to 57 of 72 patients (31 in group I, 26 in group II). In 15 patients no radiotherapy was necessary (8 in group I, 7 in group II). EORTC H&N35 questionnaires showed a statistically significant difference for the subscale 'senses' (problems with smelling and tasting), which scored worse in the irradiated group (mean 31 ± SD 29) versus the non-irradiated group (mean 13 ± SD 22) (Mann-Whitney U test $p<0,05$). The subscale 'speech' scored worse in irradiated patients (mean 36 ± SD 24 versus mean 12 ± SD 24), however, only a trend was seen since no statistical significance was reached (Mann-Whitney U test; $p=0.07$).

EORTC H&N35 questionnaires did not show significant correlations between dysfunction and follow-up time since their reconstruction. In addition, no significant relationships between known risk factors and functional outcome were found in the study group (data not shown).

Objective Data

Physical examination

Intraoral inspection in 27 of 39 patients from the group with anterior defects showed the flap had blended into the surrounding mucosa. Ten of 39 patients had a bulky flap for that anterior location. These bulky flaps seemed to further decrease tongue mobility. Moving two-point discrimination reflected protective sensitivity in 24 patients, but was larger than 12 mm in all patients. No reproducible data were obtainable in 8 patients. Three patients had hypersensitivity in their flaps causing a burning intraoral sensation.

Donor site outcome

All donor sites have been extensively investigated previously. There was no decline in range of motion of wrist and fingers. Grip strengths were within normal range in accordance to age (8).

Discussion

The current study presents perioperative flap complications as well as objective and subjective oral functional outcomes in patients who had a radial forearm free flap reconstruction after resection of intraoral malignancies over a 10-year period. The radial forearm free flap was investigated to validate its continuing use in head and neck reconstruction. The radial forearm free flap has not been fully embraced by all reconstructive surgeons mainly due to the perceived donor site limitations, but also due to excessive or limited bulk for specific indications. Alternative flaps such as the lateral arm flap or the anterolateral thigh flap have been proposed as workhorse flaps (4,5).

In this study almost two-thirds of the defects were located in the anterior part of the oral cavity (i.e. floor of the mouth, mobile tongue) and one third was used for posterior oral cavity reconstruction. Microsurgery related complications were very low with the need to reoperate due to arterial and/or venous thrombosis in 6% of cases leading to an overall flap survival rate of 99.3% (13,14). This indicates that the radial forearm free flap is a reliable and safe flap to perform. Postoperative complications like dehiscences, fistulas, and hematomas were well within the range of previously reported data using different flaps for head and neck reconstruction (15,16). Donor site complications had been thoroughly investigated in part of this group previously and shown no serious problems (8).

The EORTC H&N 35 questionnaire was used to evaluate subjective functional outcome and satisfaction of the study population, since reported validity and reliability are adequate and the various subscales are highly sensitive to difference within and between groups of patients (11). This is different from previous reports, which have mentioned objective functional outcome results in small groups for speech, deglutition, and swallowing as evaluated by videofluoroscopy (17). In our experience these objective results often do not match subjective satisfaction. We hypothesized that the group with anterior defects would have more speech problems and that the group with the posterior defects would have more swallowing problems. Both groups scored moderate impairment of speech and swallowing,

however, no significant differences were found. The lack of statistically significant differences most likely was caused by difference in individual coping of patients with their situation. This has been pointed out previously as well from patient data where defects and objective outcome data completely contradicted subjective outcome expectations (14).

In addition, all patients who had been irradiated were compared to those who had not received radiotherapy. Irradiated patients showed a statistically significant deterioration in their ability to smell or taste as well as a strong trend for impaired speech. Here too individual coping of patients seemed to play an important role for outcome. In line with Smith et al. (9) the negative effects of irradiation on functional outcome and satisfaction, however, seemed clearly present.

Non-invasive physical examination was performed in patients with anterior radial forearm free flap reconstructions only. Inspection revealed too bulky flaps, which additionally limited tongue mobility in 25.6%. Sensitivity testing proved difficult, as experienced previously (12). Protective sensitivity was not evident in every third patient, however, recurrent intraoral defects were not reported to be major a problem by patients. This unclear recovery of sensation indicates a potential benefit of using sensate flaps, which is not routinely performed at our institution.

The major flaw in this study is the diversity of the defects and associated treatments. It is impossible to perform statistical analysis for all different subgroups since they would become too small. Also, the difference in coping of different patients with a similar situation makes that some patients will be satisfied with the exact same situation with which other patients will be very dissatisfied. Nevertheless, we believe that due to the rather high number of similar reconstructions some conclusions can be justified from the present findings.

The radial forearm free flap is an easy and versatile flap with minimal donor site morbidity. It is still a valid method for reconstruction after resection of intraoral malignancies. Patient coping and postoperative irradiation influence functional outcome and patient satisfaction. Flap bulkiness plays a more limited role for anterior defects.

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Chapter 3

The Emerging Field of Tissue Engineering

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1. Introduction

Substitution of lost tissue due to trauma, congenital defects, or resections is a challenge that plastic reconstructive surgeons are confronted with on a daily basis. Technical advances in the fields of microsurgery, allogeneic and autologous grafting has put the plastic surgeon at the forefront of medical science.

A recent and rapidly growing set of tools for the reconstructive surgeon is being developed in a field known as tissue engineering. The goal of tissue engineering is to develop biological substitutes to restore, maintain or improve functionality of tissue (1). In other words; replacement of lost or damaged tissue by tissue grown in a laboratory. Engineered tissue can be manipulated and formed in a way that it meets the specific needs of a patient. Clinical use of for example cultured skin substitutes (2-4) in burn therapy and cartilage cells for repair of hyaline cartilage defects in the knee joint (5,6), illustrates the fact that tissue engineering is moving from lab bench to bed side.

The basic technique of tissue engineering can be broken down into two ingredients; cells and scaffolds (**figure 1**).

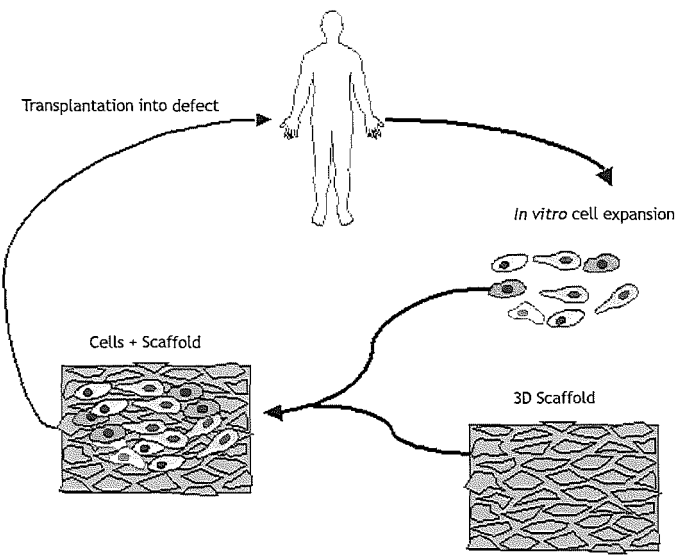

Figure 1: *Basic principles of tissue engineering. Cells are isolated from a biopsy, cultured to expand cell numbers and combined with a three dimensional scaffold in vitro. Subsequently, this construct is transplanted to the patient.*

Cells are typically isolated from a biopsy and are grown *in vitro* before use. Cell culturing times are related to dividing times of the cells and culture techniques, and will be discussed later in this chapter. For example, epidermal keratinocytes from a skin biopsy of an area of $1cm^2$ have a doubling time of approximately 24 hours and can be grown to a surface of about $1m^2$ in approximately three weeks.

Cultured cells are often combined with the second major component, a three-dimensional matrix or scaffold. Scaffolds can be composed of a number of materials with distinctive characteristics that influence the behavior of the matrix (e.g. degradation times) and of the seeded cells (e.g. cell attachment), and will be discussed later in this chapter. After settling and attachment, the cells are guided by the scaffold to establish an organized three-dimensional tissue structure.

The first part of this chapter will focus on the two basic components of tissue engineering, cells and scaffolds. The second part will discuss the current techniques used to grow and fabricate these two components into specific tissues of interest to the plastic reconstructive surgeon.

2. Scaffolds

Scaffolds in tissue engineering are either temporary or permanent three-dimensional frameworks used as a matrix for cells to attach and grow on. They are biocompatible and are usually comprised of materials that are natural or synthetic in origin. Some scaffolds are non degradable, such as Dacron or Teflon vascular constructs seeded with endothelial cells. However, most scaffolds used for implantation purposes, are composed of degradable materials. There are many scaffold materials used in tissue engineering, we will discuss some of the most popular biodegradable scaffolds, composed of natural or synthetic materials.

A. Natural scaffolds

1. Acellular tissue scaffolds

Tissue, which has been treated to remove all cells in a process known as decellularization, is used as a scaffold to seed cells. Sources of the starting tissue can be allogeneic or xenogenic in origin. Procedures such as freeze-thaw cycles and washes with detergent are used to kill and remove all immunogenic living cells in the tissue. What remains is the immunologically inert extracellular matrix of the tissue. Most of the structural proteins (e.g. collagen, and

elastin) remain intact. This acellular material can subsequently be seeded with the cells of choice. For example, acellular dermis has been used as a skin replacement in treatment of burn wounds and reconstructive procedures (7,8).

2. *Type I collagen*

Collagen is the major component of the mammalian extracellular matrix. It provides tensile strength, and flexibility in tissues, such as skin, tendon and bone. Of the 14 types described, collagen type I makes up approximately 90% of all fibrous proteins, thereby being the most abundant type. Collagen has long been used in the medical setting in the form of sponges, fleece or fibers for haemostatic purposes.

Collagen is often isolated from bovine skin, bovine tendons or rat-tail tendons by an acid extraction procedure. *In vitro*, after neutralizing the pH, the collagen can be re associated to form fibers or gels. Resorption of collagen polymers occurs by lysosomal enzymes, secreted by surrounding cells.

To adjust the characteristics of collagen fibers to specific needs, the fibers can be cross-linked to various extends to provide additional strength. Besides adding strength, cross-linking increases the degradation time. Cross-linking reduces the rate of in growth of tissue into the collagen-based scaffolds and also reduces the ability of the polymer to absorb water (9). The collagen I polymers can be crosslinked with glycosaminoglycans. Addition of glycosaminoglycans provides a tool to optimize degradation times, pore sizes and elastic characteristics of the newly formed tissue after grafting (10-13). This technique is used in the case of the dermal substitute Integra® (Integra / LifeSciences corp.) (**figure 2**).

Glycosaminoglycans are large, negatively charged molecules in the extracellular matrix. The GAG's provide compressive strength, shock absorption and turgor to the tissue by attracting water to the extracellular matrix. In the body, they are abundant in tissues such as cartilage and dermis.

Examples of GAGs are hyaluronic acid, chondroitin sulphate, dermatan sulfate, keratan sulfate and chitin. GAGs are isolated from shark cartilage (chondroitin-6-sulfate), rooster combs or are produced by a microbial fermentation process.

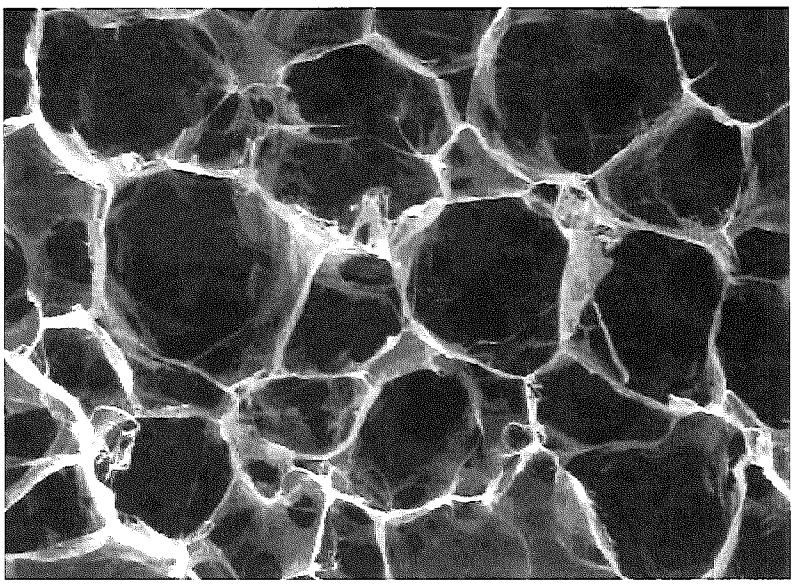

Figure 2: Highly porous sponge of bovine collagen type I and glycosaminoglycans (Picture courtesy of Integra LifeSciences Inc.)

B. Synthetic scaffolds

Certain synthetic polymers have been used to fabricate bioabsorbable sutures, pins, and fibers long before their use as a scaffold material in tissue engineering. They are usually fabricated from monomers that are of natural metabolites. Most commonly used are members of the α-hydroxy-acid family, such as glycolic acid (forming poly (α-glycolyc-acid) or PGA), lactic acid (forming poly (lactic acid) or PLA). For use in tissue engineering, the monomers can be polymerized into the form of fibers, fleeces, foams or gels.

Copolymers of two or more monomers are often used. By varying the percentages of the components, characteristics of the resulting material can be changed. For instance, PGA is hydrophilic, and has a short degradation time. PLA however, has a longer degradation time and has a more hydrophobic character. PLA has better mechanical properties than PGA. When making a copolymer of the two molecules (forming the copolymer lactic-co-glycolic acid, or PLGA), these properties can be mixed, resulting in a polymer with the mechanical properties of PLA, and the hydrophilic and resorption properties of PGA. This product is on the market as Vicril© (Johnson & Johnson).

Once placed in the body, exposure to body fluids and cells leads to degradation of the polymers by hydrolization. The degradation products of the polymers are their monomers, lactic acid or glycolic acid, which are eventually converted into CO_2 and water by the Krebs cycle (14,15). The acidic metabolites however, can cause lowering of the pH in the local environment of the scaffold, which can result in an inflammatory reaction in the surrounding tissue (16,17). Furthermore, if the surrounding tissue is not capable of removing the byproducts due to insufficient vascularization, the accumulation of osmotically active degradation products can lead to hyperosmolarity of the tissue, leading to fluid accumulation and non-specific foreign body reactions. This problem is documented in the use of PLGA screws used in bone fixation. The degradation of the PLGA was associated with a non-specific foreign body reaction in 7.9% of a population of 516 patients (18).

C. Design of scaffolds

For the design or choice of a scaffold, various aspects are taken into consideration (**table 1**). Optimization of these variables results in ideal cell seeding, cell attachment, cell growth, diffusion of nutrients, revascularization and degradation times of constructs.

For example, an increase in the total number of pores and surface area of pores per volume provides a larger surface area for cells to attach (11). Coating scaffold materials with substances, such as collagen, fibrin, or other surface adhesion proteins further improves attachment of cells. Porous structures make it possible for cells to grow into multi layers, facilitates diffusion of nutrients and ingrowth of vasculature from the surrounding tissue. Pore sizes can be varied using salt-leaching techniques. In this process, scaffold material is mixed with water-soluble salt crystals that have a size similar to the desired pore size. After polymerization, the crystals remain in the matrix and can subsequently be washed out with water, leaving behind a porous structure. The resulting pores are relatively homogenous in size and distribution. Pore sizes and shapes can be changed by using different salts .

Diffusion of nutrients is required for survival of cells in a scaffold. *In vitro,* nutrients from the culture medium feed the cells. Since tissue engineered constructs are typically avascular, during the first days after transplantation, the supply of nutrients for transplanted cells *in vivo* depends solely on diffusion from the wound bed. After grafting, formation of a new vascular network occurs by ingrowth of vasculature from surrounding tissue, providing a more solid nutrient supply to the cells. This limited distance of diffusion severely reduces the maximal size of the constructs.

Table1: Some considerations in designing and choosing scaffold materials

Biological properties	Biocompatibility
	Cell attachment
	Immunologic responses of host to scaffold materials
	Risk of disease transmission
	Sensitization host for scaffold material
	Possible bioactive properties (e.g. BMP inclusion into matrix)
Mechanical properties	Stress-strain behaviour
	Pore size
	Surface area per volume
	Number of pores per volume
	Inter-pore connections
Chemical properties	Surface chemistry (e.g. coating with cell adhesion molecules)
	Degradation rate
	Byproducts of degeneration (e.g. lactic acid in PLA degradation)
Manufacturing properties	Technical demands and costs of production
	Reproducibility of morphology and biological properties
	Possibilities for large-scale production
	Costs of raw material
	Sterility

Degradation rates vary from days to years depending on the size, structure and chemical components of the materials used (9). Half-lives of scaffolds can be adjusted to the time cells need to deposit sufficient autologous extracellular matrix material to support themselves in a three dimensional structure. Procedures, such as cross-linking and co-polymerization provide ways of manipulating this important characteristic of scaffolds.

3. Cell isolation and culture

The ability to culture and grow mammalian cells in a controlled and aseptic environment has made countless advances in research possible and has opened up the use of new, cell-based, therapies. In this part of the chapter, we describe how cells are obtained from various tissues sources, how cell cultures are initiated and kept in culture for extended periods of time and how complex liquid media that make cell culture possible are formulated.

A. Cell isolation

For isolation of a specific cell type a small piece of normal tissue is obtained by biopsy, and minced up into small pieces. These pieces are subsequently treated with proteolytic enzymes, such as trypsin or collagenase, to digest proteins involved in cell-cell contacts and in the extracellular matrix. This process frees the cells from the surrounding extracellular matrix and generates a suspension of single cells.

B. Cell culture

Cell suspensions isolated from tissues are plated into a tissue culture dish, along with a rich liquid culture medium. Cells settle, attach and spread on the surface of the tissue culture dish. This is the first or primary cell culture. After an initial lag time of 1-2 weeks, the cells become adapted to the culture environment and begin to undergo cell division that can continue for many weeks depending on the cell type.

As cell numbers increase, a state of confluence is reached, and the cells need to be plated onto new dishes. This process is known as serial passage or subculture. By using serial passage of cells, cell division can continue at a relatively constant rate for many weeks and give rise to many subcultures and a large number of cells. For example, normal diploid human dermal fibroblasts of the skin can be readily cultured *in vitro*. From only a $1cm^2$ skin biopsy it's possible to establish a primary culture of a few thousand dermal fibroblasts. Under appropriate culture conditions, human fibroblasts can be serially passaged about 10 times, which yields to a total of approximately 10^{16} cells.

Cells in culture are able to perform a wide range of highly differentiated activities characteristic of their respective roles *in vivo*. For example, dermal fibroblasts secrete and assemble collagen fibers in culture. However, cell strains may differ in their inherent ability to perform these differentiated functions under certain *in vitro* conditions. For example, unlike the fibroblasts, chondrocytes lose these differentiated functions rapidly in a two dimensional culture. The cells lose their ability to produce collagen type II and start producing collagen type I, a process known as dedifferentiation, discussed later in the chapter.

A wide variety of culture media and culture conditions have been developed over the years to facilitate the culture of specific cell types. In fact, each cell type has its preferred medium formulation and culture conditions, nevertheless, there are some fundamental common features.

Table2: Some components of a typical culture medium

Basal medium components	Function
Sodium chloride	Osmotic pressure
Inorganic salts	Provide electrolyte balance similar to blood
Sodium bicarbonate	Provides buffering capacity; with appropriate CO_2 level in the gas phase, maintains pH at 7.4
D-glucose	Source of energy, carbon
Amino acids	Source of nitrogen for protein synthesis
Vitamins	Co-factors in various intracellular biochemical reactions
Phenol red	Visual pH indicator
Serum	Provides cell growth and attachment factors, hormones, carrier proteins
Growth factors, hormones	Stimulate growth, function
Antibiotics	Prevent contamination by microorganisms

The major components of a typical liquid medium to culture cells are summarized in Table 2. Some aspects of the medium's composition (osmolarity, pH) are clearly meant to mimic blood and other physiological solutions. However, in many cases, the concentrations of nutrients and hormones/growth factors can be several folds higher than blood. Overall, culture media are typically very rich in nutrients in order to sustain growth and provide cells with an ideal culture environment. Through a process of trial and error, culture media have been optimized for the growth of different cell types.

One of the main functions of medium is to provide sources of energy to the cell. The most important sources of energy in the medium are glucose, Pyruvate, and the amino acid glutamine. Glutamine, the essential amino acids in the medium and the other amino acids are also used for protein synthesis. Addition of non-essential amino acids is often desirable since intracellular synthesis of these amino acids can be a significant drain on the cell's energy pool. Complex medium preparations may also contain nucleosides, critic acid cycle intermediates, and lipids, which may be necessary when very low serum levels are used.

One of the most common and important growth supplements is serum. Although serum is not a well-defined component and may exhibit batch-to-batch variation, it contains a wealth of factors that aid cell attachment and growth. In addition, the large load of carrier proteins provided by serum can help scavenge toxic impurities inadvertently supplied by the water, reagents, or cell culture apparatus. Serum is widely used and is generally added to the basal medium formulation in a proportion of 1 to 20% of the volume. The type of

serum most widely used is calf bovine serum, followed by fetal bovine serum that is used for more demanding cell lines. Human serum can be used for human cell lines, and can be autologous, to prevent transmission of disease.

Cells in culture can be divided into two groups, anchorage dependent and anchorage independent cells. Anchorage dependent cells grow attached to a substrate. The nature and composition of this substrate is critical for cell function, these cells do not grow unless they are attached. Chondrocytes and some blood cells are able to grow without attachment. In these cases, the cells are referred to as anchorage independent. These cells grow embedded in a semi-solid agarose gel or as small spheroids or aggregates of cells in suspension in the liquid medium. For the majority of cells, attachment and spreading on a substrate are required for survival, growth and cell function.

Knowledge of the two components has of tissue engineering has resulted in various products that are in clinical use or in development. We will describe some tissue-engineered products that are currently in use.

4. Tissue engineered skin

The standard for treating large skin defects is still closure with a full thickness or a split-thickness autograft. Harvesting sites for autologous skin can be scarce, especially in the severely burned patient, and so, ways of growing and expanding cell numbers *in vitro* have been of interest. Research towards bioengineering skin substitutes has proceeded along two important lines; (1) the optimization of *in vitro* methods for the culture and proliferation of cells of the skin and (2) the development of biomaterials which mimic important properties of the skin.

A. *In vitro* culture of epidermal keratinocytes

Since new techniques of isolating and culturing keratinocytes were discovered, it has become a standard practice to grow keratinocytes in large numbers. Keratinocytes are readily available and expansion of an epidermal surface from a biopsy size of $1cm^2$ to $1m^2$ is realized in about 3 weeks.

Keratinocytes are isolated from a full thickness biopsy of skin, approximately 1-2 cm^2. After enzymatic digestion using Dispase®, the epidermis can be separated from the dermis as an epidermal sheet. The separated sheet is further digested with the enzyme trypsin. This typically results in a single cell suspension of keratinocytes.

Epidermal keratinocytes can be cultured *in vitro* using various methods (19,20). One method widely used for the clinic employs a feeder layer of murine fibroblasts (20) that have been lethally irradiated or treated with mitomycin C to prevent their proliferation. The feeder layer forms an optimal environment for the growth of keratinocytes by conditioning the medium through the secretion of growth factors and providing insoluble extracellular matrix proteins that are required for the clonal growth of keratinocyte colonies. Using this method, small colonies of 2-4 cells form within a few days after plating the keratinocytes. Doubling times for the keratinocytes are approximately 24 hours. As the cells proliferate, the colonies continue to expand until adjacent colonies merge and form a confluent sheet of keratinocytes.

B. Skin substitutes

There are three basic types of tissue engineered skin substitutes; the epidermal sheet, a dermal substitute, or a composite skin graft composed of a dermal substitution with a top layer of cultured keratinocytes. Substitutes for the epidermis and dermis are numerous (**table 3**). Some of the more popular ones will be described.

Table3: *Overview of commercially available skin substitutes*

	Company	Product	Description
Epidermal substitutes	Genzyme Biosurgery, Cambridge, MA	Epicel®	Autologous cultured keratinocyte sheets.
Dermal substitutes	Integra LifeSciences, Plainsboro, NJ	Integra®	Composed of an upper epidermal silicone sheet and a collagen-GAG copolymer dermal substitute.
	LifeCell, Branchberg, NJ	AlloDerm®	Acellular allogeneic dermis.
	Advanced Tissue Sciences, LaJolla, CA	Transcyte®	Extracellular matrix proteins deposited by allogeneic human fibroblasts on a non-degradable matrix. Cells are killed prior to use. Top layer is a nonporous, silicone sheet.
	Advanced Tissue Sciences, LaJolla, CA	Dermagraft®	Living, cultured allogeneic fibroblasts on a biodegradable matrix.
Composite substitutes	Organogenesis Inc, Canton, MA	Apligraf®	Allogeneic cultured keratinocytes, seeded onto a dermal layer composed of allogeneic fibroblasts in a collagen gel.

1. *Substitutes for epidermis*

Epidermal sheets are made of keratinocytes that have been cultured *in vitro* using the method described above. After growing the keratinocyte colonies to confluence, the final sheet of keratinocytes is a multi-cell layered sheet of epithelium approximately 2-8 cells thick. To detach this epithelium from the culture dish, the cultures are treated with Dispase®. This results in epithelial sheets of approximately $30cm^2$ in size and 2 to 8 cell layers thick. These grafts of autologous keratinocytes are attached to petrolatum gauze, and shipped to the hospital to be transplanted back to the patient. Genzyme Biosurgery (Cambridge, Massachusetts) provides Epicel®, a service that expands autologous keratinocytes into epidermal sheets with a surface area enough to cover the entire body in 16 days. The company reports that since 1988, over 600 patients have been treated using their epidermal sheets.

Drawbacks of this technique are variable take, susceptibility of infection, fragility of the sheet during handling, and spontaneous blistering for periods up to a few months, due to the lack of a good dermal-epidermal junction.

2. *Substitutes for dermis*

There are several substitutes currently on the market. We will discuss two of the most important ones, Integra®(Integra LifeSciences, Plainsboro, NJ) and AlloDerm® (LifeCell, Branchburg, NJ).

Integra® is a bilayer construct of a porous collagen-GAG polymer sponge as a substitute for the dermis, covered with a thin silicone membrane as a substitute for the epidermis (10,11) (**figure 3**). The dermal component is a suspension of extracellular matrix molecules: bovine collagen (isolated from bovine tendon), cross-linked with glycosaminoglycans from shark cartilage during the manufacturing process. Cross-linking optimizes pore structure and pore size of 70-200 microns and degradation times and furthermore increases elasticity of newly formed the skin after healing. The product is freeze-dried and stored in 70% isopropyl alcohol until usage. The collagen-GAG dermis is not immunogenic. However, after transplantation and subsequent invasion with autologous fibroblasts and endothelial cells, it induces a mild inflammatory reaction, which degrades the matrix in about 30 days. Autologous matrix deposited by the migrated fibroblasts, replaces the degraded material.

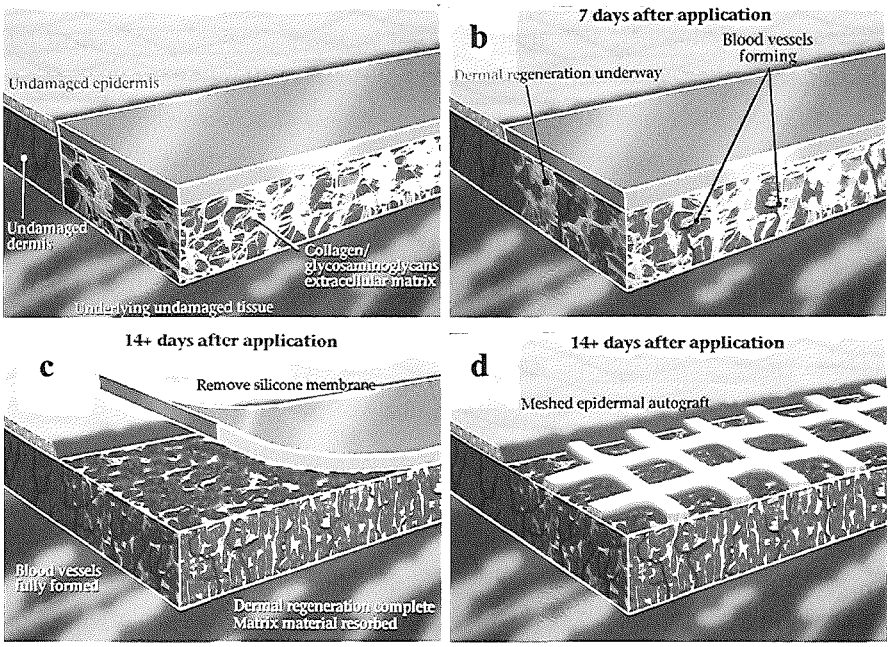

Figure 3: *The two-step use of Integra® as a skin substitute. The substitute is placed on the wound (***a***), revascularization takes place (***b***) and after two weeks, the silicone layer can be replaced by a meshed split skin autograft (***c,d***) (Pictures courtesy of Integra LifeSciences Inc.)*

The epidermal component is composed of a thin (0.009 inches) silicone layer. In addition to providing mechanical strength, this synthetic "epidermis" acts as a barrier to control loss of moisture and prevents bacterial contamination.

A major advance in the care of serious burns has been the practice of early excision therapy. However, with extensive burns these excised areas require coverage and sufficient autograft may not be available. By providing a temporary substitute for skin, Integra® Artificial Skin aids the practice of early excision therapy. Integra® is particularly useful for children and elderly patients whose skin is thin and not easily harvested for repeat auto grafting.

In a typical application, the burn wound is excised, covered with Integra® Artificial Skin, and over time the collagen-GAG dermal template promotes the formation of a vascularized neodermis. The silicone layer is peeled away (14-21 days after grafting) and the wound site is covered with an ultra thin (0.005 inches) epidermal autograft or a cultured epithelial sheet.

Another dermal substitute is AlloDerm®. This product is based on acellularized human cadaver skin. Since rejection of allogeneic skin grafts is based on immunological responses of the host immune system to the cellular components of skin, removal of the cellular components results in an immunologically inert structure, composed mainly of ECM molecules (21).

Screened cadaver allografts obtained from tissue banks are first treated with a high salt solution and the epidermis is removed and discarded. The de-epidermalized dermis is further treated with a detergent containing a de-cellularizing solution, which also serves to inactivate potential contaminating viruses. The resulting acellular dermis is washed in a freezing medium and freeze-dried under controlled conditions to avoid disruption of the matrix proteins. This material still has its rete ridges and contains collagen and elastin. Very importantly, the epidermal side still contains many of the proteins of the basement membrane such as laminin, and collagen types IV & VII, which are important for the attachment and migration of epidermal keratinocytes (22).

For usage, the acellular dermis can be meshed if necessary and is placed on a debrided wound bed, either in combination with an ultra thin meshed autograft or with a cultured epidermal layer (8). Two-step procedures are also used, where the epidermis is placed on the dermis only after initial revascularization of the dermis has taken place.

3. Cultured composite skin grafts

Composite skin grafts are composed of cultured keratinocytes and a dermal substrate (**figure 4**). Typically, the surface of a dermal substrate is seeded with a single cell suspension of trypsinised keratinocytes in medium. This construct is submerged in medium for a few days. Keratinocytes attach to the dermis and grow into a confluent epidermal layer. As a next step, the dermis, with keratinocytes, is raised to the air liquid interface of the culture system. Exposure to air results in a stratified differentiated epidermis, complete with granular and cornified layers (23).

After grafting, on a freshly debrided wound bed, fibro vascular structures invade the dermis. The keratinocytes secrete angiogenic factors (VEGF, FGF) and chemo attractant substances that support and accelerate this process (24,25).

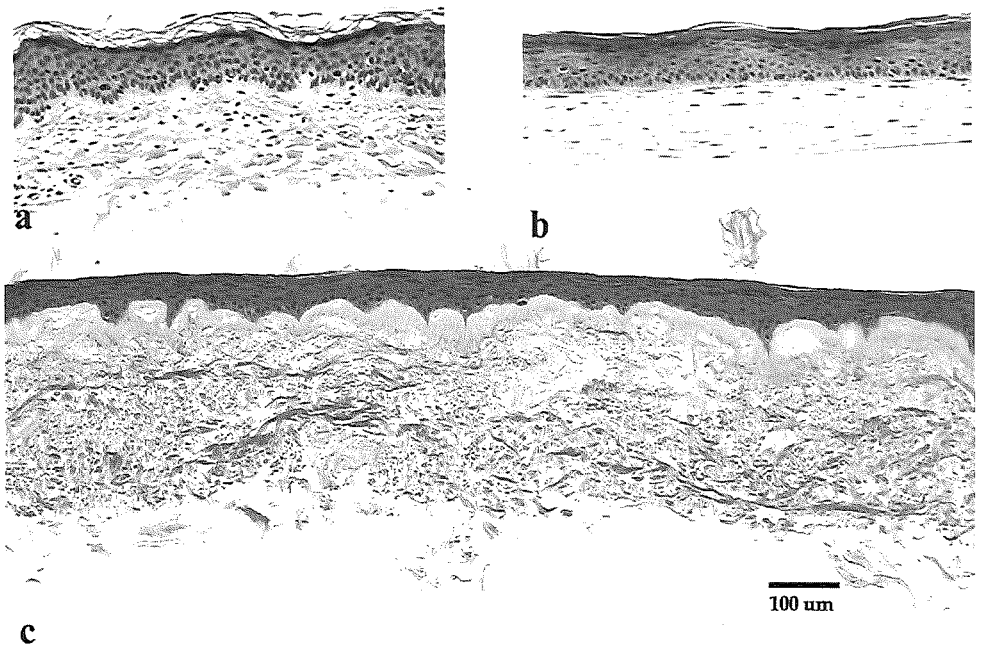

Figure 4: *Histology of normal human skin (a) compared to composite skin substitutes in the form of, Apligraf® (b), or a composite skin substitute of acellular dermis with cultured keratinocytes (c). (Pictures a and b, courtesy of Organogenesis Inc., picture c by courtesy of J.Cusick, Massachusetts General hospital/ Shriners Burns Hospital Boston)*

Organogenesis, Inc. of Canton, MA, produces another type of composite skin graft. The product, Apligraf® (**figure 4**), has an epidermal layer of cultured allogeneic keratinocytes and a dermal analogue of cultured allogeneic fibroblasts in a collagen gel. When cultured fibroblasts are mixed with a solution of bovine collagen, they form a gel. The fibroblasts remain metabolically active and over time, begin to reorganize the bovine collagen as well as produce their own collagen and matrix proteins. The cells used for Apligraf® are isolated from neonatal foreskins. The surface of this dermal equivalent is seeded with cultured allogeneic keratinocytes that spread and proliferate to form a partially differentiated epidermal covering. This bilayered skin equivalent is raised to the air-liquid interface, resulting in the differentiation of the epidermis.

According to the company, the cells obtained from one foreskin of approximately 1 cm^2 can be proliferated *in vitro* to manufacture approximately 1600 m^2 of the Apligraf® product.

Unlike grafts of autologous cells, which require 2-3 weeks preparation time for each patient, grafts of allogeneic cultured cells can be made available for immediate use. However, due to the immunological mismatch, the engraftment of allogeneic cells is not permanent, but can provide temporary coverage, which promotes wound healing.

Apligraf® is FDA approved for the treatment of venous and diabetic ulcers. Prospective randomized studies show that Apligraf® plus compression therapy improved complete wound closure compared to compression therapy alone in the case of venous ulcers. In treatment of diabetic foot ulcers, a significant increase in wound closure is observed when Apligraf® is used (26-28).

C. Future perspectives

Tissue engineered skin was one of the first tissues to enter clinical trials. Over two decades ago, the first coverage of large burn wounds with lab grown skin was reported (29). Since then, use of tissue-engineered skin has increased. However, there is still a long way to go, as illustrated by the current problems of variable take rates, long production times and high costs (30).

Research continues in the field of bioengineered skin substitutes. Many labs, as well as physicians, are searching for the ideal combination of biomaterials and cultured cells that can provide reliable, definitive wound closure. Moreover, as the clinical success of the currently available skin substitutes increases, the issues of cosmetics of the healed skin, especially for the burned patient, will grow in importance.

Efforts to reduce production times are found by use of allogeneic keratinocytes, or by mixing allogeneic cells with autologous cells in a so-called chimeric culture (31). Also, procedures where the collagen-GAG matrix has keratinocytes seeded in the pores of the matrix have been described (32,33). After grafting, the keratinocytes migrate up and repopulate the dermis, reducing production time and reducing cells necessary for providing a new epidermis.

In the future, genitally modified skin substitutes may be useful for the local synthesis, secretion and delivery of wound healing growth factors or other therapeutic agents. Keratinocytes are relatively easy to genetically modify, and have been shown to produce high levels of various growth factors; human Growth Hormone (hGH) (34), Platelet Derived Growth Factor (PDGF) (35-37), Keratinocyte Growth Factor (KGF) (38), and Vascular Endothelial Derived Growth Factor (VEGF) (39), have been described.

5. Tissue engineering of bone

Currently, bone replacement is usually accomplished by autologous or allogeneic bone grafting. Donor sites for autologous tissues are scarce and procedures, such as vascularized bone transplantation are technically challenging and time consuming. Complications of concern include unpredictable bone resorption, donor site morbidity, and infection (40). Tissue engineered bone constructs can provide an alternative source for bone.

In the following part of this chapter we describe cell types used for bone healing purposes, followed by a description of currently used scaffold materials and developing materials.

A. Cells producing bone

Cells producing bone (e.g. osteoblasts) or cells that are capable of differentiating into bone producing cells (e.g. mesenchymal stem cells) are called osteogenic cells. Mesenchymal stem cells are multipotent and can differentiate into osteoblasts, chondrocytes and other cell types of connective tissues, such as fat or muscle cells (41-46). Differentiation of mesenchymal stem cells into bone phenotype is regulated by osteoinductive factors (e.g. fibroblast Growth Factor, bone morphogenetic proteins (BMP) (47-50).

In bone, osteogenic cells are located in the bone marrow and periosteum. Osteogenic cells can be isolated by bone marrow aspiration, a relatively non-invasive, fast, simple, procedure with a high yield of osteogenic cells. The marrow can be used immediately or the mesenchymal stem cells (MSC) can be isolated and cultured *in vitro* (49,51,52). MSC can be cultured for 30 doublings, expanding cell numbers over one billion fold without losing their osteogenic potential (41). Alternative sources of osteogenic cells are periost and cancellous bone (53,54).

Bone marrow has long been recognized for its osteogenic and osteoinductive properties. It is used in the clinic for treatment of non-healing bone fractures since the 1980s (53). It can be injected into the surrounding bone as a cell suspension or combined with a carrier, such as demineralized bone. This prevents diffusion of the cell suspension, provides mass for correction of defects and in addition, the demineralized bone adds osteoconductive properties to the construct (54-57). Clinical reports describe similar mechanical and functional results using bone marrow in combination with demineralized bone compared with autologous grafting (56,58), however, more data are needed to confirm these observations.

B. Scaffold materials for bone substitutes

Two widely used scaffolds for bone substitutes are allogeneic bone and demineralized bone matrix (DMB). The latter is obtained by a hydrochloric acid treatment of bone to extract the minerals. Both materials have clear osteoconductive characteristics, meaning the material guides bone healing by enhancing attachment, migration and distribution of cells responsible for the bone healing response. Furthermore, the allogeneic bone can be produced in various forms and shapes, such as blocks, chips and powders. It is porous, has many adhesion sites for osteogenic cells that invade the material from the surrounding host tissue. In addition, the matrix material contains osteoinductive factors, embedded in the extracellular matrix, such as Bone Morphogenetic Proteins (BMPs) (59,60). For survival of these osteoinductive factors processing techniques of the bone are important. This is illustrated by the fact that if donor bone is left at room temperature for more than 24 hours before harvesting, the biological activity is lost (61).

Although allogeneic bone can be considered to be one of the optimal bone substitute by virtue of its mechanical and osteoconductive properties, drawbacks such as costs, variability of performance (e.g. resorption, incidence of stress fractures (62)), sensitization of recipients to graft specific histocompatibility antigens (63,64) and risk of transmission of disease, are significant. Therefore, alternative sources of bone replacement materials have been developed.

Materials widely used are composed of ceramic materials, such as hydroxyapatite (calcium phosphate salt) and tricalcium phosphate (TCP). Hydroxyapatite can be derived from coral. Two kinds of coral are suitable for osteoconductive bone replacement materials. Both substitutes are highly porous, with a void volume up to 66%. One type has morphology similar to cancellous bone whereas the morphology of the second type is similar to cortical bone with parallel longitudinal pores and interconnecting pores (65,66). Native coral is composed of brittle calcium carbonate, but treatment by hydrothermal exchange at 900°C results in a very pure form of hydroxyapatite, with superior mechanical properties (65). Hydroxyapatite is a natural part of bone and after the hydrothermal reaction the product is protein free, making it an immunogenically inert material.

Hydroxyapatite has a resorption rate of approximately 5 to 15% per year and therefore can be considered as a non-degradable material. It has shown its use in the clinic as an osteoconductive bone substitute (67-69) and is on the market as Pro Osteon® (Inter pore Cross, Irvine CA) (**figure 5**). Pro Osteon® is FDA approved for repair of metaphyseal defects, long bone cysts and tumor defects. Upon placement of this very porous substitute, it becomes

repopularized with fibrovascular cells, and osteogenic cells that deposit their matrix on the insoluble hydroxyapatite scaffold.

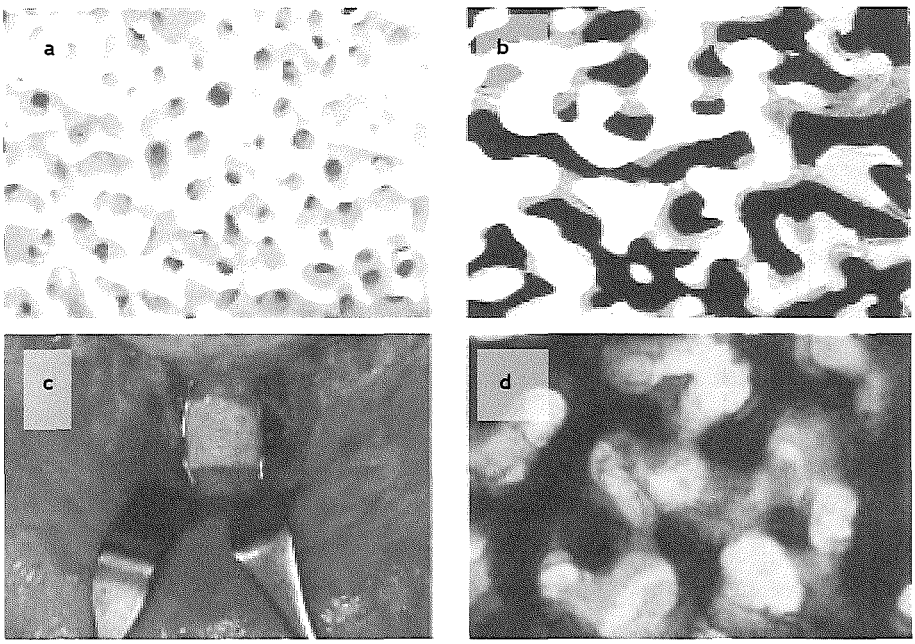

Figure5: *A Higly porous coral based product (Pro osteon 200®) is shown in low magnification (a) and scanning electron microscope (b). It can be used to augment frontal cranium (c). After 11 months, newly formed bone (gray) is deposited on the hydroxyapatite structure (white)(d).*

To shorten the long half-life of this material, the hydrothermal conversion can be partially completed, resulting in a concentric outer layer of slowly resorbed hydroxyapatite around an inner layer of more rapidly absorbing calcium carbonate. The outer layer of hydroxyapatite provides mechanical support and its slow resorption gives ingrowing cells time to attach and deposit extracellular matrix. After a few months, the outer layer becomes eroded, exposing the calcium carbonate that is resorbed at a faster rate. In a perfect setting, the newly formed bone will be matured enough to provide mechanical support before the outer layer is resorbed. Other ways of shortening resorption times include making co polymers with the more readily absorbable tricalcium phosphate.

Another scaffold material used in bone substitutes is collagen type I. Collagen type I is the most abundant extracellular matrix protein in bone and has properties that facilitate

mineral deposition in the extracellular matrix. It has been mixed with hydroxyapatite, and is currently on the market (Collagraft® / Neu Coll inc., Palo Alto, CA). This product consists of bovine skin collagen type I mixed with hydroxyapatite. Prospective, randomized trials validated the use of this product in combination with autologous bone marrow cells. The trial showed no difference between use of autologous bone transfer and the use of hydroxyapatite in combination with autogenous marrow with respect to rates of union and prevalence of complications. Twelve of the 213 patients showed positive antibodies to bovine collagen. The authors conclude that use of hydroxyapatite in combination with marrow as an alternative to autogenous bone grafts for healing fractures in long bones is justified (70). According to the company, Collagraft® has been used successfully in the combination with autologous marrow as an osteoconductive, osteoinductive, and osteogenic cancellous bone replacement in more than 150,000 patients since 1994.

C. Future perspectives.

Research and development of new bone substitutes is focusing on several areas. One area focuses on increasing osteoinductive properties by incorporating osteoinductive growth factors, such as BMP into various scaffold materials (71-75). Another area of interest is the development of injectable ceramics and polymers that polymerize *in situ*. After mixing with a catalytic agent the polymerization process is started and the polymer is injected into the site of interest. A variable period of viscosity follows, when the material can be adjusted to fit the defect while the materials harden with minimal production of heat and without producing any harmful byproducts (76). Some of the newly developed polymers secrete CO_2 during the process of *in situ* polymerization, resulting in a porous structure. In addition, the pressure generated by the CO2 generates forces the scaffold to fit the defect seamlessly (77).

Further areas of research include in the development of *in vitro* seeded natural and synthetic scaffolds (78-81). Bone constructs using cultured periosteal osteogenic cells seeded into a PLGA and PGA polymer scaffold have shown increase of bone formation in femoral shaft reconstructions (82) and in reconstruction of calvarial defects (83) in rats and rabbits. Cultured MSCs, in a coralline scaffold, have shown to increase bone regeneration in a femoral shaft defect in sheep (84).

6. Tissue engineering of cartilage

Cartilage has many characteristics that make it suitable for tissue engineering purposes. Ease of isolation and the discovery of techniques for cell expansion *in vitro* have made it possible to obtain and grow cells for transplantation purposes. Lack of vascularization, low metabolic demands and possibilities to store for long periods of time make this tissue appropriate for usage in an engineered construct and for purposes of transplantation. There is a large demand for autologous cartilage due to its limited availability and marginal capacity to repair and regenerate *in vivo*. This has led to extensive research efforts into discovering ways of *in vitro* expansion and engineering of cartilage. Tissue engineered cartilage can be used in many ways in reconstructive plastic surgery. Treatment of nose septum defects or reconstructions of the external ear are only a few of the possible uses.

A. Isolation and culture of chondrocytes

There are three types of cartilage; elastic, hyaline and fibrocartilage. Elastic cartilage can be isolated from ears, nose septum and ribs. Fibrocartilage has more collagen I in its extracellular matrix, whereas the other two have more collagen type II in their extracellular matrix. The abundance of collagen type I makes fibrocartilage less water attracting, and gives it a more fibrous character than the other two. Formation of fibrocartilage is often observed as a healing response of hyaline cartilage after trauma. Hyaline cartilage is typically isolated from joints. Enzymatic digestion using Collagenase II and trypsin is used to remove the extracellular matrix and isolate the cells for subsequent culturing (88-91). Chondrocytes are slow growing cells; dividing only once or twice a week. *In vitro,* growth can be increased using growth factors, such as fibroblast growth factor (85,86).

Cartilage mainly consists of extracellular matrix and water. This extracellular matrix is produced by chondrocytes and is composed of large polymers such as glycosaminoglycans, proteoglycans, and Collagen II and forms 95% of the tissue weight. As described earlier, these molecules are responsible for the turgor of cartilage by their ability to attract water. Cells in culture produce these molecules, such as collagen II and large aggregating proteoglycans. Production rate of these substances is used to monitor cell and tissue quality and can also be up regulated by certain growth factors, such as TGFß (85,87).

An important process in chondrocyte culture is called dedifferentiation (88). Dedifferentiation is a morphological and functional change of the cells that occurs in monolayer cultures. Cells take on a fibroblast morphology and start to secrete collagen type

I instead of collagen type II, resulting in a more fibrous tissue than the normal cartilage (89). Dedifferentiation is a reversible process. Three-dimensional culture systems, such as cell suspensions, agarose gels (87,90,91), collagen gels (92) or collagen sponges (90,91), can prevent this process or even initiate differentiation of chondrocytes. The capacity of cartilage cells to redifferentiate however is limited. Due to the problem of dedifferentiation, cells can be passed only four to six times (86).

A. Clinical applications and future perspectives

There are three tissue engineering based strategies for repairing cartilage. One uses autologous cell expansion and subsequent transplantation back into the patient (Carticel®, Genzyme Biosurgery Inc /Cambridge, MA), the second uses scaffold materials that are placed in the defect in the cartilage. Healing of the cartilage occurs by migration of the surrounding tissue into the defect. The third approach uses a combination of scaffold and cells, combined *in vitro* and placed into the organism. Only the first strategy has reached the clinic.

In 1994, the first clinical application of autologous, cultured chondrocytes for the repair of hyaline cartilage was described (6). Currently, Genzyme Biosurgery Inc (Cambridge, MA), provides Carticel® (**figure 6**), a service that cultures autologous chondrocytes commercially, using a similar approach. A healthy, autologous piece of cartilage is isolated from the patient by biopsy during an arthroscopic procedure. This piece of cartilage is shipped to the company where chondrocytes are isolated and cell numbers are expanded *in vitro*, approximately 10 fold. After cell expansion, chondrocytes are suspended in DMEM and shipped to the hospital, where the cells are placed back into the defect of the damaged joint. After accessing the knee by a small arthrotomy, the defect is debrided and covered by a periosteal flap sutured to the surrounding cartilage. This is done to seal off the defect and prevent spreading of the cells (6). Than, the cell suspension is injected under the periosteal flap. Genzyme Biosurgery reports that the Carticel® chondrocytes have been used to treat at least 3,952 patients. Recently, promising long-term results of the use of autologous cultured chondrocytes in hyaline cartilage repair have been published (5,93). Transplantation of autologous chondrocytes produces a durable and effective repair of full thickness cartilage lesions. New hyaline cartilage was observed by arthroscopic and histologic evaluation. However, comparison to other techniques requires prospective, randomized studies that are not yet available.

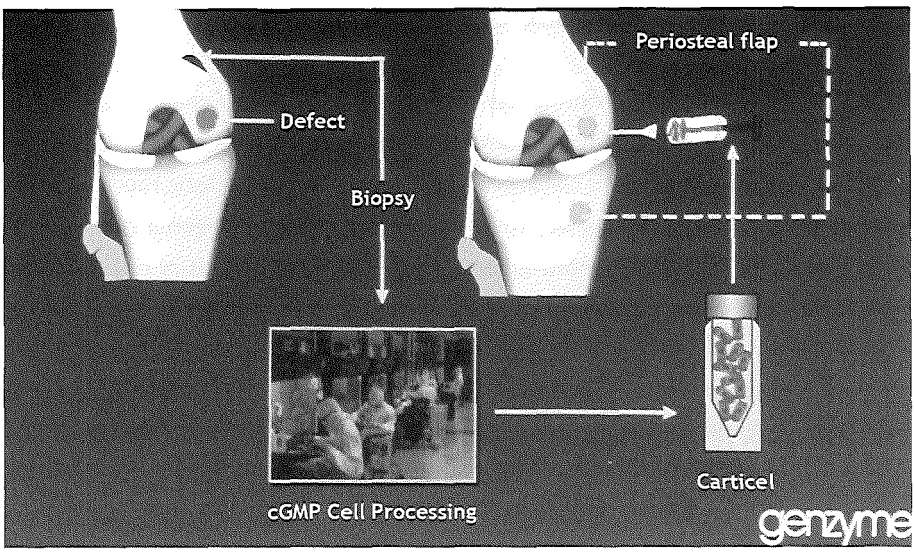

Figure 6: *Carticel®, Chondrocytes are isolated from a biopsy, expanded in culture, placed back into the injured joint, covered by a flap of periost. (Picture courtesy of Genzyme Biosurgery)*

The other two mentioned techniques are still under investigation. The first places a scaffold in a defective area of cartilage (94). Chondrocytes from the wounds edge migrate into the scaffold where they deposit their extracellular matrix. Eventually the scaffold is degraded and new functional cartilage is formed. This approach is thought to be of use especially in the healing of hyaline cartilage defects. Another approach used synthetic (e.g. PLGA or PGA (94-98)) or natural (e.g. collagen gels and sponges (100,105)) scaffolds materials, seeded with chondrocytes *in vitro*. The technique forms a tissue with similar mechanical, structural and histological properties as normal cartilage *in vitro* (100) as well as *in vivo* (101). Cartilage produced using PGA or PLGA polymers can be shaped into various forms, following the shape of the scaffold (97). An example of this is the fabrication of tissue-engineered cartilage in the shape of an ear (102). Laboratory studies using these techniques are promising with respect to culturing techniques; construct designs and transplantability of the substitutes in animals. Potential use in a clinical setting is recognized, but more research is necessary to reach this goal.

7. Future perspectives of tissue engineering

The basic principles of tissue engineering have been described in this chapter, as well as some tissue-engineered products that are currently available or will be in the near future. The potential of this emerging field is considerable, especially for the plastic reconstructive surgeon. However, there is still a long way to go until use of tissue-engineered constructs is accepted as a standard procedure. Improvements are needed in the fields of cell isolation, cell culture, scaffold design and transplantation of constructs before tissue engineering becomes widespread.

In order to provide sufficient numbers of high quality cells for the formation of tissue engineered constructs, improvements in cell isolation and cell expansion in two dimension cultures, as well as in three-dimensional cultures are necessary. Stem cells are an exciting new area of research for tissue engineering. Development of isolation techniques for stem cells from various tissues, their high proliferative potentials and possibilities to differentiate into various cell types such as cartilage, bone, fat and muscle make them theoretically ideal candidates for use in tissue engineered constructs. Another exciting area of research for tissue engineering includes the genetical modification of cells. Genetic modification of cells can be used to improve the function and performance of many types of cells. The overproduction of growth factors for wound healing or factors that improve the function of cells and tissues are being tested.

Research is also active in the development of new scaffold materials that are optimized with respect to cell adhesion. Polymers are produced that incorporate RGD (arg-gly-asp) adhesion sequences into their structures. These amino acid sequences are binding sites for a number of cell integrins involved in cell attachment. Incorporation of these sequences results in enhanced attachment of cells. The fabrication of injectable, *in situ* polymerizing polymers that can be used in a minimally invasive way, mixed with cells, and moldable before complete polymerization are also under development.

In the area of transplantation, issues such as cell density, culture time, and scaffold variables are being optimized for each individual construct. In order to transplant larger constructs, or even tissue engineered organs, ways of inducing angiogenesis and innervation are areas of active investigation.

Of the tissues discussed, skin is the only clinically approved tissue that is commercially produced in a three dimensional construct. In order to increase the number of commercially available tissue engineered cell seeded scaffold products, production costs will need to be

reduced, production times need to be shortened and new ways for large scale production need to be developed. Future products need to be practical, easy to handle and competitive if not superior in performance to the current modalities of treatment. If the field of tissue engineering meets these challenges it may reach its full potential.

Websites for Tissue Engineering

Tissue Engineering and Regerative Medicine network:
www.termis.org
wikipedia http://en.wikipedia.org/wiki/Tissue_engineering

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Chapter 4

Mucosal Keratinocyte Isolation; a short comparative study on Thermolysin and Dispase

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Abstract

Introduction: New reconstructive techniques for reconstructing large defects of the floor of the mouth include the use of cultured mucosal substitutes. The purpose of this study was to compare dispase and thermolysin for keratinocyte isolation.

Materials and methods: Keratinocyte yield per surface area of rabbit buccal mucosa was assessed by histology, cytokeratin 13 staining, seeding efficiency analysis and cell diameter quantification. Surface areas of cultured mucosa were calculated.

Results: Histology showed that treatment by thermolysin resulted in incomplete separation of epidermis from dermis. Also, the absolute number of keratinocytes per cm^2 isolated mucosa, cell yield, cell size and seeding efficiencies was higher in the dispase group. A 3.45 fold larger graft could be reconstituted using dispase.

Conclusion: The use of dispase to isolate cells from buccal mucosa is favourable to thermolysin.

Introduction

In vitro culture of oral keratinocytes is used to study various processes, such as carcinogenesis (1), or effects of e.g. tobacco (2). Moreover, this technique has been used for clinical purposes, to culture keratinocyte onto a dermal carrier in repair of intra oral defects resulting from oncological resections (3). Oral cancer is the sixth most common cancer in the world. It accounts for approximately 4% of all cancers and 2% of all cancer deaths worldwide (4).Malignant tumors of the oral cavity account for approximately 30% of all head and neck cancers. With an incidence rate of invasive lip and oral cavity tumors in The Netherlands rising to 6.6%, oral cancer is a common malignancy that often requires resection of the tumor followed by reconstruction (5).

Reconstruction is typically performed using free skin flaps such as the radial forearm flap (6). Drawbacks to the use of skin flaps include donor site defects, bulkiness and hairbearing of flaps (7). Alternative reconstructive approaches using cultured mucosa as a substitute are being developed (3). These involve the culture of keratinocytes and subsequent combination of these cells with a biomaterial that serves as a dermal carrier. The most important factor delaying clinical implementation is the culturing time necessary to expand cells to cover the average defect size of approximately 30-45 cm (8,3) Culture time, therefore, needs to be minimized. An important factor determining culture time is the number and the quality of cells harvested from the biopsy.

The predominant cell type of the epidermis is the keratinocyte. The keratinocytes located in the basal layer, are the cells with the highest proliferative capacities *in vitro* and are therefore the cells that need to be isolated to establish successful primary cultures (9,10). Basal keratinocytes have a number of characteristics that can be used for identification. They are small and have capacities to form colonies if cultured *in vitro*. Suprabasal keratinocytes, on the other hand, are larger, express cytokeratin 13 and are less efficient in forming colonies (11-13).

Widely used cell isolation techniques include explantation and enzymatic dissociation. Enzymatic protocols often involve a two-step procedure, using either dispase or thermolysin (14-18) to separate the epidermis from the dermis. Keratinocytes are typically cultured on a feeder layer of lethally irradiated fibroblasts (19). Advantages of the use of an enzymatic protocol include reduced fibroblast contamination; higher cell yields and reduced risk of bacterial contamination. Although various cell isolation procedures have been reported in the literature for skin keratinocytes, there are no reports on direct comparison of

cell qualities between dispase or thermolysin isolation for oral mucosal keratinocytes. Therefore, the goal of this study was to compare two enzymatic protocols for quality of mucosal keratinocyte retrieval. By histology, measuring cell size, colony forming efficiencies and expression of keratins in basal keratinocytes with high proliferative capacities were identified. Furthermore, the presence of fibroblast contamination was evaluated.

Materials and methods

Upon approval of the local animal ethics committee, keratinocytes were isolated from biopsies of buccal mucosa from four rabbits. The samples were treated using either thermolysin or dispase to separate epidermis from dermis. Cell characteristics, such as cell yield, size and CK13 expression were analyzed using one rabbit. As mucosal substitutes are typically fabricated using cells at passage one to two, the cell populations were analyzed again at the first passage.

Keratinocyte isolation and cell culture

Buccal mucosa was harvested from the cheek of four New-Zealand white rabbits. The mucosa was incubated in antibiotic cocktail for 1 hour (Penicillin/Streptomycin (100 U/mL / 100 µg/ml), Gentamycin (50 µg/ml), Amphotericin B (2.5 µg/ml), all Invitrogen, Breda, The Netherlands). Subsequently, six 6mm punch biopsies (0,28 cm^2) were cut from the samples. Half of the biopsies were transferred to dispase (2.5 mg/ml, (Invitrogen)) in Dulbeco's Modified Eagle Medium (DMEM (Invitrogen)) the other half in thermolysin 500ug/ml (Invitrogen) in DMEM, at 4°C overnight. Enzyme concentrations and diluents were chosen according to the manufacturers instructions. The next day, biopsies in enzyme solutions were put in 37°C for 20 minutes prior to separation of the epidermal sheath from the dermis. Subsequently, the epidermal sheaths were put in Trypsin-EDTA, 0.25% (Sigma, Zwijndrecht, The Netherlands) at 37°C for 15 minutes to obtain a single cell suspension. From two rabbits, two samples were used for histological evaluation. Three million cells of each of the three biopsies used for cell analysis were divided into three T-25 culture flasks (25 cm^2) with a feeder layer of lethally irradiated 3T3 fibroblasts (a kind gift from dr. von den Hoff, Department of Orthodontics and Oral Biology, University Medical Centre, Nijmegen, The Netherlands) in seeding medium, according to the Howard & Green protocol (19,20). Keratinocyte seeding medium was composed of a 3:1 mixture of DMEM and Ham's F12 (Invitrogen), supplemented

with 10% FBS; adenine, $1.8x10^{-4}$M (Sigma); cholera toxin, 10^{-10} M (VWR international, Amsterdam, The Netherlands); hydrocortisone, 0.4 µg/ml (VWR); insulin, 5µg/ml (Eli Lily, Houten, the Netherlands); triiodo-L-thronine, $2x10^{-9}$ M (Sigma) and penicillin-streptomycin, 100IU/ml-100µg/ml (Invitrogen). 24 Hours after seeding, mouse epidermal growth factor (EGF, Sigma) was added to a concentration of 100ng/ml. This keratinocyte culture medium (KCM) was changed every other day.

Subconfluent keratinocyte cultures were subcultured by first removing the fibroblast feeder layer with 5mM EDTA and treating the keratinocytes with trypsin-EDTA. 3T3 Fibroblasts were routinely passaged in DMEM (high glucose) supplemented with 10% FBS and penicillin-streptomycin (100IU/ml-100µg/ml) and incubated at 37°C with 10% CO_2.

Colony forming efficiency was investigated by seeding 1000 isolated cells from each of the three biopsies of one animal in triplicate onto 6 well dishes with a feeder layer in KCM. Fibroblast contamination was studied by seeding 1000 isolated cells isolated cells from each of the three biopsies in triplicate on a 6 well dish in DMEM with 10% FBS. After 21 days, 6 well dishes were rinsed using PBS and stained using eosin for 1 minute and subsequent rinsing using tap water. The number of round, single colonies was counted.

Cell count and cell size analysis

Viable cell numbers were counted after the addition of trypan blue using a haemocytometer. Cell suspensions of one representative rabbit studied into further detail by reconstituting cells to 1 million cells per millilitre. Cell size analysis was performed using a CASY® cell counter (Scharfe systems, Reutlingen, Germany). Triplicates of 75µl cell suspension (75.000 cells) per biopsy were analyzed. Cell sizes were normalized to facilitate comparison.

Histology

Samples of biopsied mucosa, as well as remnants of dermis and the epidermal sheath after enzyme treatment were used for histology. Haematoxylin and Eosin staining was performed. For immunohistochemical staining for differentiated keratinocytes, Cytokeratin 13 (CK13) was used. The CK13 protocol was as follows. Paraffin sections were deparafinnized and cooked in citric acid buffer (2.15g citric acid monohydrate in 750ml of water). Endogenous peroxidases were blocked using citric acid phosphate buffer with 30% H_2O_2 (Fluka, Zwijndrecht, The Netherlands). After a blocking step using 10% normal rabbit serum (NRS, Sanquin blood bank, Rotterdam, The Netherlands) and 10% normal goat serum (NGS, Sanquin blood bank) diluted in first step buffer (10% NRS (Sanquin blood bank) in PBS, the

primary antibody was added, mouse anti human CK13 monoclonal IgG_{2a} (Euro Diagnostica, Arnhem, The Netherlands). The next day, samples were washed using washing buffer (0.1% tween in PBS) and incubated with the secondary antibody, biotin labelled goat anti mouse immunoglobulins (Dako, Glostrup, Denmark) in secondary buffer (2% NGS, 2% NRS and 5% Bovine Serum Albumin in PBS). Following a washing step, streptavidin-HRP complex (Dako) was added in secondary buffer followed by another washing step. Next, the substrate (24µl of 30% H_2O_2 in PBS in 1.2ml 5% DAB (Sigma)) was added and the reaction was stopped using tap water. Counterstaining was performed using haematoxylin. Samples were mounted using Vectamount (Dako). Statistical analysis For comparison of the cell yield of the two protocols, we used a non-parametric Mann-Whitney test. Significance was reached with P-values <0.05.

Results

Dispase isolates more cells per biopsy than thermolysin

In two out of 10 biopsies treated with thermolysin the digestion was incomplete. The epidermis did not separate from the dermis, which is why these were excluded from analyses.

Cell counts were performed on in total 8 6mm punch biopsy specimens after thermolysin and 10 biopsies after dispase treatment (figure 1). Dispase isolation resulted in cell yields of 12.07 (±2.5 (SD)) million/cm^2 whereas thermolysin isolation resulted in an average of 3.70 (±1.9 (SD)) million/cm^2, a 3.26 fold decrease (p = 0.02,). Colony forming efficiencies, expressed as % of keratinocytes seeded onto a feeder layer forming a colony were 0.38% (± 0.13 (SEM)) for dispase and 0.31% (± 0.10 (SEM)) for thermolysin. This difference was not significant. No fibroblast colonies were found in both isolation protocols.

After seeding 1 million cells per biopsy and culturing these to passage 1, cells were counted per flask at 80% confluency. The dispase treated group had significantly more cells per flask (average 8.3 million cells) than the thermolysin treated group figure 1C (6.3 million P = 0.04). Average culture time to the first passage was 8.6 and 9.3 days for the dispase and thermolysin treated mucosa respectively and did not reach a statistically significant difference (Figure 1D).

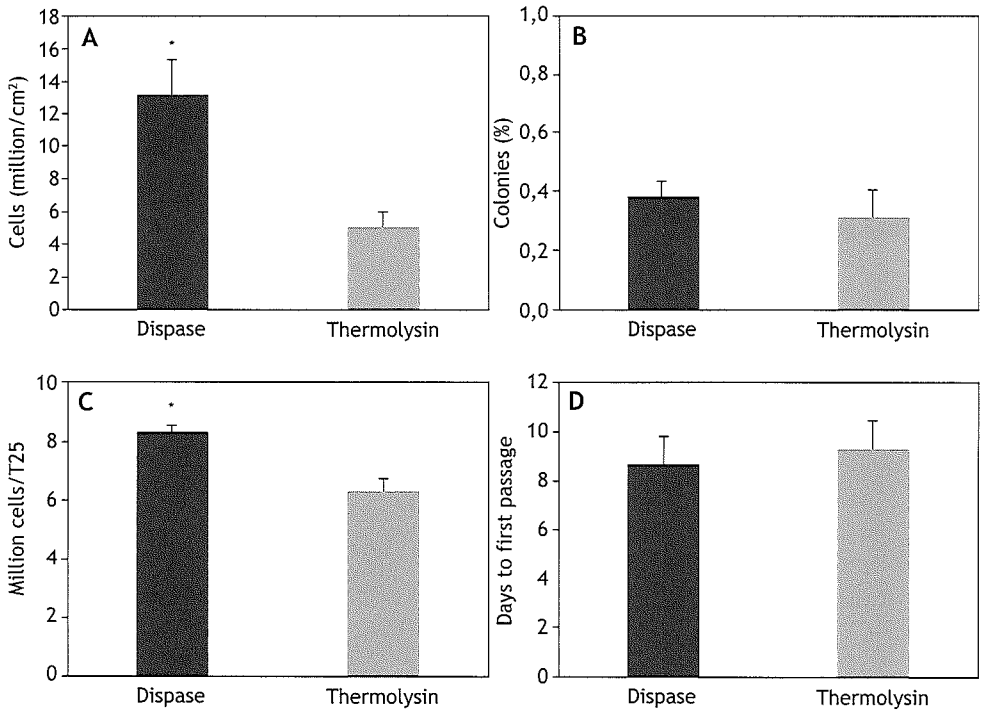

***Figure 1**: Dispase isolates more cells per biopsy than thermolysin.* **1A;** *The number of isolated cells per surface area using the dispase protocol (black, n = 8, the two biopsies where separation was Impossible were excluded) or the thermolysin protocol (grey, n = 10).* **1B;** *Colony forming efficiencies per protocol, expressed in % of 1000 seeded keratinocytes forming colonies (n = 9).* **1C:** *Cell yield per T25 culture dish at passage 1 (n = 3).* **1D:** *Days between seeding 1 million cells and first passage per isolation protocol (n = 3). * P < 0.05*

Thermolysin results in incomplete separation of epidermis and dermis As shown on H&E staining of pieces of dermis treated with dispase or thermolysin, the epidermis was separated from the dermis. Dissociation using thermolysin however, resulted in the epidermis partly remaining attached to the dermis (figure 2 A-D), in particular basal cells in the papillae of the dermis. Also, it was noted that all epidermises of the dispase treated biopsies were easily separated from the dermis. This is in contrast to the thermolysin treated biopsies, where 2 out of 10 of the epidermises did not separate at all. Moreover, the epidermises separated by thermolysin were macroscopically thin (data not shown).

Cytokeratin 13 (CK13) is expressed in suprabasal, differentiated keratinocytes of mucosa. These keratinocytes have less proliferative potential than the CK13 negative cells at the basal layer of the epidermis. For analysis of the keratinocytes remaining attached to the dermis after thermolysin treatment, biopsies were stained for CK13 expression. The remaining cells at the dermis proved to be CK13 negative, suggesting them to be basal cells with a high proliferative potential (figure 2).

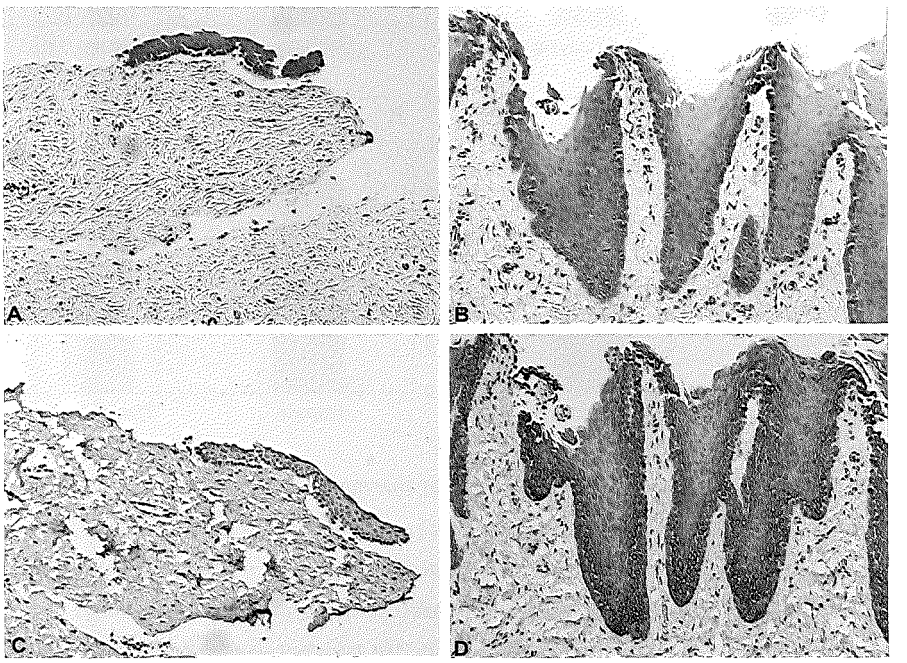

Figure 2: *Thermolysin results in incomplete separation of epidermis and dermis.* **2A:** *Haematoxyllin and Eosin staining (H&E) of dermis after dispase treatment, all but a few cells are removed upon separation of the epidermal sheath. The line of separation is the dermo-epidermal junction.* **2B:** *H&E of dermis after thermolysin treatment. Large quantities of basal cells remain attached in the papillae of the dermis. The line of separation seems to be the suprabasal layer of the epidermis. Immunohistochemical staining for differentiation marker CK13 shows basal, CK13 negative, cells remaining attached to the dermis of the thermolysin treated dermis (***2D***), whereas the dispase treated dermis (***2C***) shows hardly any attached CK13 negative keratinocytes. (magnification 200x)*

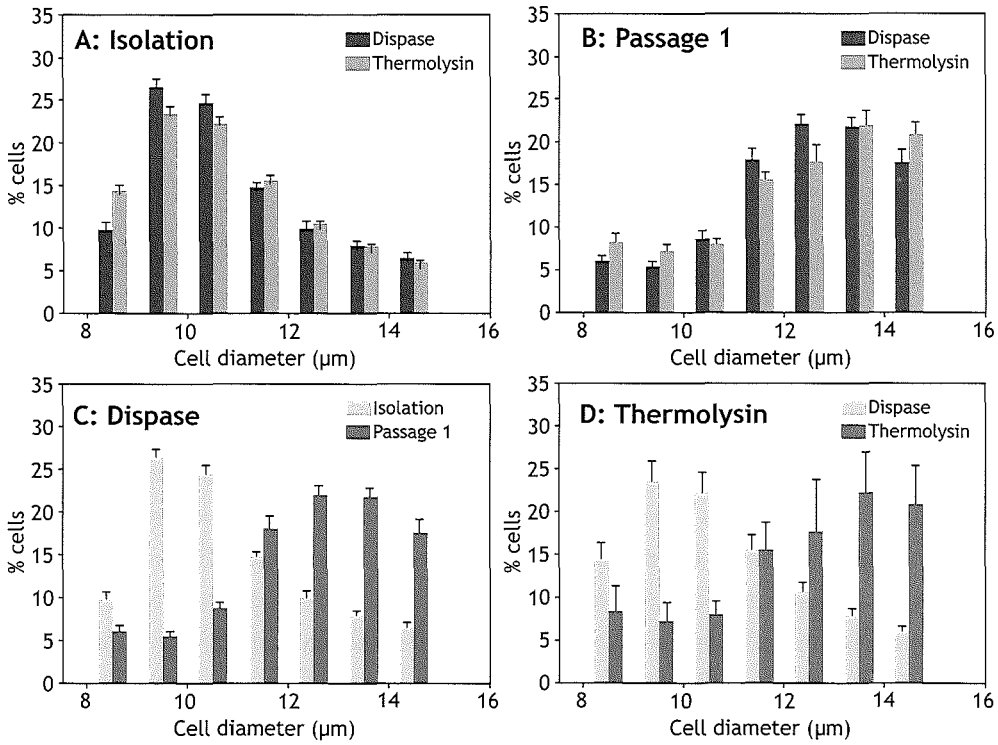

Figure 3: *Cell Sizes.* **3A:** *CASY analysis was performed to measure cell diameters of dispase treated biopsies (dark grey) or thermolysin treated biopsies (light grey) at isolation .* **3B:** *Cell diameter distributions for both enzymatic protocols were compared at passage1. Similar distributions were observed in both protocols. Figure* **3C** *and* **D** *show a cell diameter increase upon upon in vitro propagation of keratinocytes isolated by dispase or thermolysin treatment respectively. Bars represent the SEM of triplicate measures of three biopsies, a total of 9 measurements per protocol. Measurements were expressed as percentages to assess relative size distributions.*

Dispase results in an increased number of small keratinocytes per cm^2 Basal keratinocytes with high proliferative potentials are small. Typically their diameters are less than 14 µm. In figure 3, cell sizes of the cell population less than 15µm are plotted as percentages at isolation and first passage of the cells. As illustrated in figure 3A size distributions at isolation are similar between the dispase protocol and the thermolysin protocol. The absolute amount of small keratinocytes per surface area of biopsy therefore is 3.26 fold higher.

Upon isolation, cells are cultured and start to differentiate and cell size should increase. This was confirmed by our data (figures 3C and D). However, similar cell size distributions between the two isolation groups were observed at passage 1 (figure 3B), indicating no influence of isolation protocol on cell size differentiation during culture.

Discussion

In establishing primary cultures from epidermis a number of problems can be encountered. Fibroblast overgrowth occurs as a result of fibroblasts isolated as passenger cells. Other problems include bacterial contamination as sterilizing the contaminated oral cavity prior to the biopsy is technically challenging. Several approaches in tackling these issues have been developed. The use of specific media formulations (21), as well as the use of a radiated fibroblast feeder layers have been developed to discourage fibroblast growth and selectively stimulate the keratinocytes to proliferate (19). Also, the use of proteases that cleave the epidermis from the dermis at the dermo-epidermal junction were introduced (17). After cleaving the epidermis from the dermis, the epidermis is trypsinized separately. This approach results in a decrease in fibroblast contamination, and is less laborious than previous enzymatic protocols where biopsies are minced rigorously and processed in trypsin in spinning flasks for long times. Moreover, it is the experience of our laboratory that bacterial contamination is reduced, as clumps of cells and matrix are removed prior to culture facilitating the antibiotics to penetrate the tissues.

In order to choose an optimal protocol to isolate mucosal keratinocytes for expansion *in vitro*, thermolysin and dispase were tested. These enzymes have been well characterized for their use in isolation from keratinizing skin and are routinely used by many well-established laboratories (17). Unlike the use for keratinocyte isolation from keratinizing epidermises, a comparative study using the two most popular enzymes for non-keratinizing epidermises has not been found.

In the present study, both enzymes proved to be convenient and suitable for cell isolation shortly after harvesting of biopsies, ensuring optimal viability of the cells. Keratinocytes were successfully cultured using either of the two enzymes. Additionally, no fibroblast contamination was observed in any of the protocols.

The results of this study show a clear advantage of the use of dispase. First of all, cell yields per surface area of biopsy were 3.26 times higher in the dispase treated groups. This

was shown by cell counts and confirmed by histology. Moreover, the decrease in cell yield per surface area of biopsy seemed to be caused by a supra-basal separation level of the epidermis and dermis. Analysis of expression levels of differentiation marker CK13 (11) in the keratinocytes remaining attached to the dermis upon thermolysin treatment proved the remaining keratinocytes to be basal cells with high proliferative capacities. Also, the enzyme proved to be unpredictable in its effectiveness as only part of the epidermises separated following overnight incubation. Other reports on the use of thermolysin in keratinizing skin report separation at the dermo-epidermal junction. Supra-basal split of the epidermis using thermolysin in keratinizing skin has been reported previously (14). No histological analysis has been reported on the use of thermolysin for non-keratinizing mucosa.

The data indicating no relative difference in proliferative capacities were confirmed by the data obtained by measuring the colony forming efficiencies and cell size analysis. The colony forming efficiency test showed a non-significant increase in number of colonies using the dispase protocol. Percentages correspond to previous reports using cultured keratinocytes isolated from skin biopsies (15,16).

Cell size has been a long used method to identify basal keratinocytes. Small cells with diameters between 9 and 14μm have high nucleus/cytoplasm ratios and are considered keratinocyte stem cells with high proliferative potentials. Although DNA content as well as nuclear size remain constant, a differentiation dependent increase in cell diameter concurs with appearance of differentiation specific proteins such as involucrin and differentiation-specific keratins, as well as the disappearance of basal cell markers such as β1 intergrins (12,13). In the present study, an increase in size was seen as a result of culturing the cells as cells differentiate, corresponding to earlier reports on cell diameter changes of keratinocytes in culture (22,23). Our data show similar cell size distributions between both tested protocols suggesting that no differences occur upon culture *in vitro*.

In conclusion, we measured the differences between two enzymatic protocols in isolation of mucosal keratinocytes. Although relative differences between cell populations were minimal, the 3.26 fold difference in cell yields per surface area and the significant increase in surface area of cultured keratinocytes justify the use of dispase over thermolysin.

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Chapter 5

Fibroblasts Accelerate Culturing of Mucosal Substitutes

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Abstract

Reconstruction of large mucosal defects of the floor of the mouth is typically performed with keratinizing skin. Drawbacks include donor site defects and hair bearing of the flaps. Cultured mucosal substitutes (CMS) have been developed for clinical use to replace keratininzing skin. A-cellular dermis is often used as a dermal carrier for autologous cells, as it reduces wound contraction and is easier to handle by the surgeon than e.g. collagen gels. A major problem of CMS using a-cellular dermis is variation in epidermal quality.

To improve the quality of the CMS, human fibroblasts were incorporated into the a-cellular dermis and seeded with human keratinocytes. To study the role of the fibroblasts on epidermal morphology and basement mebrane formation, CMS were stained for differentiation markers $ß_1$ integrin, cytokeratin 10 and involucrin after one and two weeks in culture. Basement membrane formation was analysed by laminin 5 and collagen IV and VII staining, proliferation by Ki-67 staining.

Epidermis of fibroblast-containing CMS matured faster into a well-organized epithelium. A 52.7% increase in basal cells, a 53.5% increase in mitosis index and a 78% increase in keratinocyte cell-layers was observed.

Addition of fibroblasts reduced culturing time, enhanced proliferation, maturation and quality of the epidermis.

Introduction

Oral cancer is the sixth most common cancer in the world. It accounts for approximately 4% of all cancers and 2% of all cancer deaths worldwide. Malignant tumors of the oral cavity account for approximately 30% of all head and neck cancers. With an incidence rate of invasive lip and oral cavity tumors in The Netherlands rising to 6.6%, oral cancer is a common malignancy that often requires resection of the tumor followed by reconstruction (1,2).

Reconstruction of large defects of mucosa in the mouth is typically performed using free or pedicled skin flaps. Drawbacks to the use of skin flaps include donor site defects, bulkiness, sweating and hair bearing of the flaps (3,4). The feasibility of culturing oral mucosa *in vitro* for use in reconstructive surgery has been described. The first successful clinical trials, using collagen gels and membranes, report significant contraction of the cultured mucosal substitutes (CMS) as a challenging problem (5-7).

Dermal carriers reportedly reduce contraction compared to the use of keratinocyte sheets alone. Moreover, clinical reports on the use of cultured skin substitutes in patients with extensive skin loss, such as in burns, report reduced wound contraction and enhanced wound healing upon the addition of fibroblasts to a dermal matrix (8-10). Furthermore, the addition of fibroblast to the dermal carrier seems to enhance quality of the cultured epidermis and determines the phenotype of keratinocytes (11,12). In addition, *in* vivo studies have shown that fibroblasts enhance angiogenesis (8).

In case of cultured mucosal substitutes, the effect of fibroblasts on reconstituted epidermis has been illustrated using collagen gels. Clinical application of collagen gels, however is limited due to a number of problems. A collagen gel is mechanically weak; it cannot be sutured in place, in particular in the oral cavity where the tongue as well as the cheeks are constantly moving. Secondly, a collagen gel contracts more than other dermal scaffolds, such as a-cellular cadaver skin, and is therefore less feasible for transplantation. On the other hand, a-cellular cadaver skin is commercially available as Alloderm® and is clinically approved. Basement membrane proteins remain present at the basal membrane site of the dermo-epidermal junction (DEJ), as well as in the remnants of the vascular basal membranes. This facilitates in vitro keratinocyte attachment (13), as well as migration of endothelial cells invading the dermis upon transplantation (14). The DEJ is responsible for resistance to shear forces that could separate the epidermis from the dermis, such as in the case of a blister. Furthermore, DEJ proteins facilitate migration of keratinocytes e.g. in wound margins (15). Recently, one of the authors developed a technique to centrifuge

fibroblasts into a-cellular dermis(16). A clear improvement of epidermal morphology and a better formation of the DEJ as a result of the incorporation of fibroblasts in skin substitutes were seen using keratinizing skin keratinocytes. Moreover, fibroblasts obsoleted the addition of growth factors, such as KGF and EGF (16,17). These results suggest that incorporation of fibroblasts in non-keratinizing mucosal substitutes, might lead to important improvement of the quality of the cultured mucosa.

To date, no reports on the specific effects of bioactivation of a-cellular dermis using viable autologous fibroblasts on the epidermis was found in the literature. Therefore, the aim of this study was to evaluate whether incorporation of fibroblasts affects mucosal epidermal morphogenesis. This was done by evaluating the expression of several keratin, integrin and proliferation markers and the expression of basement membrane proteins.

Materials and methods

Culture of human keratinocytes

Upon approval of the local medical ethics committee, healthy human buccal mucosa was harvested. The mucosa was incubated in an antibiotic cocktail for 1 hour (Penicillin/ Streptomycin (100 U/ML/100 µg/ml), Gentamycin (50 µg/ml), Amphotericin B (2.5 µg/ml), all Invitrogen, Breda, The Netherlands). The biopsy was transferred to dispase (2.5 mg/ ml, (Invitrogen)) in Dulbeco's Modified Eagle Medium (DMEM (Invitrogen)), at 4°C overnight. Next day, the biopsy in dispase was put in 37° for 20 minutes prior to gentle separation of the epidermal sheet from the dermis. The dermis was used to isolate fibroblasts as described below. Subsequently, the epidermal sheet was put in Trypsin-EDTA, 0.25% (Sigma, Zwijndrecht, The Netherlands) at 37°C for 15 minutes. After neutralisation of the trypsin by DMEM with 10% Fetal Bovine Serum (FBS) (Sigma) a single cell suspension was obtained by gentle pipetting. Cells were seeded in a T75 culture flask onto a feeder layer of lethally irradiated 3T3 fibroblasts (a kind gift from dr. von den Hoff, Department of Orthodontics and Oral Biology, St. Radboud University Medical Centre, Nijmegen, The Netherlands) in seeding medium, according to the Rheinwald &Green protocol (18-20). Keratinocyte seeding culture medium was composed of a 3:1 mixture of DMEM, high glucose and Ham's F12 medium (Invitrogen), supplemented with 10% FBS; adenine, $1.8x10^{-4}$M (Sigma); cholera toxin, 10^{-10} M (VWR international, Amsterdam, The Netherlands); hydrocortisone, 0.4 µg/ml (VWR); insulin, 5 µg/ml (Eli Lily, Houten, the Netherlands); triiodo-L-thronine, $2x10^{-9}$ M (Sigma)

and penicillin-streptomycin, 100IU/ml-100µg/ml (Invitrogen). 24 Hours after seeding, proliferation medium was added to the cells. Proliferation medium is composed of seeding medium supplemented with 10 ng/ml mouse epidermal growth factor (EGF, Sigma). The medium was changed every 2 days.

Isolation and culture of human fibroblasts

Upon separation of the epidermis from the dermis, the dermis was washed once more in PBS, minced and put into DMEM with 1.5 mg/ml collagenase I and 2.5 mg/ml dispase at 37°C for 90 minutes. Subsequently, the enzyme solutions were filtered using a 100 µm cell strainer prior to centrifugation at 1200 RPM (200 G) for 5 minutes. Next, cells were seeded onto culture dishes in 10% FBS in DMEM and cultured at 37°C, 10% CO_2. Fibroblasts at passage three to five were used for seeding into a-cellular dermis.

Preparation of a-cellular dermis

Human cadaver skin (CMV, HIV and HBV negative), cryopreserved in 10% glycerol, was obtained from the European Skin Bank, Beverwijk, The Netherlands. The epidermis was pealed off the dermis after overnight incubation in 10x PBS with antibiotic cocktail and ethylene-diamine-tetra-acetic-acid (EDTA). The remaining dermis was acellularized using irradiation of the skin by 35Gy using gamma irradiation. Next, the dermis was maintained in antibiotic cocktail at 4°C for an additional week prior to centrifugal seeding of fibroblasts.

Centrifugal seeding of fibroblasts

To incorporate mucosal fibroblasts into the acellular dermis, a centrifugal seeding technique for fibroblasts was used, as described elsewhere (16,17). In short, 1.3 cm^2 pieces of dermis were placed with their basal membrane side down onto 35ml of DMEM / 1% agarose gel in a 50 ml Falcon tube (BD biosciences, Alphen aan den Rijn, The Netherlands) at room temperature. Next, 1 ml of fibroblast suspension containing 800.000 cells was added. Grafts were centrifuged at 200g for 60 minutes before keratinocytes were seeded onto the papillary side of the dermis. In order to estimate the number of cells incorporated into the dermis, cells in the supernatant were counted.

Culturing mucosal substitutes

After centrifugal seeding of fibroblasts, pieces of a-cellular dermis were placed with the papillary side up into 6 well dishes. A stainless steel seeding ring with a 1cm diameter

was placed on the dermis and subsequently filled with keratinocyte suspension containing 250.000 third passage keratinocytes in seeding medium (composed of DMEM/F12 (3:1), FBS 1%, cholera toxin 10^{-10}M, hydrocortisone 200 ng/ml, insulin 5µg/ml, ascorbic acid 50 µg/ml (Sigma) and penicillin-streptomycin 100IU/ml-100 µg/ml, as described elsewhere (21,22)). Grafts were produced in duplicate for two separate experiments. The next day, culture medium was changed to priming medium, which was the same as seeding medium but supplemented with bovine serum albumin (BSA) 24 µM (Sigma), fatty acid cocktail (oleic acid 25µM. linoleic acid 15 µM, arachidonic acid 7 µM, palmitic acid 25 µM) (Sigma), L-carnitine 10 µM (Sigma l) and L-serine 1 mM (Sigma). Grafts were maintained in this medium submerged for an additional 2 days. The next day, grafts were placed on a stainless steel mesh in a 6 well dish and were raised to the air-liquid interface for 7 or 14 days to generate an epidermis. The air-liquid interface medium was composed of serum-free priming medium supplemented with 1 ng/ml EGF (Sigma). The medium was changed every 2 days.

Morphology and Immunohistochemistry

Duplicate grafts per group of two separate experiments were harvested and cut in half. Subsequently, one half was snap frozen and one half embedded in paraffin. Paraffin sections (6 µm) were deparafinized, rehydrated and prepared for morphological or immunohistochemical analysis of K10, K13, β_1 chain, involucrin and vimentin. Immunohistochemical analysis of K16, K17, K10, α_6 chain, β_4 chain, laminin 5, and collagen type IV and VII was performed using 5 µm frozen section, which after sectioning at -20°C, were air-dried overnight, fixed in acetone for 10 minutes. The primary antibodies used in the present study are listed in table 1.

In brief, the following procedure was used for the paraffin sections: for the β_1- integrin antigen retrieval was done by cooking in citric acid buffer (2.15g citric acid monohydrate in 750ml of water) for 15 minutes in a microwave. After a cooling down for a period of 60 minutes in the citric acid buffer, sections were washed in PBS. For the vimentin and K10 protocols, a protease digestion step was introduced by exposing the samples to 0.5mg/ml trypsin in PBS for 10 seconds before neutralizing using 1% FCS in PBS for 5 seconds. After washing, endogenous peroxidases were blocked for 15 minutes using citric acid phosphate buffer with 30% H_2O_2 (Fluka, Zwijndrecht, The Netherlands. Thereafter, another washing step using PBS, aspecific binding was blocked using blocking buffer, composed of 10% normal rabbit serum (Sanquin blood bank, Rotterdam, The Netherlands) and 10% normal goat serum

(Sanquin blood bank) diluted in first step buffer (10% normal human serum (Sanquin blood bank) in PBS, for 30 minutes at room temperature.

Table 1: Primary antibodies used for immunohistochemical staining of tissue section

Sections	Antibody designation	Source*
Paraffin embedded	ß1 chain(CD29)	Lab Vision, Duiven, The Netherlands
	CK 13	Euro Diagnostica, Arnhem, The Netherlands
	CK 10	Euro Diagnostica
	Involucrin	Lab Vision
	Vimentin	Euro Diagnostica
Frozen	Ki-67	DAKO, Heverlee, Belgium
	K16 (LL0025)	Dr. I.M. Leigh, London, England
	K17 (CK-E3)	Sigma, Saint Louis, Missouri
	K10 (DE-K10)	ICN Biochemicals Inc, Ohioa
	ß6 chain (JEB5)	Dr. A. Sonnenberg, Amsterdam, The Netherlands
	ß4 chain(3E1)	Biomol, Hamburg, Germany
	Laminin 5 (P3E4)	Chemicon, Temecula, CA
	Collagen type IV (PHM12)	Chemicon
	Collagen typeVII (LH7.2)	Dr. I.M. Leigh

*Antibodies not purchased from indicated sources were personal gifts from the investigator named.

All primary antibodies are listed in table 1. All primary antibodies were monoclonals. Mouse-anti-human-CD29-IgG_1 (1:200 dilution, Lab Vision, Duiven, The Netherlands) for $ß_1$-integrin, CK13 (1:25 mouse anti human CK13 monoclonal IgG_{2a} (Euro Diagnostica, Arnhem, The Netherlands), CK10 (Mouse- anti-human-keratin 10 IgG_1 (1:50, Euro Diagnostica), involucrin (1;1600, mouse-anti-human-involucrin IgG_1, Lab Vision) vimentin (1:100, mouse-anti-human-vimentin IgG_1, Euro Diagnostica) and Ki-67 (1:200, mouse-anti-human-Ki-67 IgG_1, Heverlee, Belgium) were added in first step buffer and incubated at 4°C overnight. As controls, isotype controls were added as a primary antibody, as well as no primary antibody.

The next day, samples were washed using washing buffer (0.1% tween in PBS) and incubated with the secondary antibody, biotin labelled goat anti mouse immunoglobulins (Dako) in secondary buffer (2% normal goat serum, 2% rabbit serum and 5% Bovine Serum Albumin in PBS) for 30 minutes at room temperature. Biotin labelled goat anti-mouse Ig-G was used for $ß_1$-integrin staining under the same conditions. Following a washing step using washing buffer, streptavidin-HRP complex (Dako) was added 1:300 in secondary buffer for

30 minutes at room temperature followed by a washing step with washing buffer. In the case of vimentin, streptavidin-ABC-HRP (Dako) was used under the same conditions. Next, the substrate (24µl of 30% H_2O_2 in PBS was added to 1.2ml 5% DAB (Sigma)) was added to the samples for 5 minutes before stopping the reaction using tap water. Next, counterstaining was performed using haematoxylin for 10 seconds. The samples were mounted using Vectamount (Dako) after drying.

Cell counting and statistical analysis

For analysis of cell layers and number of basal cells, 6 fields of view per graft were counted using a 100x magnification. Duplicate grafts of two separate experiments were quantified. For statistical analysis a non-parametric Mann-Whitney test was used.

Results

Fibroblasts remain present in the dermis upon centrifugation

Success of the seeding protocol was confirmed by vimentin staining to identify the fibroblasts in the dermis. Fibroblasts were homogeneously incorporated in the seeded dermises (Figure1 A, B). High power magnification of the incorporated fibroblasts in the dermis show vialble cells with normal nuclear morphology and nucleus/cytoplasma ratios (figure 1C). Control samples without fibroblasts showed no dermal vimentin staining. Quantification of the number of fibroblasts in supernatant of centrifuged cell suspensions showed 220.000 cells remained in the dermis per cm^2 graft.

Fibroblasts Enhance Epidermal Morphology

Fibroblasts were centrifuged into the reticular side of acellular dermis to study the effect of fibroblast on epidermal quality. This was followed by seeding of keratinocytes onto de papillary side of the dermis and rising of mucosal constructs to the air-liquid interface. Histological analysis of 7 days samples revealed a clear epidermis of differentiated keratinocytes with basal, spinal and granular layers.

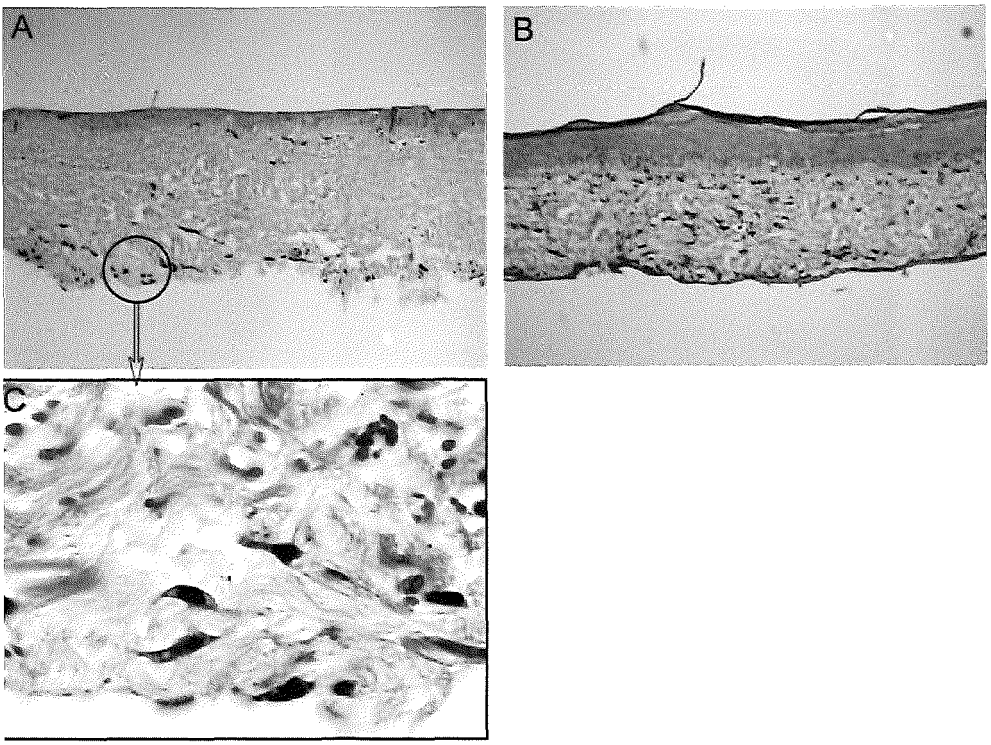


Figure 1: *Fibroblasts remain present in the dermis upon centrifugation. Vimentin staining shows fibroblast incorporation in to the a cellular dermisat at day7 and day 14 of culture at the air-liquid interface (magnification A and B, 40x). Color images available online at www.liebertpub.com/ten.*

Histogenesis of mucosal constructs in the presence of fibroblasts at the 7th and 14th day at air-liquid interface (figure 2 a and b) showed a much more developed epidermis than samples without fibroblasts. Quantification of the number of basal cells per surface area showed an increase in grafts with fibroblasts incorporated of 52.7% at the 7th day (P = 0.03) and 41.1% at the 14th day (P = 0.02). In addition, the number of cell layers increased 78.2% (P = 0.03) and 49.0% (P = 0.02) respectively (figure 3a and b) as a result of fibroblast incorporation. Furthermore, it was noted that the number of basal cells had significantly decreased after 14 days in the grafts without fibroblasts (P = 0.02). Fibroblast addition resulted in a stable number of basal cells per surface area. A significant decrease in cell layers of keratinocytes was observed (P = 0.02) in the constructs without fibroblast. Fibroblast incorporation, on the other hand, resulted in a significant increase in cell layers over time (P = 0.05).

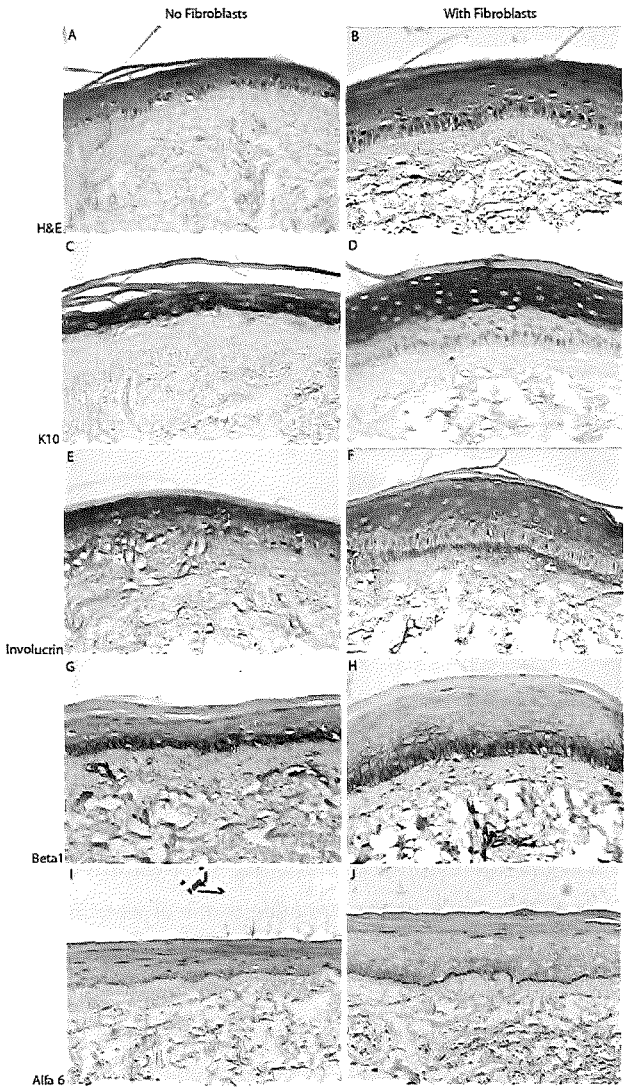


Figure 2: *Histology of cultured mucosal substitutes at 14 day sculturing at the air-liquid interface. The left column indicates grafts without fibroblasts, and the right column has fibroblasts incorporated into the dermis. (***A,B***) Hematoxylin and eosin staining showing a better - organized epidermis upon fibroblast incorporation. (***C,D***) and (***E,F***) Cytokeratin 10 and involucrin staining for differentiated keratinocytes showing fibroblast incorporation delays keratinocyte differentiation. (***G,H***) and (***I,J***) ß1 integrin and its a_6 subunit staining confirming delayed keratinocyte differentiation in the fibroblast-incorporated group of dermises (magnification A-J, 200x).Color images available online at www.liebertpub.com/ten.*

Fibroblasts Delay Differentiation

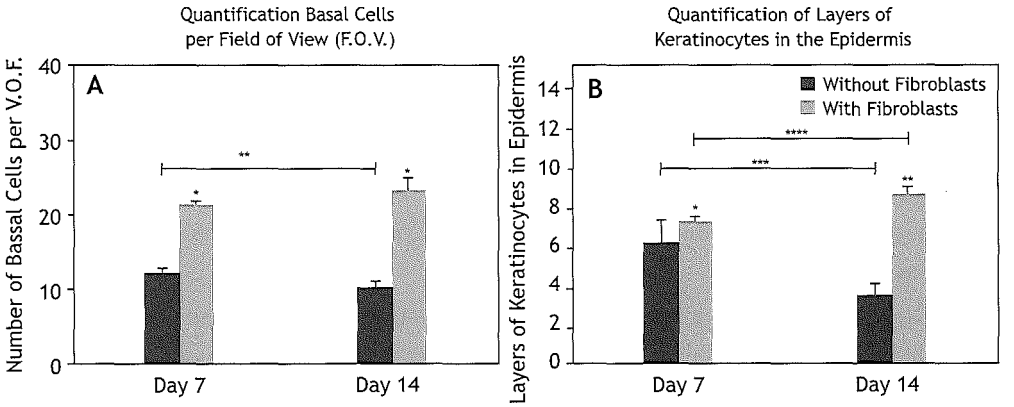
The role that fibroblasts play in epidermal regulation of differentiation was investigated by examining expression of proteins related to keratinocyte differentiation. K10 and involucrin expression (figure 2 c-f) in mucosal constructs containing fibroblasts showed suprabasal expression in all samples, as can be observed in natural mucosal tissues. In contrast, fibroblast free cultures showed expression of K10 in the basal cells as well as in suprabasal keratinocytes.

Expression of integrin subunits $ß_1$, $α_6$ and $ß_4$ was evaluated as well (figure 2 g-j). A clear increase in number of positive cells per cm^2 of graft and expression levels of these cells at the basal layer was observed in the CMS with fibroblasts. In addition, expression of the integrin at suprabasal levels of the epidermis was observed in the fibroblast containing CMS.

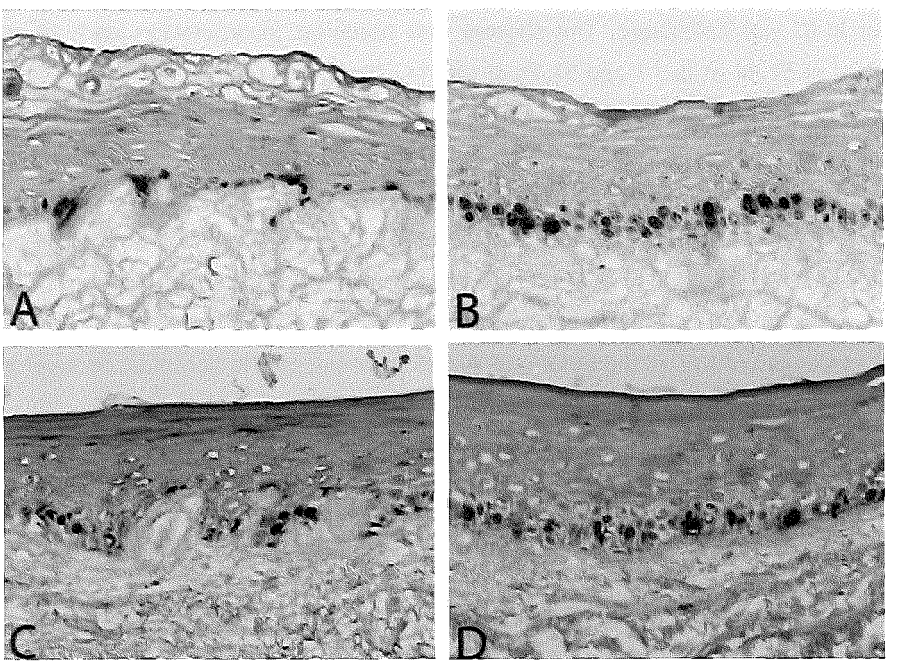
Fibroblasts Enhance Proliferation in the Epidermis

Ki-67, a marker expressed in proliferating cells, was used to quantify the number of proliferating basal cells (figure 4). The mitosis index (MI), expressed as % of basal cells positive for Ki-67 was calculated. This showed that fibroblast incorporation leads to an increase in MI of 52.8% at day 7 and 53.5% at day 14. These differences were statistically significant with P values of 0.03 and 0.02 respectively. Other indicators of hyper proliferation include suprabasal cells expression of Ki-67 as well as ß1 integrin or its subunits. This was only observed in grafts with fibroblasts.

Concurring with these data are the levels of expression of hyperproliferation-associated marker K17 (figure 5 f-h). At day 7, K17 is expressed less in grafts without fibroblasts than in the grafts with fibroblasts. At day 14, K17 expression was noted in both groups, although a decreased expression of K17 at the basal segment of the epidermis in fibroblast containing CMS was observed compared to its day 7-time point.



Figre 3: Quantification of the number of basal cells pe rhigh-power field (A) and the number of epidermal cell layers (B). At all time points, the incorporation of fibroblasts into the dermis resulted in an increase in basal cells, as well as an increase in cell layers. Data represent mean ± standard error of the mean. Six fields of view were counted in duplicate grafts per time point of 2 separate experiments.



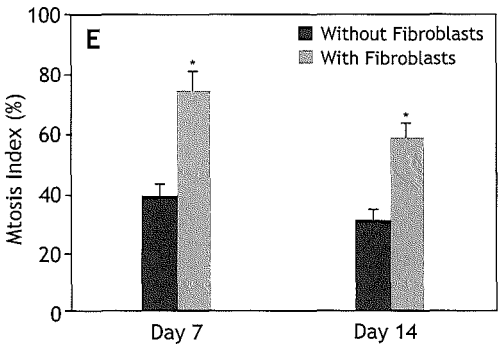


Figure 4: *Ki-67 staining at day 7 (A,B) and day 14 (C,D) shows that the incorporation of fibroblasts (rightcolumn) results in enhanced proliferation in the basal cells. Furthermore, suprabasal proliferation was observed in the fibroblast group. Quantification of the prolife rating basal cells (E) using high-power magnification showed an increase of up to 30% as a result of fibroblas tincorporation. Data represent mean standard error of the mean. Six fields of view we recounted in duplicate grafts per time point of 2separate experiments (magnification A-D, 200x). Color images available online at www.liebertpub.com/ten.*

Fibroblasts Enhance Basement Membrane Protein Deposition

Several proteins were evaluated to study whether fibroblasts played a role in basement membrane development. Staining for basal membrane components, such as collagen type IV and laminin-5 showed that the incorporation of fibroblasts did not affect expression of these basement membrane markers. Collagen type VII at day 7 however (figure 5 a-d), was expressed only in fibroblast containing CMS. In 7 days old fibroblast-free constructs collagen type VII is not expressed. At day 14 this difference was less pronounced. The expression of all analysed BM proteins was confined to the DEJ. In addition, presence of laminin-5 was noted in the remnants of vasculature basement membrane proteins in the a-cellular dermis.

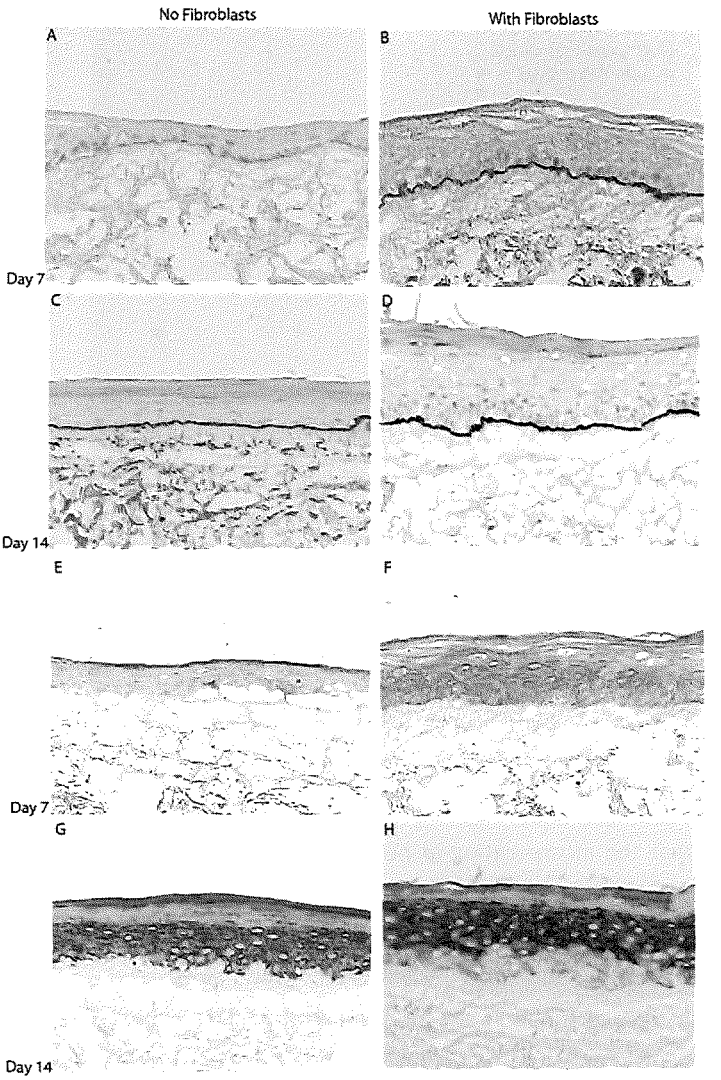


Figure 5: *Staining for basal membrane componen tcollagen VII and cytokeratin 17. Cultured mucosal substitutes without fibroblasts are indicated in the left column, and grafts with fibroblast are shown in the right column. Collagen VII staining showed that, at day 7, the air-liquid interface (***A,B***) and, at day 14 (***C,D***), the incorporation of fibroblasts results in enhanced deposition of this protein at the basal membrane. Hyperproliferation- associated marke rcytokeratin 17 (***E-H***) was enhanced as well as are sultof incorporation of fibroblasts. At day 14, however, the fibroblast group showed less expression in the basal cells (magnification A-J, 200x). Color images available on line at www.liebertpub.com/ten.*

Discussion

The objective in mucosal engineering is to have a well-differentiated epidermis, which is firmly attached to the dermal carrier at the basement membrane. Furthermore, certain *in vivo* characteristics play key roles in successful transplantation. Contraction should be minimal and angiogenesis maximal for optimal vascularisation ensuring the delivery of nutrients, cells and antibiotics to the grafted tissue. For successful clinical application the graft should be easily handled and sutured in place by the surgeon. Finally, culture time should be minimized.

Problems of blistering, fragility and wound contraction in early ventures for clinical use of cultured epidermal substitutes without dermal carriers showed the necessity of a dermal component for clinical use (23). The use of collagen gels as dermal carriers encountered problems in handling and wound contraction. Of the various clinically approved dermal analogues, an a-cellular dermis ensures minimum wound contraction and optimal mechanical properties. Furthermore, the vasculature can invade into the dermis from the wound bed using the basement membrane proteins present in the remaining proteins of basal membranes of the donor vasculature (12,14,24,25). A problem encountered in our laboratory in producing CMS using a-cellularized cadaver skin, however, is varying quality of the newly formed epidermis.

Studies using collagen gels showed the beneficial effects of addition of fibroblasts on epidermal quality of a construct (12,17). Incorporation of fibroblasts into an a-cellular dermis, however, has proved to be technically challenging and its effects have not been described for mucosal substitutes. From our study, a number of observations illustrate the extent to which fibroblast determine the quality of the epidermis.

In this study it became clear that fibroblasts incorporated into an a-cellular dermis improves epidermal morphogenesis. A better-organized epidermis at day 7 and day 14 in the fibroblast supplemented grafts, as well as a higher number of cell layers and better cell alignment at the basal layer illustrated this. Another indication of improved epidermal development by the addition of fibroblast was the increase in cell layers and stable number of basal cells over time.

Evaluation of integrin subunits β_1, α_6 and β_4 revealed suprabasal integrin expression while in the fibroblast free matrices expression was confined to the basal layer. Also Ki-67 staining for proliferative cells showed suprabasal proliferation in the CMS with fibroblasts incorporated. In addition, it showed a significant increase in MI of the basal keratinocytes.

Finally, hyperproliferation associated marker K17 was expressed, irrespective of the absence or presence of fibroblasts. These results indicated a hyperproliferative state of the epidermis. This finding indicates that the grafts are in an activated state. Hyperproliferation of the epidermis is commonly seen as the first phase in wound healing and is considered beneficial to the process (15,26,27). Moreover, decreased proliferation in the epidermis, one of the effects of ageing, is correlated to impaired wound healing (28,29).

The observed activated state of the epidermis might be caused by paracrine factors secreted by the fibroblasts. A number of studies described the paracrine pathway by changing the phenotype of epidermis by heterotypical recombination experiments. Examples include studies performed by Okazaki et al. (11,30), where nail matrix fibroblasts were combined with skin keratinocytes. The keratinocytes started to express nail specific keratins. Also, they have shown the expression of mucosa specific keratins when mucosal fibroblasts were combined with skin keratinocytes and vice versa (30,31). An important paracrine growth factor that is known to affect the epidermis is Keratinocyte Growth Factor (KGF). KGF is a member of the FGF family and is also known as FGF-7. It is produced by fibroblasts, not by keratinocytes and it affects keratinocytes through the receptor that is expressed on keratinocytes. It is cytoprotective and known to be a mitogen and motogen (21,32,33).

Another growth factor that might be responsible for the activated epidermis is EGF (Epidermal Growth Factor). This growth factor has been supplemented to the culture media and documented earlier as an epidermal activator (34,35). Interestingly, the epidermis formed in fibroblast-seeded matrices resembled the epidermis that can be seen in cultured skin substitutes, which are stimulated using EGF or KGF (20,21,33). One more explanation might be the high amount of fibroblasts incorporated into the matrices. El Ghalbzouri et al. (16) has shown in skin substitutes that a specific number of fibroblasts incorporated per 1 ml collagen results in epidermal normalization (absence of K6, K16 and K17). For mucosal substitutes the optimal fibroblast concentration should be established as well. As for the preparation of C.M.S., hyperproliferation is probably advantageous to the fabrication process, as it reduces culture time. Expression of hyperproliferative markers is expected to normalize upon transplantation (24).

Fibroblasts promoted early maturation of the dermo-epidermal junction (DEJ). The DEJ plays an important role in prevention of separation of the epidermis from the dermis. In disorders such as inherited Junctional Epidermolysis Bullosa, the deposition and attachment of keratinocytes to basal membrane proteins, such as laminin 5 is disturbed (36). Here the D.E.J. is incomplete, resulting in blisters. Additionally, basement membrane components

play a role in re-epitheliasation. Proteins, such as laminin 5 and collagen VII, a component of the anchoring fibrils, are deposited by fibroblasts and keratinoctytes (37,38). Furthermore, laminin 5 plays an important role in the restoration of the basement membrane, as it stimulates keratinocyte migration and proliferation (39,40). In our *in vitro* study a more rapid maturation of the DEJ was observed as a result of fibroblasts being present in the dermis. This concurs with data from other authors. El Ghalbzouri showed this effect for keratinizing skin by culturing grafts in fibroblast-conditioned medium (37,38). This suggests that this phenomenon is of a paracrine origin. The observation has clinical implications as a more mature DEJ might result in a firmer anchoring of the epidermis to the dermis, resulting in reduced problems of blistering or loss of the epidermis upon transplantation into the mechanically challenging oral environment.

Another positive *in vivo* effect can be expected from seeding dermal carriers with dermis-derived fibroblasts *in vitro*, as it provides a carrier for the proper type of fibroblast to the wound upon transplantation. In the case of a construct without fibroblasts, fibroblasts migrate into the dermis from the wound bed. The wound bed derived fibroblasts often originate from fat or scar tissue, which contains a large number of myofibroblasts. Others have shown, that addition of fat derived fibroblast or myofibroblasts to a dermis leads to enhanced dermal contraction by α-smooth-musle actin expressing myofibroblasts (8-10). Therefore, the addition of dermal fibroblasts to our CMS prior to transplantation could result in reduced scar contraction and better wound healing (12). In addition, Erdag et al have shown that the addition of fibroblasts to the constructs enhances angiogenesis and improves graft survival.

Conclusion

From these observations presented in this study we conclude that the addition of fibroblasts to an acellular dermal carrier resulted in a clear improvement of epidermal morphology. Addition of fibroblast resulted in an increase of basal cells per surface area and early ripening of the DEJ, facilitating early transplantation. The in vitro data predict a possible improvement of in vivo behavior of these grafts. From our data it can be concluded that development of epidermis and basal membrane takes significantly less time, which is an important step forward towards clinical implementation of these techniques (41). his might have consequences for the culture time prior to transplantation. Further studies

will be performed looking at the optimal time for transplantation for the construct with fibroblasts.

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Chapter 6

Effect of Hypoxia *in Vitro* on Cultured Mucosal Substitutes

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Abstract

Cultured mucosal substitutes (CMS) have been developed for clinical use. Upon transplantation of CMS, a period of hypoxia occurs as vasculature invades the a-vascular dermis. This makes the CMS fragile and susceptible to e.g. infection.

In this report, the effect of hypoxia on epidermal morphology, proliferation and differentiation was studied using reconstructed mucosal substitutes as a three-dimensional model. Hypoxia was shown using a pimonidazole staining after 24 up to 72 hours in hypoxic culture using 1.5% oxygen. CMS were stained for the differentiation marker cytokeratin 10 (K10) and the integrin subunit β_1. Epidermal proliferation was examined by a Ki-67 staining. Finally, the secretion of VEGF was examined by ELISA

The results show that hypoxia disturbed epidermal architecture and decreased β_1 integrin expression in basal keratinocytes, while the differentiation program (K10 expression) was altered as well. In addition, mitotic indices decreased after hypoxic treatment. Finally, a transient upregulation of VEGF secretion was observed the first 48 hours.

In conclusion hypoxia delays differentiation, reduces proliferation, and decreases metabolic activitity.

Introduction

Oral cancer is the sixth most common cancer in the world. It accounts for approximately 4% of all cancers and 2% of all cancer deaths worldwide. Malignant tumors of the oral cavity account for approximately 30% of all head and neck cancers. With incidence rates of invasive lip and oral cavity tumors in the Netherlands rising to 6.6%, oral cancer is a common malignancy that often requires resection of the tumor followed by reconstruction (1,2).

Reconstruction of large intraoral mucosal defects is typically performed using free or pedicled skin or muscle flaps. Drawbacks to the use of skin flaps include donor site defects, bulkiness, sweating and hair bearing (3,4). The feasibility of culturing oral mucosa *in vitro* for use in reconstructive surgery has been described. Clinically used Cultured Mucosal Substitutes (CMS) typically are composed of cultured mucosal keratinocytes seeded onto an a-vascular dermal carrier such as a-cellularized cadaver skin.

Clinical use of CMS using non-keratinizing keratinocytes have been troubled by problems such as infection and partial graft take. Upon transplantation, a phase of plasmatic inbibition occurs prior to vasculature invading the a-vascular dermis. Cells are depending on diffusion to provide oxygen and nutrients. During this period grafts are hypoxic and vulnerable to infection.

Hypoxia has a number of well-documented effects on cells and tissues. It enhances the secretion of survival enhancing factors such as Hypoxia Inducible Factor 1 (HIF-1), which in turn induces the secretion of angiogenic factors, such as VEGF i-NOS and PDGF-B (5,6). Furthermore, hypoxia induces metabolic changes enhancing production of glycolytic enzymes favouring anaerobic glycolysis and cell survival (7,8). On the other hand, hypoxia induces cell cycle arrest (9) and finally apoptosis through e.g. P53 pathways (10,11).

In contrast to hypoxia, hyperbaric oxygen therapy seems to be beneficial to survival of skin grafts by increasing oxygen tension in the grafts and reducing bacterial contamination (14-17). Also, overexpression of VEGF by genetic modification of cultured skin grafts has been shown to result in enhanced survival of grafts and angiogenesis (18,19).

To date, published reports discussed changes in two-dimensional systems. No data have been published on the effect of hypoxia on three-dimensional CMS. Therefore the aim of this study was to characterize the CMS in hypoxic conditions *in vitro* as a model for the early period post transplantation. More specifically, the effect of hypoxia on epidermal morphology, differentiation and proliferation, as well as secretion of angiogenic growth factors in CMS has been evaluated.

Materials and methods

Culture of human keratinocytes

Upon approval of the local medical ethics committee, human buccal mucosa was harvested from patients during scheduled ablative intra oral procedures. The mucosa was incubated in an antibiotic cocktail for 1 hour (Penicillin/Streptomycin (100 U/ML/100 µg/ml), Gentamycin (50 µg/ml), Amphotericin B (2.5 µg/ml), all Invitrogen, Breda, The Netherlands). Biopsies were transferred to dispase (2.5 mg/ml, (Invitrogen)) in antibiotic cocktail, at 4°C overnight. After which they were put at 37° for 10 minutes prior to gentle separation of the epidermal sheets from dermis. The dermis was used to isolate fibroblasts as described below. Subsequently, epidermal sheets were put in Trypsin-EDTA, 0.25% (Sigma, Zwijndrecht, The Netherlands) at 37°C for 20 minutes. After neutralisation of trypsin by DMEM with 10% Fetal Bovine Serum (FBS) (Sigma) a single cell suspension was obtained by gentle pipetting. Keratinocytes were seeded into a T75 culture flask onto a feeder layer of lethally irradiated 3T3 fibroblasts (a kind gift from dr. von den Hoff, Department of Orthodontics and Oral Biology, University Medical Centre, Nijmegen, The Netherlands) in seeding medium, according to the Howard & Green protocol (20-22). Keratinocyte seeding culture medium was composed of a 3:1 mixture of DMEM, high glucose and Ham's F12 medium (Invitrogen), supplemented with 10% FBS; adenine, $1.8x10^{-4}$M (Sigma); cholera toxin, 10^{-10} M (VWR international, Amsterdam, The Netherlands); hydrocortisone, 0.4 µg/ml (VWR); insulin, 5µg/ml (Eli Lily, Houten, the Netherlands); triiodo-L-thronine, $2x10^{-9}$ M (Sigma) and penicillin-streptomycin, 100 IU/ml-100µg/ml (Invitrogen). 24 Hours after seeding, proliferation medium was added to the cells. Proliferation medium was composed of seeding medium supplemented with 10ng/ml mouse epidermal growth factor (EGF, Sigma). The medium was changed every 2 days.

Isolation and culture of human fibroblasts

Upon separation of epidermis from dermis, the dermis was minced and put into DMEM with 1.5 mg/ml collagenase I and 2.5 mg/ml dispase at 37°C for 90 minutes. Subsequently, enzyme solutions were filtered using a 100µm cell strainer prior to centrifugation at 1000 RPM (200 g) for 5 minutes. Next, cells were seeded onto culture dishes in 10% FBS, penicillin-streptomycin 100IU/ml-100µg/ml and gentamycin (50 µg/ml) in DMEM and cultured at 37°C, 10% CO_2. Fibroblasts at passage three to five were used for experiments.

Preparation of a-cellular dermis

Human cadaver skin, cryopreserved in 80% glycerol, and CMV, HIV, HBV negative, was obtained from the European Skin Bank, Beverwijk, the Netherlands. The epidermis was pealed off the dermis after overnight incubation in 10x PBS with antibiotic cocktail and ethylene-diamine-tetra-acetic-acid (EDTA). Remaining dermis was acellularized by gamma irradiation (35Gy) and cut into pieces of 1.5 cm^2 and stored in DMEM supplemented with penicillin-streptomycin and gentamicin.

Centrifugal seeding of fibroblasts

To seed mucosal fibroblasts into the acellular dermis, a protocol was optimized using a centrifugal seeding technique and a-cellular dermis, as described elsewhere (23,24). 1.5 cm^2 pieces of dermis were placed with their basal membrane side down onto 40 ml of DMEM / 1% agarose gel in a 50 ml Falcon tube (BD biosciences, Alphen aan den Rijn, The Netherlands) at room temperature. Next, 1 ml of fibroblast suspension containing 800,000 cells was added. Grafts were centrifuged at 200 g for 90 minutes.

Culturing mucosal substitutes

Upon centrifuging fibroblasts into dermis, pieces were placed with basal membrane side up onto 6 well dishes. Grafts were produced in duplicate. A stainless steel seeding ring with a 1 cm diameter was placed on the dermis and subsequently filled with keratinocyte suspension containing 250,000 third passage keratinocytes in seeding medium, composed of DMEM/F12 (3:1), FBS 1%, cholera toxin 10^{-10} M, hydrocortisone 200 ng/ml, insulin 5 µg/ml, ascorbic acid 50 µg/ml (Sigma) and penicillin-streptomycin 100 IU/ml-100µg/ml, as described elsewhere (25,26). Next day, priming medium, which is seeding medium supplemented with bovine serum albumin (BSA) 24µM (Sigma), was added, fatty acid cocktail (oleic acid 25 µM, linoleic acid 15 µM, arachidonic acid 7 µM, palmitic acid 25µM) (Sigma), L-carnitine 10 µM (Sigma) and L-serine 1 mM (Sigma). Grafts were maintained in this medium submerged for an additional 2 days. Next, grafts were placed on a stainless steel mesh in a 6 well dish and were raised to the air-liquid interface for 7 or 14 days. Air-liquid interface medium was composed of serum-free priming medium supplemented with 1 ng/ml epidermal growth factor (EGF)(Sigma). Medium was changed every 2 days.

Hypoxia protocol

After 14 days at air-liquid interface, medium was changed and 5ml of medium was added to CMS on grids in 6 well plates remaining at the air-liquid interface. Next, six grafts were placed in 1.5% oxygen, 5% CO_2, and six grafts were placed in a regular stove using 20% oxygen, 5% CO_2. At time points 24, 36, 48, 60 and 72 hours media samples (1.5ml) were harvested from all CMS cultures and 1.5ml of fresh media was added. Harvested media samples were centrifuged and stored in -20°C until analysis using ELISA was performed. At time points 0, 24, 48, and 72 hours, duplicate grafts were harvested and cut in two parts for paraffin embedding and cryosectioning.

Morphology and Immunohistochemistry

Protocols for morphology and immunohistochemistry were performed according to protocols as published earlier (27,28). In short, harvested mucosa cultures were snapfrozen or washed in PBS, fixed in 4% paraformaldehyde, dehydrated and embedded in paraffin. Sections (6 µm) were cut, deparaffinized in ethanol and rehydrated in preparation for morphological or immunohistochemical analysis of keratin 10, 16, integrin subunit β_1 and Ki67. Immunohistochemical analysis of K16 and K10 was performed using 5 µm frozen sections, which after sectioning at -20°C, were air-dried overnight, fixed in acetone for 10 minutes. The primary antibodies used in the present study are listed in Table 1.

Table 1: Primary antibodies used for immunohistochemical staining of tissue sections

Antibody designation		Source[a]
Paraffin-embedded		
	Keratin 10 (CK-10)	Euro Diagnostica, Arnhem, The Netherlands
	β_1 chain (CD29)	Lab Vision, Duiven, The Netherlands
	Hypoxiprobe-1	Chemicon International, Huizen, The Netherlands
Frozen		
	Ki67 (Mb67)	DAKO, Heverlee, Belgium
	Keratin 1 6 (LL0025)	Dr.I.M.Leigh, London, England

[a]Antibodies not purchased from indicated sources were personal gifts from the investigator named.

After incubation with primary antibodies, sections were stained with avidin-biotin-peroxidase complex system (streptABcomplex/HRP, DAKO), as described by the suppliers with the following minor modifications: phosphate-buffered saline was used instead of Tris-buffered

saline and for Ki67 staining antigen retrieval was performed by immersing slides in 0.1 M citrate buffer (pH 6.0) for 30 min at 100°C followed by slow cooling to room temperature for at least 3 hours prior to staining of the sections. All sections were counterstained with hematoxylin.

Visualization of Hypoxic Cells

For visualization of hypoxic cells, a Hypoxiprobe-1 kit was used (Chemicon international, Huizen, The Netherlands). This kit contains Pimonidazole and stains hypoxic cells using immunohistochemistry. Parafinized slides were used according to company's instructions.

ELISA for FGF and VEGF

Culture media were assayed for basic Fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) using duo sandwich ELISA kits (R&D systems, Abbingdon, UK). Samples of each 12 hours were assayed in triplicate, according to the company's instructions.

Mitosis Index

For analysis of proliferation, Ki-67 positive cells in the basal layer of 6 fields of view per graft were counted using a 1000x magnification. Duplicate grafts per group were quantified. The resulting data were expressed as the mean ±SD.

Statistical analysis

For statistical analysis a non-parametric Mann-Whitney test was used for both Ki-67 and ELISA data.

Results

Mucosal grafts are hypoxic in 1.5% oxygen

Grafts were cultured at air liquid interface for 14 days. After 14 days one half of the grafts were cultured under hypoxic conditions in 1.5% oxygen up to 72 hours. To test whether this treatment resulted in hypoxia a pimonidazole staining was used. Normoxic cultures (figure 1A) showed no hypoxic cells. After 24 hours hypoxic cultures show an intense staining in epidermis as well as in dermal fibroblasts that were centrifuged into the dermis, indicating intracellular hypoxia in 1.5% oxygen (figure 1B),

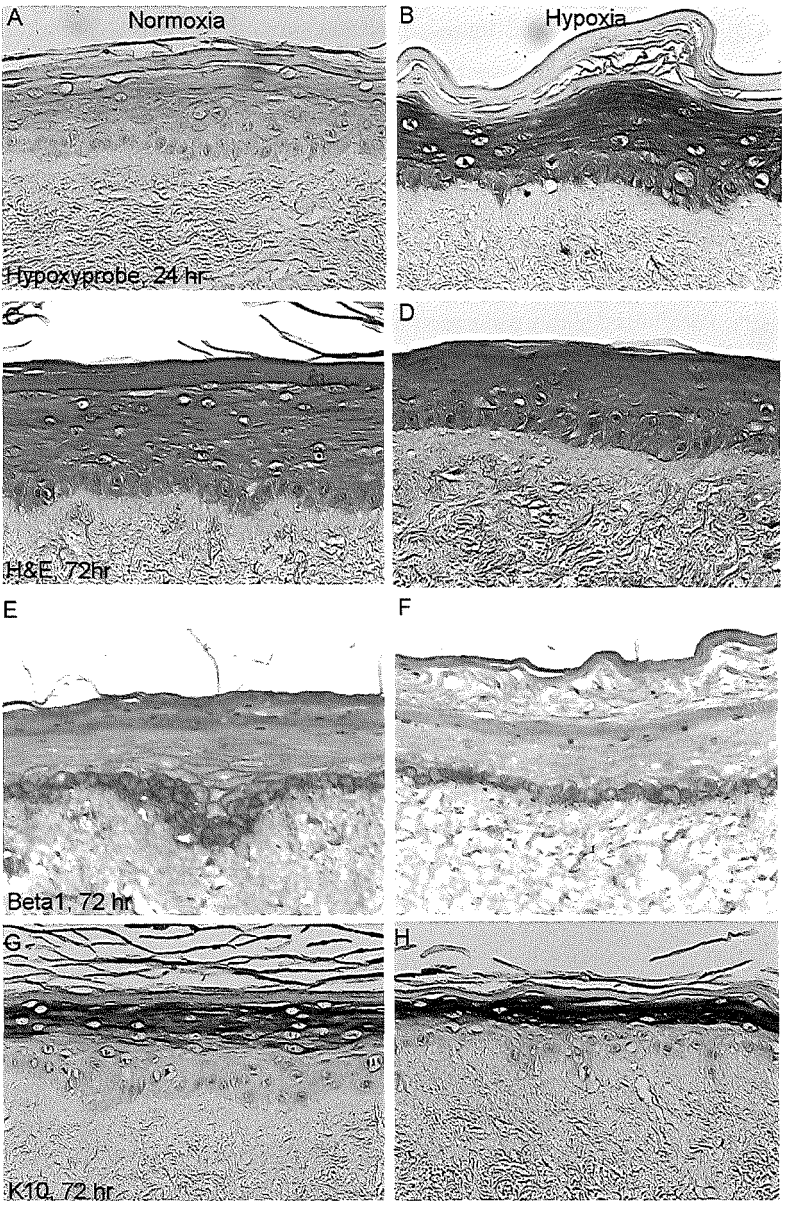

Figure 1: **A-B:***Hypoxic cultures show intense staining, even after 24 hours, pimonidazole, indicating hypoxia (figure 1B). Normoxic cultures showed no hypoxic cells (figure 1A).* **C-D:** *Hematoxyllin and Eosin stainging showed a disturbed architecture of basal cell layers in hypoxic grafts, Normoxic grafts (left column) displayed 1-2 well aligned rows of basal cells with round-*

shaped nuclei. Hypoxic grafts (right column) seemed to have only 1 row of basal cells after 24 hours. This row was not well-organized and showed reduced numbers of cells per layer of keratinocytes. Moreover, after 72 hours in hypoxic culture the number of cell layers in hypoxic grafts proved to be more than in the epidermises of normoxic grafts. **E-F:** *Integrin ß1 was expressed at the suprabasal cell layers of normoxic CMS. Hypoxic conditions on the other hand induce differentiation after 48 hours B1 expression was reduced to the basal layer. Figure* **1G-H:** *K10 expression in mucosal constructs showed suprabasal expression in all normoxic mucosal tissues. Hypoxic cultures showed increased differentiation of the epidermis as jugged by the expression of K10 in the basal cells as well as in suprabasal keratinocytes. Magnification 200x*

Hypoxia reduces number of viable cell layers

After confirming hypoxia, morphologic analyses were performed. H&E staining showed a disturbed architecture of basal cell layers in hypoxic grafts. While normoxic grafts (**figure 1C**) displayed 1-2 well-aligned rows of basal cells with round-shaped nuclei; hypoxic grafts seemed to have only 1 row of basal cells (**figure 1D**). This row was not well-organized and showed reduced numbers of cells per layer of keratinocytes. Moreover, after 72 hours in hypoxic culture the total number of cell layers in epidermises of hypoxic grafts proved to be less than those of normoxic grafts.

Hypoxia enhances K10 expression and reduces ß1 integrin expression

After showing altered morphology, epidermal differentiation patterns were studied. Integrin subunit $ß_1$ was used as a marker for basal cells while K10 was used to show differentiation. The integrin $ß_1$ was expressed at basal and suprabasal cell layers of normoxic CMS as was described previously (27). However, after 48 hours hypoxia, $ß_1$ levels were reduced, indicating differentiation (figure 1E,F).

K10 expression (figure 1G,H) in mucosal constructs showed suprabasal expression in all normoxic mucosal tissues. Concurring with our $ß_1$ data, K10 staining showed hypoxia induced differentiation of keratinocytes as K10 was expressed in basal as well as in suprabasal keratinocytes. Both K10 and $ß_1$ are correlated to proliferative qualities of keratinocytes, hence results indicated a decrease in keratinocyte proliferation.

Hypoxia results in a decrease in proliferation

To evaluate whether hypoxia affects the epidermal proliferation process, we examined the expression of Ki-67 and K16 (hyper-proliferation associated keratin) in hypoxic and

normotoxic cultures (**figure 2 A** and **B**). Kertinocyte proliferation was only observed in the basal cell layers under both conditions. Mitosis index (MI) was calculated and showed a significant decrease in percentage of proliferating basal keratinocytes per surface area after 24 hours of hypoxia from 88.8% (±1.19) in normoxia to 55.1% (± 0.57) in hypoxia ($P<0.05$). This difference increased up to a 51.6% reduction, from 84% (± 5.79) in normoxia to 40.7% (± 1.35) after 72 hours ($P < 0.05$, figure 3c). No differences were observed in the expression of the hyperproliferation associated protein K16 in hypoxic and normoxic cultures (data not shown).

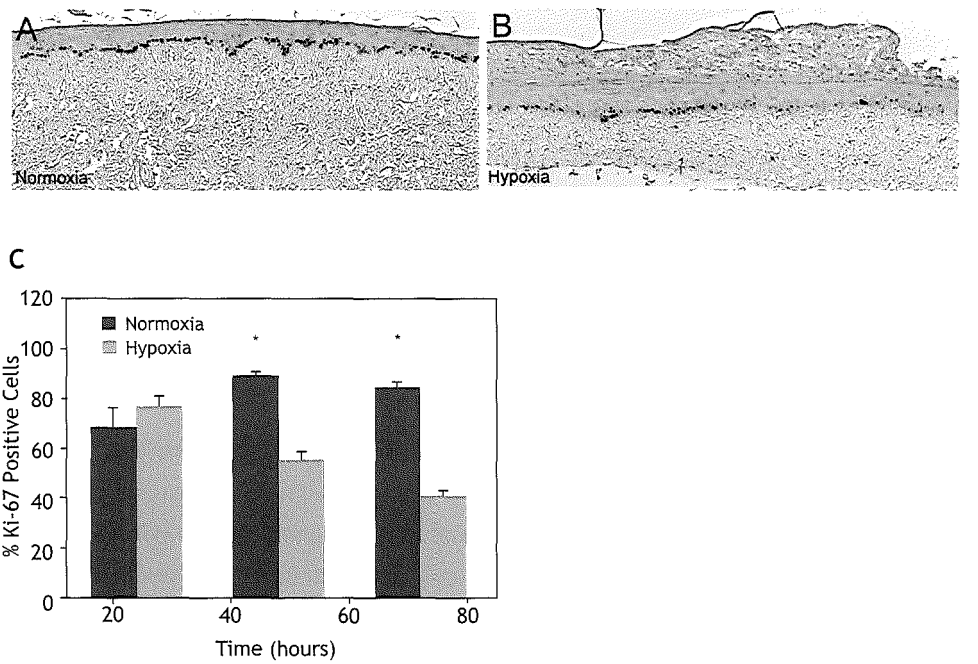

Figure2: *Proliferation marker Ki-67 showed proliferation confined to the basal cell layers (Figure* **2A***). From gross morphology at 100x magnification after 72hrs in hypoxic culture (figure* **2B***), a obvious decrease in proliferation can be observed. Mitosis indices (MI) were calculated and confirmed a significant decrease after 24 hours of hypoxia up to a 51% reduction after 72 hours (figure* **2c***, magnification 200x, *= P < 0.05).*

Hypoxia transiently upregulates VEGF expression

The effect of hypoxia on secretion of angiogenic growth factors that play a role in vascularisation was studied, since hypoxia is thought to stimulate angiogenesis partly by

up-regulation of VEGF, We analyzed the presence of VEGF and FGF in the medium of the cultures using ELISA (figure 4). Results showed an immediate difference in VEGF secretion by hypoxia. This difference was significant up to 48 hours ($P < 0.05$). Differences were up to 1.72 fold after 24 hours in hypoxic culture conditions. Concentrations of VEGF in the media of hypoxic cultures rises rapidly in the first 24 hours after which concentrations remain stable. The VEGF concentrations in normoxic culture media on the other hand rises until 72 hours, resulting in a reduction of the difference between normoxic and hypoxic conditions to 2%. Concentrations of FGF did not differ between normoxic and hypoxic conditions (data not shown).

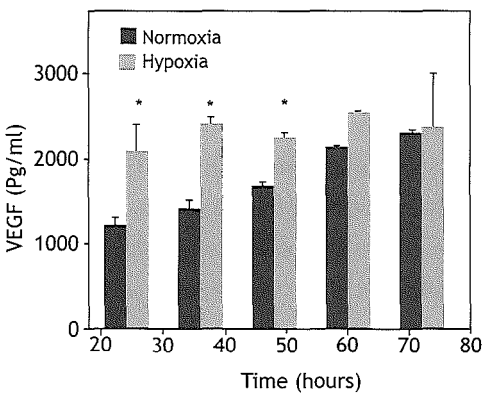

Figure 3: *Culture medium was analyzed for VEGF concentration. The results show an immediate and significant increase in VEGF secretion in hypoxia within the first 48hrs of up to 1.72 fold. VEGF Concentrations in normoxic culture media rise over time, resulting in reduction of these differences to 2% at 72 hours (, *= $P < 0.05$).*

Discussion

The aim of this study was to characterize responses of three-dimensional cultured mucosal substitutes to hypoxic conditions. This is the first report on the clinically highly relevant behavior of CMS under hypoxic conditions. Others have reported on the effect of hypoxia using two dimensional cell culture. In contrast to a two-dimensional system, a three-dimensional construct, has a natural multi-layered structure, where cells interact with

eacht other as well as with the extracellular matrix. Also exposure of individual cells to their environment is different, depending on cell position in the epidermis.

It has been demonstrated that epidermal hypoxia occurs frequently under physiological conditions (29). For example, wound margins become hypoxic immediately after wounding due to a reduced dermal blood supply by intravascular cloth formation (30). Also, in bone marrow, progenitor cells are physiologically subjected to hypoxic conditions (31). *In vitro*, keratinocytes in the CMS survive on diffusion of oxygen from the air and culture media. Upon transplantation oxygen levels will decrease while the (semi) occlusive dressings render oxygen levels to a minimum.

Pimonidazole staining showed epidermises to be hypoxic in the 1.5% oxygen culture conditions. Pimonidazole is a marker for hypoxia, forming protein adjuncts in hypoxic cells. It has been well-established to study hypoxia wound healing (32) tumors (33) and tissue engineered constructs (34).

Hypoxia altered epidermal morphology. Architecture of the epidermis was disturbed and thickness of the epidermis was reduced as less cell layers were formed. This may be a result of reduced proliferation, a hypothesis that was confirmed by our immunohistological data. Expression of the integrin subunit β_1 was reduced while differentiation marker K10 was enhanced. β_1 expression is typically considered to be confined to proliferative, basal cells in the epidermis while K10 expression concurs with differentiation and loss of proliferative capacities (35), On top of this our Ki-67 data showed a decrease in mitosis indices by hypoxia. K16, a marker for hyper proliferation, was equally expressed by the keratinocytes, indication that this process is not altered by hypoxia. An explanation for this observation might be the presence of EGF in the medium. EGF is considered to activate the epidermal proliferation program (24). Our data on proliferation concur with others who have shown in two-dimensional cell culture that hypoxia *in vitro* leads to growth arrest of cultured keratinocytes. Moreover, some groups have shown this arrest to be temporary and reversible upon re-oxygenation (9).

In our three-dimensional model, FGF was not enhanced by hypoxic conditions, which concurs with recent data on mesenchymal stem cells in hypoxic culture (37). No reports on skin or CMS have been published on this subject. VEGF secretion on the other hand was increased in hypoxic cultures and reached its maximum after 36 hours in culture. At later time points, VEGF levels remained constant while secretion in normoxic grafts seems to increase. This observation is in line with data obtained by others on cultured keratinocytes (6,38,39).

The current study validated the use of CMS under hypoxic conditions as a model for the early period post transplantation. From our data it was seen that hypoxia results in decreased metabolism as well as differentiation. Also, VEGF was rapidly but transiently up regulated. Whether these two effects enhance survival by decreasing demand for nutrients and oxygen while angiogenesis is enhanced in the wound bed is one hypothesis that warrants further research.

Acknowledgements

We gratefully acknowledge Dr T.L.M. ten Hagen, dept of Surgical Oncology, Erasmus MC, Rotterdam, the Netherlands, for providing the hypoxic culturing facilities. Funding from the Nuts-Ohra foundation and the 'Drie Lichten Foundation' was gratefully accepted.

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Chapter 7

Quantitative Analysis of Radiation-Induced DNA Break Repair in a Cultured Oral Mucosal Model

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Abstract

Oral mucositis is a major side effect of radiation therapy. Development of strategies for reduction of this problem calls for quantitative models. The goal of the present study was to test the feasibility of detecting double-strandbreaks (DSBs) and DSB repair proteins upon radiation of mucosa in a 3-dimensional culture system using morphology and immunohistochemistry.

Human oral keratinocytes and fibroblasts were seeded onto and into anacellular dermal carrier to produce a cultured mucosal substitute (CMS). CMSs were gamma-irradiated with 0,2, and 12Gy. One group received 4 Gythrough 2 Gyfractions with a 24-h interval.

Radiation-induced damage was quantified using hematoxylinandeosin (H&E). DSBs and DSB repair proteins were visualized and quantified using antibodies against P53 binding protein 1 (53BP1), MRE11, and RAD51. As in cell culture, CMSs showed intranuclear loci of damage and repair, mostly in the proliferative basal cell layers. Maximum percentages of damaged basal layer keratinocytes were 54.8% using H&E (12Gy) up to 78.9% (12Gy)for 53BP1.

This study shows the feasibility of DNA repair markers to quantify radiation damage. This is an important step forward in the study of mucositis and the development of treatment and prevention strategies, proving once more the power and clinica limportance of tissue engineering.

Introduction

Oral mucositis is considered a major side effect and complication in patients receiving chemotherapy, bone marrow transplantation, and radiotherapy for head and neck tumors. Clinical symptoms may vary from mild erythema to severe, painful ulcers in the mouth, resulting in malnutrition and placement of a feeding tube (1). Incidences of 60% in patients receiving radiotherapy have been reported. For many of these patients, it necessitates breaks in therapy for periods up to several days or weeks and pain relief using morphine (2). This allows the defects to heal, although at the same time, oncological therapy is compromised because of repopulation by tumor cells (3). Also, long-term effects of radiation therapy (e.g., osteoradionecrosis, mucosal atrophy) are related to the severity of the acute radiation response (4,5).

Mucositis is a clinical diagnosis of multifactorial origin (4-6). Damage to the epidermis is a result of direct DNA damage from radiation or indirect DNA damage from re-active oxygen species released upon radiation. Other causes of indirect damage include the release of cytokines and matrix metalloproteinases by epidermal and dermal cells, such as endothelial cells and fibroblasts, inducing an inflammatory response and edema. This reduces tissue vascularization and oxygenation. The final result is ulceration of the mucosa and colonization of the ulcer by the commensal bacteria that are present in large quantities in the oral cavity. No *in vitro* model is available to study radiation-induced damage to mucosa. Such a model potentially holds a large number of advantages over *in vivo* models. Tissue engineering might be useful in the development of an *in vitro* model.

Radiation primarily affects proliferating cells, causing highly proliferative tumor cells to be more radiosensitive than non-dividing cells in healthy tissues. Mucosa, however, is a tissue with a physiologically high cell turnover rate, mak-ing the cells susceptible to radiation damage. Damage can be observed especially in the basal layer of the epidermis, where proliferation of the epidermis occurs. Radiation re-sults in a number of different DNA lesions, the DNA double-strand break (DSB) being a particularly genotoxic one. A DSB induces cell-cycle arrest, giving time for the cell to recruit repair proteins. Alternatively, the cell goes into apoptosis and dies.

Cells have a number of guarding mechanisms that locate damage and initiate a suitable repair mechanism (7). One of the proteins involved in the detection of DSB is P53 binding protein 1 (53BP1), a protein that rapidly locates at the site of a DSB, as early as 5 min after irradiation. Generally, 12 to 14 h after irradiation, protein localization returns to normal (8).

DSBs are repaired using processes of the error-prone non-homologous end joining (NHEJ) or the error-free homo-logous recombination (HR) (9). The choice of mechanism depends in part on the phase of the cell cycle or, more spe-cifically, the number of chromatid copies (10). In G1 and early S phase, a single copy is present, and the cell uses NHEJ as a repair mechanism of choice. During S and G2 phase, how-ever, chromosomes are duplicated such that they consist of 2 identical sister chromatids. HR repairs a DSB on 1 sister chromatid, using the other intact chromatid as a template. In the processes of NHEJ and HR, a vast array of proteins are involved (7,9,11). A number of these proteins locate at the site of the DSB and can be used to observe sites of gamma irradiation-induced DSB repair.

MRE11 is part of a protein complex with the RAD50 and NBS1 proteins. Mutations in genes encoding for these pro-teins cause an ataxia-telangiectasia-like disorder or Nijmegen breakage syndrome (12). Patients with this disorder or syndrome are radiosensitive and cancer prone. A biologi-cally relevant activity of the MRE11 complex is bridging of broken DNA ends early in DSB repair (13-16). This activity can be important in the NHEJ and HR pathways of DSB repair, even though they are mechanistically distinct.

The RAD51 protein is another protein playing a key role in DSB repair, specifically in the HR pathway. RAD51 mediates DSB repair by promoting pairing of the intact identical se-quence on the sister chromatid (17-19). Mutations in HR's in-teracting partner, the product of the breast cancer-associated 2 gene (BRCA2), reveal the clinical importance of HR medi-ated by RAD51. Cells with mutated BRCA2 are unable to activate the RAD51 protein properly, and patients have a life-time risk of approximately 80% of developing breast cancer, demanding prophylactic mastectomy (20).

Upon gamma irradiation of cells, 53BP1, RAD51, and MRE11 localize in sub-nuclear structures. They accumulate in large quantities at the site of a DSB, forming ionizing radiation-induced foci (IRIF) in the cell nucleus. These DSB-marking foci can be visualized using immunofluorescence. *In vitro* studies have shown the number of cell nuclei with IRIF to be dependent on radiation dose using 2-dimensional cultures on plastic (8,21,22).

The goal of the present study was to test the feasibility of detecting DSBs and DSB repair proteins upon radiation of mucosa in a 3-dimensional (3D) culture system using morphology and immunohistochemistry. Antibodies that detect 53BP1, MRE11 and RAD51 were tested.

Materials and methods

Culture of human keratinocytes

Upon approval of the local medical ethics committee, human buccal mucosa was harvested. The mucosa was incubated in an antibiotic cocktail for 1 h (penicillin/strep-tomycin (100 IU/mL/100 µg/mL), gentamycin (50 µg/mL), amphotericin B (2.5 µg/mL), all Invitrogen, Breda, the Netherlands). The biopsy was transferred to dispase (2.5 µg/mL, Invitrogen) in antibiotic cocktail at 48°C overnight. The next day, the biopsies in the enzyme solutions were held at 37°C for 10 min before gentle separation of the epidermal sheets from the dermis. The dermis was used to isolate fibroblasts as described elsewhere below. Subsequently, the epidermal sheets were put in trypsin-ethylenediaminetetraacetic acid EDTA, 0.25% (Sigma, Zwijndrecht, The Netherlands) at 378C for 20 min. After neutralization of the trypsin using Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS) (Sigma) a single-cell suspension was obtained by gentle pipetting. Keratinocytes were seeded into a T75 culture flask onto a feeder layer of lethally irra-diated 3T3 fibroblasts (a kind gift from Dr. von den Hoff, Department of Orthodontics and Oral Biology, University Medical Centre, Nijmegen, the Netherlands) in seeding medium, according to the Howard and Green protocol (23-25).

Keratinocyte seeding culture medium was composed of a 3:1 mixture of high-glucose DMEM and Ham's F12 medium (Invitrogen) supplemented with 10% FBS, adenine $1.8x10^{-4}$M (Sigma), cholera toxin 10^{-10} M (VWR inter-national, Amsterdam, the Netherlands), hydrocortisone 0.4 µg/mL (VWR), insulin 5 g/mL (Eli Lily, Houten, the Netherlands), triiodo-L-thronine $2x10^{-9}$M (Sigma), and penicillin/streptomycin 100 IU/mL/100 µg/mL (Invitrogen). Twenty-four h after seeding, proliferation medium com-posed of seeding medium supplemented with 10 ng/mL mouse epidermal growth factor (Sigma) was added to the cells. The medium was changed every 2 days.

Isolation and culture of human fibroblasts

Upon separation of the epidermis from the dermis, the dermis was washed once more in PBS, minced, and put into DMEM with 1.5 mg/mL collagenase I and 2.5 mg/mL dispase at 37°C for 90 min. Subsequently, the enzyme solutions were filtered using a 100-µm cell strainer before centrifugation at 1000 RPM (200 g) for 5 min. Next, cells were seeded onto culture dishes in 10% FBS, penicillin/strepto-mycin 100 IU/mL-100 µg/mL and gentamycin 50 µg/mL in DMEM and cultured at 37°C, 10% carbon dioxide (CO_2). Fibroblasts at passages 3 to 5 were used for experiments.

Preparation of a cellular dermis

Human cadaver skin, cryopreserved in 85% glycerol and cytomegalovirus, human immunodeficiency virus, and hepatitis B negative, was obtained from the European Skin Bank (Beverwijk, the Netherlands). The epidermis was peeled off the dermis after overnight incubation in 10xPBS with antibiotic cocktail and EDTA. The remaining dermis was acellularized using gamma irradiation (35Gy). The dermis was cut into pieces of 1.5 cm^2 and stored in DMEM supplemented with penicillin /streptomycin and gentamicin.

Centrifugal seeding of fibroblasts

To seed mucosal fibroblasts into the acellular dermis, a protocol was optimized using a centrifugal seeding techni-que and acellular dermis, as described elsewhere (26,27) Pieces of 1.5-cm^2 dermis were placed with their basal membrane side down onto 40 mL of DMEM/1% agarose gel in a 50 mL Falcon tube (BD Biosciences, Alphen aan den Rijn, the Netherlands) at room temperature. Next, 1 mL of fibroblast suspension containing 800,000 cells was added. Grafts were centrifuged at 200 g for 90 min.

Culturing mucosal substitutes

After the fibroblasts were centrifuged into the dermis, pieces were placed with the basal membrane side up in 6-well dishes. Grafts were produced in duplicate for 3 separate experiments. A stainless steel seeding ring with a 1-cm diameter was placed on the dermis and filled with keratinocyte suspension containing 250,000 third-passage keratinocytes in seeding medium composed of DMEM/ Ham's F12 (3:1), FBS 1%, cholera toxin 10^{-10}M, hydro-cortisone 200 ng/mL, insulin 5 µg/mL, ascorbic acid 50 µg/mL (Sigma), and penicillin/ streptomycin 100 IU/mL/100µg/ mL, as described elsewhere (28,29). The next day, culture medium was changed to priming medium, which is the same as seeding medium but supplemented with bovine serum albumin (BSA) 24 µM (Sigma), fatty acid cocktail (oleic acid 25 µM, linoleic acid 15 µM, arachidonic acid 7 µM, palmitic acid 25 µM) (Sigma), L-carnitine 10 µM (Sigma), and L-serine 1 mM (Sigma). Grafts were maintained in this medium submerged for an additional 2 days. Next, the grafts were placed on a stainless steel mesh in a 6 well dish and were raised to the air-liquid interface for 7 or 14 days. The air-liquid interface medium was composed of serum-free priming medium supplemented with 1 ng/ mL epidermal growth factor (Sigma). The medium was changed every 2 days.

Radiationprotocol

After 13 days at the air-liquid interface, 3 CMSs per group were gamma-irradiated at dosages of 0, 2 and 12 Gy. One group received a fractionated dose of 4 Gy by 2 doses of 2 Gy with an interval of 24 h. Subsequently, grafts were incubated for 6 h at 37°C, 10% CO_2. Next, the CMSs were harvested and cut in 2 parts for paraffin embedding and cryo-sectioning.

Morphology and immunohistochemistry

Harvested CMSs were snap frozen or embedded in paraffin. Paraffin sections (5 µm) were deparaffinized, re-hydrated, and prepared for morphological or immunohistochemical analysis. Cryo-sections (6 µm) were fixed with 2% para-formaldehyde. Next, samples were processed for immunohistochemical detection of radiation damage and repair, apoptosis, and proliferation.

Histology

Hematoxylin and eosin (H&E) staining was performed on the paraffinized sections.

Visualization of DNA breaks and repair proteins

DSBs were detected using 53BP1, and repair proteins were detected using antibodies against MRE11 and RAD51. After sectioning, cryo-sections were fixed with 2% para-formaldehyde. Mesenchymal stem cells were permeabilized with 0.1% Triton X-100 in PBS and washed with PBSþ(0.5% BSA and 0.15% glycine) to prevent aspecific binding of the antibodies. The following antibodies were used: rabbit anti-human 53BP1 (rabbit polyclonal antibody, Novus Bio-logicals, Littleton, CO), rabbit anti-human RAD51 (nr. 2307, rabbit polyclonal antibody) (30), rabbit anti-human MRE11 (nr. 2244, rabbit polyclonal antibody) (31). Samples were incubated for 90 min at 37°C in a humidified chamber and washed with 0.1% Triton X-100 in PBS followed by a quick washing step with PBS. Subsequently, the secondary antibody (Alexa Fluor 594, goat α-rabbit immunoglobulin G; Molecular Probes, Inc., Leiden, the Netherlands) was applied for 60 min at 37°C in a humidified chamber and washed with 0.1% Triton X-100 in PBS and PBS. The sections were cover-slipped with 40'6-diamidino-2-phenylindole /DAPCOA/ Vectashield and sealed.

Quantification of radiation damage

Radiation damage was quantified using morphology and immunohistochemical staining. In H&E samples, the percentage of atypical, pyknotic nuclei was quantified in the basal, proliferative layer. A nucleus was considered pyknotic when it showed cytoplasmic shrinkage

as well as nuclear condensation or fragmentation (**figure 1**). As for DNA damage and repair proteins, the percentage of nuclei with one or more IRIF was calculated in the basal layer. The percentage was also determined in the suprabasal layers, where no clear dose response was observed (data not shown). Six fields of view were counted per graft at high-power magnification (1000x). Triplicate grafts per time point were counted.

Apoptosis assay

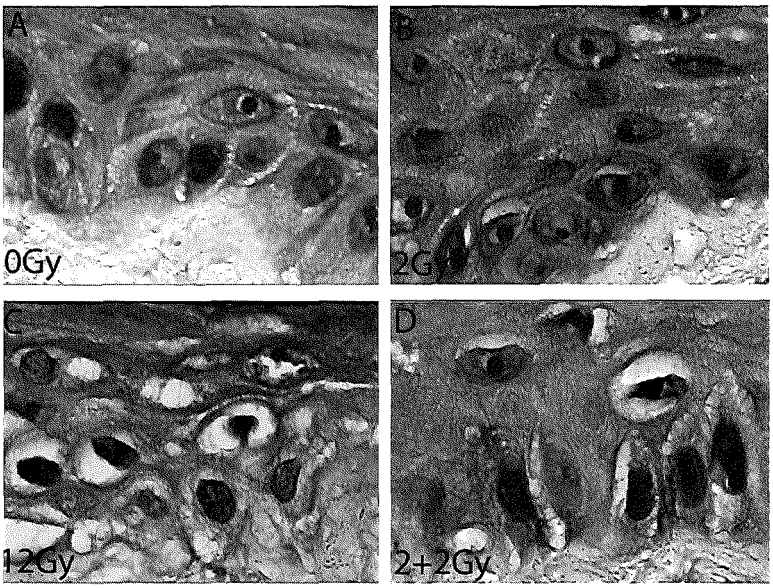
For detection of apoptotic cells in the CMSs at various radiation doses, a TUNEL assay (DeadEnd Colorimetric TUNEL System; Promega, Leiden, the Netherlands) was used as described by the manufacturer. After incubation with the DAB substrate, the sections were counterstained using hematoxylin.

Cellproliferation

For detection of cells in G1, S, G2, or M phase of the cell cycle, Ki-67 was used (Dako, Heverlee, Belgium). The secondary antibody, biotin-labelled goat anti-mouse immunoglobulins (Dako) in secondary buffer (2% normal goat serum (Sanquin Bloodbank, Rotterdam, the Netherlands) 2% human plasma (Sanquin), and 5% BSA (Sigma) in PBS) for 30 min at room temperature. Streptavidin-ABC-HRP (Dako) in PBS was used under the same conditions. Next, the substrate (1.2 mL 5% DAB (Sigma) with 24 µL of 30% hydrogen peroxide in PBS) was added to the samples for 5 min before stopping the reaction using tap water. Counterstaining was performed using hematoxylin for 10 s. After drying, the sections were coverslipped using Vectamount (Dako) and sealed with nail polish. Mitotic indices were expressed as a percentage of positive basal cells per high-magnification field of view.

Cell counting and statistical analysis

For analysis of the percentage of basal cells with IRIF, one author counted cells in the basal layer of 6 fields of view per graft using 1000x magnification. Cells were considered positive when more than 2 IRIF were present. Triplicate grafts per group were quantified. Statistical analysis was performed using the Mann-Whitney test. Statistical significance was reached if $p < 0.05$.



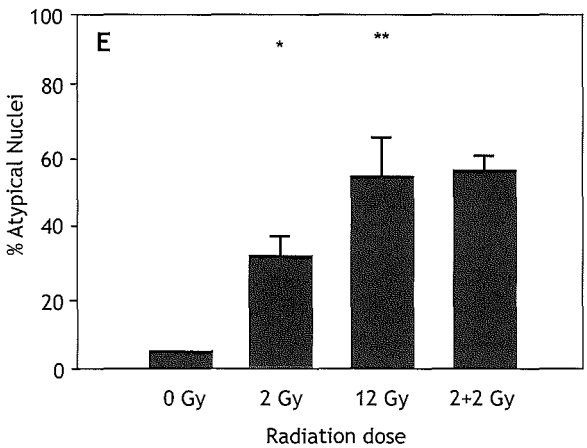


Figure 1: *(**A-D**) High magnification of hematoxylin and eosin-stained basal epidermal layers revealed the appearance of py-knotic nuclei from the dose of 2 Gy (original magnification 1000x). (**E**) Quantification of the number of pyknotic nuclei per surface area at various radiation doses was performed. A dose response was observed. Error bars represent standard errors of the mean of 3 grafts per group; approximately 120 cells per graft were counted (* $p < 0.05$). Color images available online at www.liebert pub.com/ten.*

Results

Effect and quantification of radiation on epidermal morphology

Radiation damage in the epidermis was studied invitro using 3D cultures of human oral mucosal keratinocytes. To determine the effect of radiation on quality of the epidermis in a CMS, H&E staining was performed (**figure 2 A-D**). The unirradiated samples showed a well-differentiated, multi-layered epidermis after 14 days of culturing the CMS at the air-liquid interface. The epidermis of the CMS resembled the natural mucosal epidermis (32) and was well organized into a basal layer and a spinous layer. Upon irradiation, a number of changes were observed. Gross morphology did not change significantly at doses of 2 Gy or at a fractionated dose of 4Gy administered by 2 times 2 Gy with a 24-h interval (2 + 2 Gy). At 12 Gy, however, the epidermis appeared to be severely damaged, resulting in drastic changes in morphology.

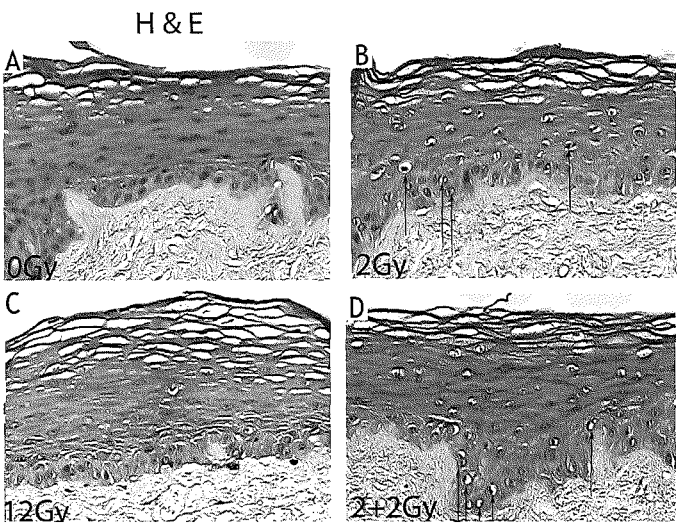


Figure 2: *Hematoxylin and eosin stain of cultured mucosal sub-stitutes at various radiation doses. (A) Unirradiated epidermises show differentiated keratinocytes with basal, spinal, and granular layers, as can be seen in natural mucosal tissue. (**B-D**) From an irradiation dose of 2 Gy, pyknotic nuclei, composed of severely condensed material, appear in the keratinocytes of the proliferative basal layer (black arrows). Original magnification 200x, bars re-present 50 um. Color images available online at www.liebertpub .com/ten.*

Next, we examined the effect of radiation on the mor-phology of the CMS in more detail. Upon radiation, cells with highly condensed, pyknotic nuclei started to appear in the basal and spinous layers of the epidermis (**figure 1 A-D**). To quantify the effect of radiation in CMS, the number of pyknotic cells in the proliferative, basal compartment of the epidermis were determined (**Figure 1E**) relative to the total cell number per high-power field of view. Percentages were dose dependent, rising from 4.4% (±1.1) in the unirradiated group to 53.5% (±16.5) in the 12 Gy irradiated group. In samples that received 2 + 2 Gy, the number of pyknotic cells was 54.8% (±9.1).

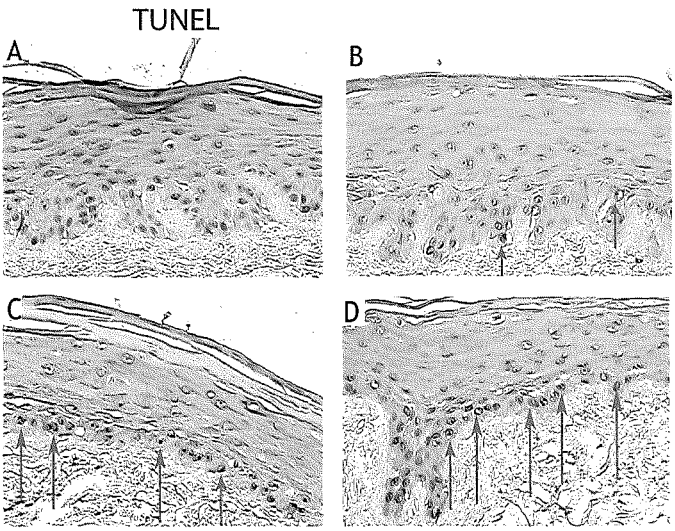


Figure 3: *TUNEL stain (right column) TUNEL staining showed that positive cells were most prevalent in the basal cells of the epidermis, most conspicuously in the 2 + 2 Gy groups. The nuclei staining positive for the apoptosis marker TUNEL were pyknotic (grey arrows). Bars represent 50 um. Color images available on-line at www.liebertpub.com/ten.*

To extend observations from morphological changes in-dicative of apoptosis, biochemical analysis was performed using TUNEL as a marker (**Figure 3 A-D**) to visualize whether radiation induced apoptosis. TUNEL-positive, apoptotic cells were observed in the basal layers, most prominent in the pyknotic nuclei. As was observed in morphology, there was a dose-dependent increase in TUNEL-positive cells as radiation doses increased.

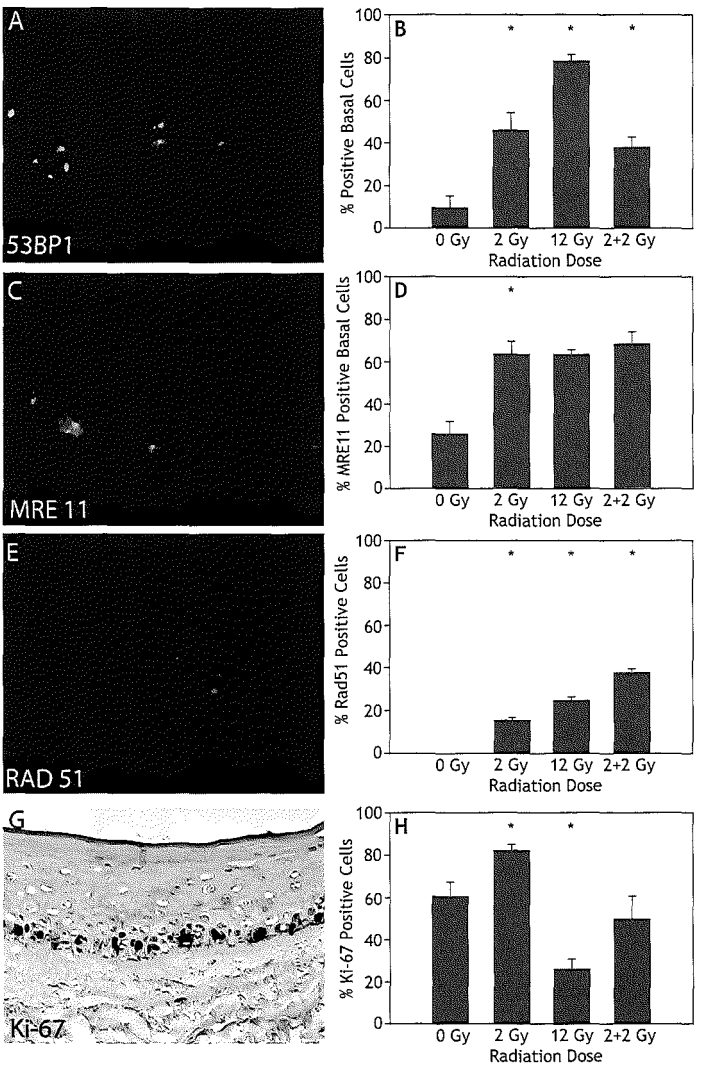


Figure 4: *(**A**) Radiation damage was detected using an antibody for P53 binding protein 1 (53BP1), a protein involved in the detection of double-strand breaks (DSBs). Intranuclear radiation induced foci (IRIF) for 53BP1 were observed. (**B**) Quantification of the percentage of cells with IRIF in the basal layer showed a response to increasing radiation doses. (**C**) Radiation damage repair was detected in the keratinocytes by staining for MRE11. IRIF for MRE11 were observed most clearly in the basal cells. (**D**) Quantification of number of MRE11-positive nuclei at the basal layer of the epidermis shows a response of expression to radiation doses. The number of nuclei with IRIF, however, seems to reach a maximum after 2 Gy, as the response flattens out. (**E**) DSB repair*

*was shown using the RAD51 antibody for homologous recombination. (F) Quantification of the percentage of cells showing IRIF for RAD51 showed a maximum in deoxy-ribonucleic acid repair in the group receiving 2 doses of 2 Gy. (G) Because homologous recombination only occurs in the late S phase, mitosis indices were measured using Ki-67 staining. (H) Quantification showed an up-regulation of mitosis index at a dose of 2 Gy. Overall, however, cell proliferation decreased as radiation doses increased. Error bars represent standard errors of the mean of 3 grafts per group; approximately 120 cells per graft were counted (*p < 0.05). Color images available online at www.liebertpub.com/ten.*

Quantification of doserelated radiation damage using 53BP1 IRIF

To develop a model for the quantification of radiation damage, 53BP1 was used as a marker for DSBs. 53BP1 is one of the first proteins that can be observed at the site of damage in the cascade of DSB repair. Therefore, we used 53BP1 to quantify radiation damage in the epidermis. Upon irradiation, 53BP1 was observed as IRIF in the proliferative basal layer (**Figure 4A**) and, less evidently, in the suprabasal cells. Quantification of the percentage of basal cells with IRIF showed a response to increasing doses of radiation (**Figure 4B**). Starting at 9.9% (±8.7) in the unirradiated group and increasing to 46.1% (±13.5) in the 2 Gy group and 78.9% (±3.5) in the 12 Gy group; 2 + 2 Gy resulted in 38.3% (±7.7), similar to a single 2 Gy dose. Quantification of the number of IRIF per nucleus showed a response as well, starting from 4.9% (±3.3) in the control group, 9.9% (±3.4) in the group receiving 2 Gy, and 11.9% (±0.6) in the group receiving 12 Gy. The fractionated-dose group showed an average of 5.7% (±2.5) IRIF per positive nucleus, again comparable with that of the group receiving a single dose of 2 Gy. Similar responses were observed for the suprabasal layers. These responses were less marked than in the pro-liferative basal layer (data not shown).

Quantification of doserelated radiation damage using MRE11 IRIF

Next, MRE11 was used to reveal DSB repair in the cell nucleus, and cells with IRIF were quantified. In all samples, IRIF were observed in the basal layer (**Figure 4C**). IRIF were also present in the suprabasal layer. The number of IRIF-positive nuclei in the suprabasal layer, however, did not change as radiation doses increased (data not shown). Quantification of the percentage of cells with IRIF in the basal layer showed a rapid response to increase in radiation dose (**Figure 4D**) between 0 and 2 Gy, starting at 25.7% (±1.9) in the unirradiated group and rising to 63.4 (±10.1) and 63.3 (±3.1) for the groups that received 2 Gy and 12 Gy, respectively. For the group that received 2 + 2 Gy, a percen-tage of 68.6% (±7.5) was measured. The response seemed to level off at a dose of 2 Gy.

Quantification of dose-related radiation damage using RAD51 IRIF

RAD51 was used to study IRIF in S and G2 phase when HR is performed. Upon irradiation, RAD51 IRIF were observed. No IRIF were formed in the unirradiated samples.

The IRIF were only present in the proliferative, basal cell layers of the epidermis (**Figure 4E**), with none in the suprabasal levels of the epidermis. Quantification of the percentages of cells with IRIF showed a response to increasing doses of radiation (**Figure 4F**). In the irradiated groups, the percentage of IRIF nuclei was 14.7% (±1.9) for the group that received 2 Gy, rising to 24.1% (±2.0) for the group receiving 12 Gy. The groups receiving a fractionated dose of 2 + 2 Gy showed the highest response: 37.3% (±2.8).

Dose dependent decrease in mitosis

Ki-67 was used to determine the proliferative activity in the epidermis (**Figure 4G**). Proliferation was observed in the basal layers exclusively. Mitotic indices were calculated. Overall, a decrease in the mitotic index was observed as radiation doses increased (**Figure 4H**) from 60.6% (±11.2) in the unirradiated group to 26.3% (±7.0) in the group receiving 12Gy. Remarkable, however, was an increase in mitosis index after 2 Gy radiation to 81.9% (±4.5). Apparently, the 2 Gy dose results in enhanced proliferation of the epidermis.

Discussion

The goal of the present study was to develop a 3D *in vitro* model to quantitatively study radiation damage in cultured mucosa, using morphology as well as markers for DNA damage and repair proteins.

Changes in cell and tissue morphology occurred as ra-diation doses increased. Cell nuclei became small and py-knotic. Close observation of the cell nucleus in our 3D mucosa revealed an increase in number of pyknotic nuclei in the basal layer, where the proliferative cells reside, as radiation doses increased. TUNEL staining showed these atypical nuclei to be apoptotic cells. It is well documented that cells go into apoptosis if the DNA damage is too severe, as an escape mechanism for malignant transformation. Moreover, the appearance of pyknotic nuclei resembles the morphology of so-called sun burn cells (SBCs), observed in ultraviolet-irradiated skin (33,34) and skin substitutes (35-37). SBCs develop as cells are damaged by ultraviolet light. Morphologically, y pyknotic nuclei with cytoplasmic shrinkage, mostly observed in the supra basal and mid epidermal layers, characterize SBCs. In the

case of gamma-irradiated mucosal substitutes, these cells appeared in the basal layer as well as the suprabasal layers.

Gamma irradiation results in cell damage. Particularly important are DSBs of DNA. An alternative for apoptosis is DNA repair. A number of biochemical markers for DNA damage and repair have been developed and are used extensively in cell culture. Moreover, in cell culture, intra-nuclear presence of damage and repair proteins was correlated to radiation (8,21,22). The present study tested 53BP138 for suitability in detecting radiation-induced DNA damage in CMS. For visualization of DNA repair, MRE11 and RAD51 were used. All antibodies proved to be useful.

Being visible early in the DNA damage response, 53BP1 was useful for quantification of radiation damage. Quantification of IRIF revealed a dose response to increasing doses of radiation. After 2 fractions of 2 Gy, similar damage was observed as in the 2 Gy group. This might be explained from the results of cell culture studies where the number of cells with IRIF decrease to normal levels 12-24 h after irradiation.In addition to 53BP1 H2AX was tested as an early marker for DSB. IRIFs were observed in the irradiated tissues. However, due to rapid bleaching of the IRIFs, quantification proved to be impossible.

Subsequent markers in the DNA DSB repair cascade in-clude MRE11 and RAD51. As for MRE11, this marker showed an early saturation of the number of nuclei with IRIF, making it a less suitable marker for quantification of radiation dose responses. The number of MRE11 induced IRIF in tissue culture has shown to reach a plateau at 6 Gy (22). The percentage of cells with IRIF was similar, 60%, con-curring with the data on 3D cell culture. The accumulation of damage-load to its maximum most probably explains the plateau at 2 Gy.

Quantification of HR using RAD51 showed increasing counts from 2 to 12 Gy. No RAD51 IRIF were observed in the unirradiated CMS. Two and 12 Gy showed higher counts of positive cells. The group of grafts receiving 2 fractions of 2 Gy showed the highest counts of IRIF. Concurring with these data are the data on the mitotic indices. This showed a proliferative peak in the group receiving 2 Gy. This peak might be a sign of repopulation, as was observed in mouse tongue mucosa (5,39). Upon small doses of radiation, basal stem cell keratinocytes start proliferating to repopulate the epidermis. The recruit-ment of cells 24 h after irradiation from G0 to S and G2 concurs with the introduction of HR as a repair mechanism of choice. In addition, the RAD51 protein has been shown to remain present for up to 24 h. HR repairs damage caused by a second fractional dose of 2 Gy to the cells that left G0 phase. Repopulation has been studied in the mouse tongue model. Here it was shown to start 3 to 7 days after irradiation (40,41). We found an increase in proliferative index as

soon as 8 h after radiation. This might be a result of symmetrical stem cell proliferation, or abortive proliferation by damaged stem cells, in which each damaged cell shows a number of abortive di-visions before differentiation and dying (5,42).

Changes observed in the presented *in vitro* model resemble changes observed *in vivo* in animals and humans, in which radiation damage also is most prevalent in the basal layers of mucosa. Similar morphological cell changes are observed, including nuclear condensation and pyknosis (41,43). Future projects will include the study of the effect of radiation-preventive cytokines or strategies (44). Also, more-detailed studies of long-term proliferative dynamics, such as cell cycle time, the number of cells in abortive divisions, the number of cells in repopulating divisions, and the percentage of surviving cells, are ongoing.

Conclusion

The authors conclude that the use of DNA repair markers to quantify radiation damage in a 3D invitroculture is feasible. Use of these markers in tissue-engineered constructs might lead to further insights in molecular mechan-isms of radiation damage in a 3D organo-typical structure. The presented model can be considered to be an important step forward as a tool in the study of the development, as well as the treatment or prevention, of mucositis in a more quantitative way, proving once more the power and clinical importance of tissue-engineering techniques.

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Chapter 8

General Discussion

General Discussion

The aim of this thesis was to develop, improve and test a CMS as a step towards clinical application in therapy for head and neck oncological procedures. To take a next step from lab bench towards bedside a number of goals were set and addressed in the various chapters (table 1).

Table 1: Goals in project Cultured Mucosal Substitutes, from Lab Bench Towards Bedside?

1.	Study the performance of current reconstructive techniques to identify possible issues that can be improved and challenges that the CMS will meet upon transplantation.
2.	Choose an optimal isolation protocol
3.	Choose an optimal design of the CMS by choosing a dermal carrier and decide on the cell types included
4.	Study behavior CMS to clinically relevant environments *in vitro*
	- Hypoxia
	- gamma irradiation

Head and neck cancer is increasingly common and Intra-oral reconstructive procedures are performed frequently. As mucosa is scarce, reconstruction of large mucosal defects is generally performed using free flaps, composed of keratinizing skin flaps. A number of workhorse flaps, including the radial forearm flap are used. Flaps should be thin in the anterior part of the mouth in order support mobility of the (remnant of the) tongue for important functions such as speech. In the posterior part of the mouth, on the other hand, more bulky flaps facilitate swallowing of food and saliva.

Patients require post-reconstructive radiotherapy in 80% of cases

The majority of our patients (79.9%) required post-operative radiotherapy so flaps should be well-vascularized to withstand irradiation. **Chapter 2** discusses clinical experience and quality of life outcomes using the Radial Forearm Flap in intra-oral reconstruction of the floor of the mouth. Two groups were described, based on location of the flap either in the anterior or posterior part of the oral cavity. Reconstructions, in particular in the anterior part of the mouth resulted in 26% of cases in too much bulk which impaired tongue function. The combination of skin and irradiation gave additional problems with dry mouth and swallowing.

Donor site morbidity is a problem. Damage to the superficial radial nerve results in decreased sensibility and cold intolerance. Furthermore, wound problems, tendon exposure and an unattractive and large donor-site scars can occur in up to 28% of patients (1-4). Improvement in functional outcome and reduced donor site morbidity would be expected if resected mucosa could be replaced with the same tissue according to the principle "replace like with like"(5, 6). Therefore, tissue engineering strategies were explored to assess feasibility in reconstructive procedures of large mucosal defects.

CMS can provide In vitro answers to clinical and experimental questions

Besides for clinical application, CMS as well as cultured keratinizing skin substitutes have become important *in vitro* models to study highly relevant subjects including carcinogenesis and congenital disorders such as Junctional Epidermolysis Bullosa (7,8). Others have performed important work studying the effect of various growth factors and other proteins using genetically modified keratinocytes(9-11). These techniques use human cells and new insights are gained, without the use of animals.

Setting up a CMS production line takes time

Cultured mucosal substitutes have been developed as described in this thesis. A number of problems are recognized in both the production phase and in the transplantation phase. Generally, a CMS is fragile. One should consider the production process of a CMS to be like a card house. Before one can test clinically relevant questions a large number of conditions have to be met (table 2). Cells should be isolated, seeded and grafts should be cultured. Problems can occur at every step of the protocol, and contamination is a constant danger. Infection in vitro means the CMS is prematurely destroyed, leading to frustration of the culturing crew, waste of money and time. Culturing CMS demands a learning period in which all these risks are encountered, recognized and dealt with. The first step in setting up a line is to test cell isolation protocols.

Dispase is the enzyme of choice in keratinocyte isolation

Generally, cell expansion *in vitro* happens exponentially. The rate at which this happens depends on the cell cycle of the cell type of interest. Keratinocytes have a cycle of approximately 24 hours. For clinical application, culture time should be reduced. One of the first steps to be optimized is the number of cells isolated per biopsy. In order to choose an optimal protocol for isolation and expansion in vitro, thermolysin and dispase were tested

(**chapter 4**). These enzymes are routinely used by many well-established laboratories (12). The results showed a clear advantage for the use of dispase. First of all, cell yields per surface area of biopsy were 3.26 times higher in the dispase treated groups. All of the dispase treated biopsies separated completely and at the level of the dermo-epidermal junction. Thermolysin on the other hand proved to be unpredictable in its effectiveness for two reasons. First, only part of the epidermal layers separated at all following overnight incubation. Secondly, epidermises that did separate showed a supra-basal separation level of the epidermis and dermis. Analysis of expression levels of differentiation marker CK13 (13) in the keratinocytes remaining attached to the dermis upon thermolysin treatment proved that remaining keratinocytes were basal cells. Basal keratinocytes are undifferentiated epidermal cells with the highest proliferative capacities, also the primary cells responsible for successful culturing and cell expansion necessary to be able to proceed to the phase of seeding onto a dermal scaffold.

Table 2: Determinants of successful culturing and clinical application of CMS

		Factors for success
In vitro	Isolation protocol	Straight-forward surgical protocol Communication between laboratory and surgeon Planning of isolation before reconstructive procedure Bacterial and fungal contamination rates Fibroblast contamination Cells/cm^2 biopsy % stem cells/cm^2 biopsy
	Costs	Culture time - Isolation protocol - Technical difficulty protocol - % of successfully produced CMS - Infection rates
In Vivo	Surgery	Communication between laboratory and surgeon Logistics, e.g. transportation to OR Mechanical properties, handling of CMS for surgeon Resistance of CMS to handling by the surgeon Straight-forward surgical protocol Straight-forward wound protocol for surgical and nursing staff
	Graft take	Angiogenesis Resistance to hypoxia Resistance to shearing forces Susceptibility to Infection
	Long term	Wound contraction Resistance to radiation Impairment of intra-oral functions such as speech

Of the various clinically approved dermal analogues, an a-cellular dermis ensures minimum wound contraction and optimal mechanical properties. Furthermore, the vasculature can invade into the dermis from the wound bed using the basement membrane proteins present in the remaining proteins of basal membranes of the donor vasculature (14-17). A problem encountered in our laboratory in producing CMS using conventional methods with a-cellularized cadaver skin and cultured keratinocytes without the use of fibroblasts was varying quality of the newly formed epidermis.

Recently, a technique was developed to centrifuge fibroblasts into a-cellular dermis (18). In keratinizing skin substitutes, a clear improvement in epidermal morphology and a better formation of the DEJ was a result of incorporation of fibroblasts. Moreover, fibroblasts made the addition of growth factors, such as Keratinocyte Growth Factor (KGF) and Epidermal Growth Factor (EGF) unnecessary (18,19). It has been shown that addition of fibroblasts to constructs enhances angiogenesis *in vitro* and improves graft survival. These results suggest that incorporation of fibroblasts in non-keratinizing mucosal substitutes might lead to important improvement of the quality of the cultured mucosa.

Addition of fibroblasts to CMS enhances morphology and quality of the CMS

To test this hypothesis, in **Chapter 5** human fibroblasts were incorporated into the a-cellular dermis of CMS and seeded with human keratinocytes. CMS were stained for H&E for gross morphology and differentiation markers $ß_1$ integrin, cytokeratin 10 and involucrin after one and two weeks in culture. Basement membrane formation was analysed by laminin 5 and collagen IV and VII staining, proliferation by Ki-67 staining.

Addition of fibroblasts to CMS enhances proliferation of keratinocytes and reduces culturing time

Our study showed that fibroblasts incorporated into an a-cellular dermis indeed improves epidermal morphogenesis and function. Gross morphology showed a better organized epidermis with more cell layers in fibroblast containing dermises. Evaluation of integrin subunits $ß_1$, $α_6$ and $ß_4$ revealed a higher number of basal cells per surface area. This was confirmed by Ki-67 staining showing suprabasal proliferation and statistically higher prolifation rates, which are advantageous to production, as it reduces culture time.

Addition of fibroblasts to CMS enhances maturation of the dermo-epidermal junction

Fibroblasts promoted early maturation of the epidermis as well as of the dermo-epidermal junction (DEJ). The DEJ plays an important role in prevention of separation of the epidermis from the dermis. Early ventures using cultured keratinocyte sheeths without dermal components in burns surgery showed significant problems of blistering, underscoring the importance of adding a dermal component to the construct. Combination of epidermis and a dermal analogue in vitro results in the formation of this DEJ. Moreover, in a-cellularized dermis, the DEJ proteins responsible for anchoring the epidermis, such as laminin, remain present after decellularization. A better DEJ formation by fibroblasts has clinical implications as a more mature DEJ might result in a firmer anchoring of the epidermis to the dermis, reducing blistering or loss of the epidermis upon transplantation into the mechanically challenging oral environment.

The observed effects on the epidermis by dermal fibroblasts in believed to be of paracrine origin. Growth factors, such as KGF, also known as FGF-7, play important roles in the development and maturation of an epidermis. Especially of interest is KGF, which is secreted only by fibroblasts, while keratinocytes express receptors for this growth factor. This means this growth factor is not present in grafts without fibroblasts and are introduced into the grafts with fibroblasts by the fibroblasts. Addition of KGF has dramatic consequences in vitro as well as *in vivo* (9,20). These changes are remarkably similar to the observed changes by addition of fibroblasts to the dermis.

Hypoxia occurs in physiological conditions

Epidermal hypoxia occurs frequently under physiological conditions (21). For example, wound margins become hypoxic immediately after wounding due to a reduced dermal blood supply by intravascular cloth formation (22). Also in bone marrow, progenitor cells are physiologically subjected to hypoxic conditions, preserving their undifferentiated status (23). *In vitro*, keratinocytes in the CMS survive on diffusion of oxygen from the air and culture media. Upon transplantation oxygen levels are expected to decrease while (semi) occlusive dressings render oxygen levels to a minimum.

The aim of our hypoxic experiment (**chapter 6**) was to characterize three-dimensional cultured mucosal substitutes in hypoxia. It was hypothesized that culturing in hypoxic conditions could have a beneficial effect post transplantation due to upregulation of angiogenic factors. To test this hypothesis CMS were grown for periods up to 72 hours in

2% oxygen and were compared to CMS cultured in ambient oxygen concentrations. It is the first report on clinically highly relevant behavior of three-dimensional CMS under hypoxic conditions.

Hypoxia decreases proliferation, delays differentiation in keratinocytes of CMS

Hypoxia altered epidermal morphology, development and function. Architecture of the epidermis was disturbed and thickness of the epidermis was reduced as less cell layers were formed. This is the result of reduced proliferation, a hypothesis that was confirmed by our immunohistological data. Expression of the integrin subunit β_1 was reduced while differentiation marker K10 was enhanced. Confirming Ki-67 data showed a decrease in mitosis indices by hypoxia. Our data on proliferation concur with others in two-dimensional cell culture where hypoxia *in vitro* led to growth arrest of cultured keratinocytes. Moreover, some groups have shown this arrest to be temporary and reversible upon re-oxygenation (24).

Hypoxia induces early and transient upregulation of VEGF secretion

In our three-dimensional model, VEGF secretion was increased in hypoxic cultures and reached its maximum after 36 hours in culture. At later time points, VEGF levels remained constant while secretion in normoxic grafts seem to increase. This observation is in line with data obtained by others on cultured keratinocytes (25-27).

This study validated the use of CMS under hypoxic conditions as a model for the early period post transplantation. Whether these two effects enhance survival by decreasing demand for nutrients and oxygen while angiogenesis is enhanced in the wound bed is a hypothesis that warrants further research.

After angiogenesis has occurred and grafts become normoxic by adequate supply of blood and oxygen, a new phase starts in the clinically applied CMS, involving post-reconstructive irradiation. As described in chapter 2, most of the patients receive postoperative radiotherapy.

Typical protocols use doses of 2Gy up to a cumulative dose of 66 or 70Gy. Oral mucositis is considered a major side effect and complication in patients receiving radiotherapy for head and neck tumors. In addition, mucositis is highly relevant for patients undergoing chemotherapy and bone marrow transplantation. Clinical symptoms may vary from a mild erythema to severe, extremely painful ulcers in the mouth resulting in malnutrition and

placement of a feeding tube (28). Incidences of 60% in patients receiving radiotherapy have been reported. For many of these patients it necessitates breaks in therapy for periods up to several days or weeks and pain relief using morphine (29). This allows the defects to heal, while on the other hand oncological therapy is inevitably compromised due to repopulation by tumor cells (30). Also, long term effects of radiation therapy, e.g. osteoradionecrosis, mucosal atrophy are related to the severity of the acute radiation response (31,32).

Mucositis is caused by a number of changes induced by radiation in the various anatomical layers of mucosa (31-33). One of the causes is radiation damage to keratinocytes in normal mucosa as this is one of the only constantly proliferating cell types. Proliferation maintains the epidermis as a protective layer. Mucositis still is a clinical diagnosis. No scoring systems use histological or molecular markers. To date, no three-dimensional in vitro model is described to study radiation induced damage to mucosa. Such a model potentially holds a large number of benefits.

Radiation results in dramatic epidermal changes in CMS

In **Chapter 7**, tissue engineering proved to be useful in studying radiation damage. CMS were irradiated by gamma radiation at doses of 2Gy, 2x2Gy with a 24 hour interval and 12Gy. Results showed dramatic changes in cell and tissue morphology increasing at higher doses. Cell nuclei became small and picnotic. Also, micronuclei were observed as a sign of radiation damage. Next we were able to quantify these changes.

Morphological changes can be used to study radiation damage quantitatively

Close observation revealed an increase in number of picnotic nuclei especially in the basal layer, where proliferative cells reside. TUNEL staining showed these atypical nuclei to be apoptotic. Quantification of these damaged basal cells in H&E processed grafts proved to be possible, moreover, a dose-response curve was shown. To confirm our histological data, we tested various proteins involved in DNA damage and DNA repair to show and quantify DNA damage and DNA repair at various radiation doses.

CMS can be used to study DNA damage and Repair quantitatively

Gamma irradiation results in DNA damage. Of the various types of DNA damage, gamma irradiation typically results in double-strand DNA breaks (DSB) where both strands of the DNA are broken. DNA damage can lead to a number of cellular responses. One of the first possibilities is malignant transformation of the cell as its genetic information might be

altered. In order to prevent this, apoptosis is an escape mechanism that often occurs if the DNA damage is too severe. An alternative for apoptosis is DNA repair.

A number of biochemical markers for DNA damage, as well as repair have been discovered and were tested extensively in cell culture studies (34-36). In **Chapter 7** a number of markers for both DNA damage as well as for DNA repair were successfully tested for the first time in a three-dimensional tissue, the CMS. For quantification of radiation damage the early marker 53BP1 was useful in visualizing and quantifying DNA damage. Of subsequent markers in the DNA DSB repair cascade RAD51 was most useful for quantification. Moreover, dose response curves were seen, validating a model measuring radiation damage and potentially measuring the effects various radiation protocols and radiation damage preventing agents, such as Vitamin C.

Radiation induces cell proliferation at a single low dose

In this study, mitotic indices were determined. Results showed a proliferative peak in the group receiving 2Gy, a dose that is typically used in the clinical situation. This peak might be a sign of repopulation as was observed in mouse tongue mucosa (32,37). Upon small doses of radiation, basal stem cell keratinocytes start proliferating to repopulate the epidermis. Repopulation has been studied in the mouse tongue model. Here it was shown to start 3 to 7 days after irradiation (38,39). We found an increase in proliferative index as soon as 8 hrs after radiation. This might be a result of symmetrical stem cell proliferation, or abortive proliferation by damaged stem cells, where each damaged cell shows a number of abortive divisions prior to differentiation and dying (32,40).

In vitro histological and proliferative changes upon radiation resemble observed changes *in vivo*

Changes observed in the presented *in vitro* model resemble changes observed *in vivo* in both animals and humans where radiation damage is most prevalent in the basal layers of mucosa. Similar morphological cell changes are observed including nuclear condensation and picnosis (39,41). Therefore, the model can be considered to be an important step forward as a tool in the study of etiology, as well as treatment or prevention of mucositis, proving once more the power and clinical importance of tissue engineering techniques.

Where are we now, Future Perspectives

As can be concluded from this thesis, CMS has earned its place in experimental, *in vitro* studies for various subjects. The strength of three dimensional cultures is the fact that they behave largely physiologically, as illustrated by our hypoxia and radiation studies. This means that clinically relevant questions can be studied closely in vitro. Other purposes can be optimization. How long should we treat grafts using hypoxia or hyperoxic condition prior to transplantation? What radiation dose results in most rapid restoration of normal cells in CMS and compare this to CMS with tumor cells. Adding other cell types than fibroblasts and keratinocytes, such as endothelial cells, mast cells and inflammatory cells to the construct will increase physiological relevance of the model. Therefore it can be expected that CMS will be used increasingly.

From Lab Bench to Bedside. There have been people using CMS in clinical applications such as intra-oral reconstruction. Especially intra-orally the environment is demanding. A number of hurdles remain to be conquered as discussed elsewhere in the thesis and in Table 1.

We do believe the aforementioned experiments put CMS a step closer toward the clinic. A robust protocol is developed and more importantly tested in clinically relevant conditions. The next step can be studying the performance of CMS *in vivo*. The question remains how CMS responds to large numbers of bacteria, enzymes and constantly moving tissues intra-orally.

A technique to cope with these threads is to use a two-step protocol of transplantation using a prelaminated flap. In this protocol the CMS is placed subcutaneously on a fascia, such as the radial forearm fascia. Here, vascularisation can take place in the first two weeks in a sterile environment with a well vascularized wound bed. Next, the fascia with the graft firmly attached and vascularized can be grafted to the intra-oral environment using free flap techniques.

We think our CMS is ready for testing *in vivo* and opt for the technique described and hope the CMS will prove to be an addition to the armamentarium of the reconstructive plastic surgeon.

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Chapter 9

Nederlandse Samenvatting

Doelen project

Het doel van deze promotie was het ontwikkelen en testen van een nieuw soort kunst slijmvlies (mucosa) voor gebruik bij reconstructieve ingrepen in de mond na bijvoorbeeld het verwijderen van een tumor. Dit gekweekte mucosale substituut (CMS) wordt vervaardigd van een klein biopt (bijvoorbeeld 1cm^2) van niet aangetast slijmvlies. In het labarotorium (*in vitro*) kan deze 1cm^2 worden uitgegroeid tot enkele honderden cm^2 in het verloop van enkele weken. Na deze periode kan men dit lichaamseigen slijmvlies terug transplanteren naar de patiënt. Om dit CMS vanuit het labarotorium lijkt me prima om een stap dichterbij te zetten de kliniek te krijgen hebben wij ons tevoren een aantal doelen gesteld (tabel 1), die uit worden gediept in de diverse hoofdstukken van dit proefschrift.

Tabel 1: Doelstellingen in projekt Cultured Mucosal Substitutes, from Lab Bench Towards Bedside?

1.	Bestudering van de kwaliteit van huidige reconstructieve technieken in de hoof-hals oncologie
	- Identificatie van verbeterpunten
	- Identificatie van milieufactoren voor het CMS.
2.	Kiezen van het beste isolatieprotocol
3.	Optimalisatie van het ontwerp van het CMS door verkiezing van dermal carrier en celtypes.
4.	Bestudering van het gedrag van CMS in klinisch relevante condities *in vitro*
	- Hypoxie
	- gamma bestraling

Mond-Keel kanker is veel voorkomend

Hoofdhals kanker komt steeds meer voor. Reconstructieve procedures na het verwijderen van tumoren worden frequent verricht. Bij grote defecten in de mond, bijvoorbeeld na verwijdering van een halve tong is er een tekort aan slijmvlies. Het verwijderde slijmvlies is dun, plooibaar, vochtig en onbehaard. Grote defecten kunnen over het algemeen niet worden gesloten met slijmvlies. In plaats hiervan wordt vaak huid van de arm of het been gebruikt waarvan de bloedvaten met behulp van microchirurgische technieken in de hals worden aangesloten op locale vaatjes (vrije lap). Eigenschappen van de ideale lap zijn afhankelijk van de plaats van het defect. In de voorste mondholte is een dunne lap ideaal, met name om de bewegelijkheid van de tong niet te beperken. Achterin de mond is een meer volumineuze lap idealer om slikken van speeksel en voedsel te ondersteunen. Over het

algemeen kan worden gesteld dan huid van de gebruikelijke lappen stugger is dan mucosa, uit verhoornend epitheel bestaat zweetklieren bevat en behaard is. Om de resultaten van huidige reconstructieve technieken te bestuderen hebben we allereerst een studie verricht bij patiënten die reconstructie hebben ondergaan met huid van de arm, de zgn Radial Forearm Flap.

Patiënten worden in 80% van de gevallen nabestraald

Hoofdstuk 2 behandelt de klinische ervaring, resultaten en kwaliteit van leven van het gebruik van de Radial Forearm Flap (FRFF) in intra-orale reconstructie. De populatie (n=72) werd onderverdeeld naar locatie van de tumor in voorste mondholte (n=39) en achterste mondholte (n=33). De studie liet zien dat, met name in de voorste mondholte, reconstructie vaak te volumineus waren en in 39% van de gevallen gepaard gingen met verminderde tongfunctie. Daarnaast werd de meerderheid van de mensen (79.9%) postoperatief bestraald hetgeen vaak in een droge mond met slikproblemen resulteerde.

De FRFF wordt geoogst van de onderarm. Deze zogenaamde donor site kan vervolgens problemen (morbiditeit) geven, donor site morbiditeit. Een bekend probleem is beschadiging van een gevoelszenuw terplaatse, de onderarm radialis superficialis wat kan leiden tot verminderd gevoel in de duimrug, koude intolerantie of neuroomvorming. Andere problemen zijn blootliggende pezen en grote littekens op de pols in 28% van de gevallen (1-4).

Een aantal van deze problemen verwacht men te kunnen verminderen door het gebruiik van gekweekt slijmvlies. Immers, gekweekt slijmvlies verminderd de hoeveelheid benodigde huid van de arm. Daarnaast wordt mucosa vervangen door mucosa volgens het principe 'replace like with like' (5,6).

CMS kan in het laboratorium klinische vragen beantwoorden

Naast ontwikkeling van CMS om in de kliniek te gebruiken is gebleken dat CMS dusdanig lijken op echte mucosa in gedrag *in vitro* dat ze steeds meer worden gebruikt om klinische vragen te beantwoorden in het laboratorium zonder dat proefdieren of mensen nodig zijn. Voorbeelden hiervan zjin de bestudering van aangeboren afwijkingen zoals epidermolysis bullosa waarbij er een aanlegstoornis is van de basaal membraan wat resulteert in ernstige blaarvorming. Ander voorbeelden zijn de bestudering van werkingsmechanismen van kankerverwekkende stoffen/omstandigheden zoals UV licht (7,8). Veel hedendaagse en veelgebruikte kennis omtrent de effecten van groeifactoren is met behulp van gekweekte weefsels opgedaan, bijvoorbeeld door gebruik van genetische modificatie van cellen in huid en mucosa susbstituten (9-11).

De opzet van een CMS lijn vergt tijd

CMS zijn ontwikkeld zoals onder andere beschreven in dit proefschrift. Voor een succesvolle en snelle productie zijn een aantal problemen herkend (tabel 2) in zowel de productie als de transplantatie fase. Over het algemeen kan gesteld worden dat een CMS buitengewoon fragiel is.

In de opzet van een productielijn zullen al deze problemen voorkomen en het team zal hier op gepaste wijze mee om moeten leren gaan.

Tabel 2: Determinanten kweek en klinische applicatie CMS

		Factoren voor succes
In vitro (in het lab)	Isolatie protocol	Robuust biopterings protocol Goede communicatie chirurg/verpleging met labarotorium Periode voor transplantatie moet lang genoeg zijn Lage infectie percentages in het lab Weinig contaminatie van fibroblasten in keratinocytenkweek Hoeveelheid cellen/cm^2 biopsie % stam cellen/cm^2 biopsy
	kosten	Kweektijd - Isolatieprotocol - Moeilijkheid kweekprotocol - Succespercentage kweek CMS - Infectiepercentage
In Vivo	Chirurgisch	Communicatie chirurg met labarotorium Logistiek (transport lab-OK) Mechanische kwaliteit CMS (kan het gehecht worden?) Makkelijke applicatie door chirurg Robuust wond nabehandelingsprotocol
	Take	Angiogenese Bestand tegen hypoxie Bestand tegen schuifkrachten Infectiegevoeligheid
	Lange termijn	Wondcontractie Bestralingsgevoeligheid Beperking orale functies zoals spreken en slikken

In de productie is de eerste stap de cel isolatie uit het biopt. Vervolgens moeten de cellen worden gekweekt om daarna te worden gebruikt voor het vervaardigen van kunstmucosa. Op elk punt van deze cascade kan iets misgaan en met name infectie met gisten, schimmels en bacteriën zijn een constante bedreiging. In het geval van infectie wordt alles weggegooid, hetgeen leidt tot tijdsverlies, geldverspilling en bovenal frustratie van het team.

Dispase is het enzym van keuze voor keratinocyten isolatie

Cel expansie is een exponentieel proces. De snelheid waarmee dit gebeurt is afhankelijk van de celcyclus van het betreffende celtype. Keratinocyten (epitheelcellen) hebben een cyclus van ongeveer 24 uur. Zoals in tabel 2 is genoemd is een voorwaarde van klinische applicatie van CMS minimalisatie van de kweektijd. Een van meest efficiente en eenvoudige wijzen om dit te realiseren is om de hoeveelheid cellen waar je mee start te optimaliseren. Hierom werden in **hoofdstuk 4** twee veel gebruikte enzymen vergeleken, Thermolysine en Dispase (12).

De resultaten lieten zien dat Dispase te prefereren is boven Thermolysine om een aantal redenen. Ten eerste was het aantal geïsoleerde cellen per oppervlakte biopt 3.26 maal groter. Dispase behandeling liet in alle gevallen een totale separatie van epidermis van de dermis zien. Thermolysine resulteerde vaak in incomplete of geen separatie van de epidermis van dermis waarmee dit enzym minder betrouwbaar was. De partieel gesepareerde epidermissen bleken bij microscopisch onderzoek de basale keratinocyten op de dermis te hebben laten staan. Analyse met immunohistochemische kleuringen (CK13) (13) wees uit dat de achterblijvende keratinocyten basale cellen waren met hoge proliferatieve capaciteiten.

Van de diverse dermale substituten die tegenwoordig commercieel verkrijgbaar heeft acellulaire dermis als kenmerk minimale wondcontractie in combinatie met goede mechanische eigenschappen. Daarnaast kunnen de bloedvaten gebruik maken van de resten van basaalmembraaneiwitten om vanuit het wondbed in te groeien(14-17). Een van de problemen waar wij mee geconfronteerd werden bij het testen van deze dermis met gekweekte keratinocyten was de variabiliteit van de kwaliteit van de CMS. Dit probleem was dusdanig ernstig dat wij hebben gezocht naar een oplossing.

Recent is er een techniek beschreven waarbij voor het kweken van huidsubstituten fibroblasten in de dermis gecentrifugeerd werden (18). De resultaten waren een drastische verbetering in kwaliteit van de huidsubstituten. Deze verbetering was dusdanig dat zelf het gebruik van bepaalde groeifactoren in het medium, zoals Keratinocyten Groei Factor (KGF) overbodig bleken te zijn(18,19). Daarnaast hebben andere groepen laten zien dat het gebruik van fibroblasten de vaatingroei (angiogenese) sterk verbetert na transplantatie van huidsubstituten. Aan de hand van deze studies hebben wij gekeken of de toevoeging van fibroblasten een gunstig effect zou hebben op de CMS die wij maken.

Toevoeging van fibroblasten aan de dermis geeft een sterk verbeterde morfologie en kwaliteit van CMS

In hoofdstuk 5 hebben wij deze hypothese getest. Humane fibroblasten werden toegevoegd aan a-cellulaire dermis. Hierop werden vervolgens keratinoycten gezaaid. Histologie en immunohistochemie om de differentiatie patronen van de epidermis te bestuderen werd verricht. Voor morfologie werd een H&E kleuring gebruikt. Voor differentiatie patronen zijn de markers $ß_1$ integrine, cytokeratine 10 and involucrine gebruikt. Vorming van de neo-basaalmembraan werd bestudeerd met kleuringen voor LamininеV en CollageenIV en VII. Cel proliferatie werd aangetoond met een Ki-67 kleuring.

Fibroblasten stimuleren proliferatie en reduceren productietijd

Onze studie heeft laten zien dat toevoeging van fibroblasten aan een a-celluaire dermis de epidermale kwaliteit verbeterd. Morfologisch werd een beter georganiseerde epidermis gezien met meer cellagen dan de dermis zonder fibroblasten. Evaluatie van differentiatiepatronen met de genoemde markers lieten meer basale cellen per oppervlakte zien, hetgeen suggereert dat er meer proliferatie mogelijk zou kunnen zijn. Hierom werd nog een proliferatie studie gedaan met Ki-67. Deze kleuring liet naast suprabasale proliferatie meer proliferatie zien, iets wat de kweektijd aanzienlijk kan bekorten.

Fibroblasten stimuleren de maturatie van de dermo-epidermale hechting

Toegevoegde fibroblasten zorgden voor een snellere vorming van de epidermis en ook van de dermo-epidermale hechting (DEJ). Deze DEJ is de lijmlaag tussen de epidermis en de dermis en heeft een belangrijke functie. Een goede DEJ voorkomt scheiding van epidermis en dermis (blaarvorming). Deze eigenschap is met name in de mond van belang waar de tong en voedsel bijvoorbeeld constant over het slijmvlies schuren.

Hypoxie is een fysiologisch verschijnsel

Naast mechanische uitdaginen wacht de CMS in de mond een periode van lage zuurstof-concentratie (hypoxie). Epidermale hypoxie gebeurt onder fysiologische omstandigheden. Na een verwonding worden de wondranden hypoxisch door verminderde doorbloeding van de dermis na intravasculaire trombosering (22). Indien de CMS worden getransplanteerd naar een wondbed is dus de verwachting dat transplantaten hypoxisch worden, wat nog verergerd wordt door het gebruik van (semi) occlusieve wondbedekkers. Over het gedrag van CMS in vitro onder hypoxische omstandigheden is nog weinig bekend.

Dit was de reden om in hoofdstuk 6 ons CMS in een hypoxische milieu te bestuderen. Naast observatie was er een hypothese dat hypoxische preconditionering het succes van transplantatie zou kunnen verbeteren doordat hypoxie secretie van angiogenetische groeifactoren stimuleert. De CMS werden tot 72 uur in 21% zuurstof gekweekt en vervolgens vergeleken met een controle groep van CMS die in kamerlucht gekweekt waren.

Hypoxie verminderd proliferatie en vertraagt differentiate in CMS

Hypoxie veranderde ontwikkeling, morfologie en functionaliteit van de epidermis. De verdeling van verschillende epidermale lagen werd verstoord en er werden minder lagen aangelegd. Dit bleek het resultaat van verminderde proliferatie zoals aangetoond met immunohistochemische kleuringen.

Differentiatiemarker K10 kwam meer tot expressie terwijl de marker voor basaal cellen, het β_1 integrine, minder tot expressie kwam. Analyse van mitose indices met behulp van proliferatie marker Ki-67 bevestigde dat proliferatie minder werd onder hypoxie.

Hypoxie geeft een voorbijgaande toename van VEGF secretie

VEGF productie in ons drie-dimensionale model werd gestimuleerd door hypoxie en was maximaal na 36 uur. Na 36 uur bleef de concentratie constant en bleek de concentratie VEGF in normale kweek condities te stijgen.

Deze studie demonstreerde dat CMS voor bepaalde vraagstellingen gebruikt kunnen worden. Of VEGF stimulatie en verminderde celdeling overleving verbeteren en angiogenese stimuleren is iets wat uiteindelijk toch *in vivo* zal worden getest.

Na volledige take van de CMS met adequate doorbloeding *in vivo* breekt een volgende fase aan waarin, zoals in hoofdstuk 2 beschreven bestraling een grote rol speelt. De meeste bestralingsprotocollen gebruiken dagelijkse doses van 2Gy tot een totaal van 66 of 70Gy. Naast beschadiging van kwaadaardig weefsel geeft bestraling als neveneffect schade aan gezond slijmvlies. Deze beschadiging wordt ook wel mucositis genoemd en komt ook veel bij mensen voor die met chemotherapeutica worden behandeld. Incidenties tot 60% zijn beschreven voor radiotherapie (29). Het beeld gaat gepaard met blaarvorming, ulceratie en is extreem pijnlijk. Vaak is het zo pijnlijk dat de therapie noodgedwongen wordt onderbroken voor klinsche pijnstilling en voeding met een voedingssonde (28) tot genezing van de ulcera. Gedurende deze pauzes kunnen naast normale cellen kwaadaardige cellen zich herstellen, hetgeen niet wenselijk is (30).

Bovenop korte termijn complicaties bestaan er lange termijn complicaties van bestraling zoals osteoradionecrosis, een soort verlittekening van de (neo)onder kaak gepaard gaand met pijn en fisteling en breuken in het bot. Ontstaan van lange termijn complicaties is gerelateerd aan het optreden van korte termijn complicaties (31,32).

Mucositis ontstaat door beschadiging van verschillende lagen in de mucosa(31-33). Met name cellen die actief aan het delen zjn zijn gevoelig voor straling en chemotherapie. Een van de meest gevoelige celtypen is de keratinocyt in de epidermis omdat deze constant aan het delen is.

Tot op vandaag is mucositis een klinische diagnose. Er bestaan geen histologische of moleculaire markers die worden gebruikt voor klinische kwantificatie van schade. Daarnaast bestaat er geen drie-dimensinaal model om bestralingsgerelateerde schade te bestuderen. Hierom hebben we ons CMS model bestraald om bestralingsschade te bestuderen.

Bestraling resulteert in forse epidermale veranderingen van het CMS

Het doel van **hoofdstuk 7**, was het bestuderen van veranderingen in CMS na bestaling. Daarnaast hadden we als hypothese dat we DNA schade en reparatiemechanismen en kleuringen konden gebruiken om schade te kwantificeren. Hiertoe werden CMS bestraald met verschillende doses. Bestraling leidde tot grote morfologische veranderingen. Celkernen werden klein en picnotisch. Er ontstonden micronuclei als teken van stralingsschade. De veranderingen leken toe te nemen met toenemende doses bestraling.

Morfologie kan worden gebruikt om stralingsschade te kwantificeren

Kwantificatie bleek mogelijk. Bij bepaling van het percentage picnotische cellen in de basale (proliferatieve) laag van de epidermis bleek dat dit percentage hoger werd naarmate het CMS aan meer straling was blootgesteld. Om deze picnotische cellen nader te karakteriseren is er een apoptose (= geprogrammeerde celdood) kleuring gedaan met een zgn TUNEL assay. De picnotische cellen bleken apoptotische cellen te zijn. Naast morfologische veranderingen hebben wij ook geprobeerd met kleuringen voor eiwitten betrokken bij de detectie en het herstel van DNA schade bestralingsschade in drie-dimensionale weefsels aan te tonen en te kwantificeren.

Met CMS kun je DNA schade en reparative kwantitaief bestuderen

Bestraling is schadelijk op een aantal manieren. Een van de manieren is dat het schadelijk is voor DNA. Bestraling geeft in DNA zogenaamde 'Double Strand' breuken (DSB). Dit betekend

dat beide strengen DNA gebroken zijn. Er zijn een aantal mechanismen beschreven hoe de cel met deze schade om kan gaan. In het kort kan hij afsterven (apoptose), of hij kan na detectie van de schade deze repareren.

In deze detectie-reparatie cascade is een aantal markers beschreven die uitgebreid in celkweek zijn getest (34-36). **Hoofdstuk 7** beschrijft het aantonen van deze eiwitten in drie-dimensionale kweek. Bovendien was met name 53BP1, een vroege detectiemarker, succesvol in kwantificatie van stralingsschade. Van de markers van DSB reparatie bleek rad51 zeer bruikbaar. Van de eiwitten werd een dosis-respons curve aangetoond. Dit is met name belangrijk en bruikbaar voor het testen van bestralingsschade beschermende protocollen en stoffen zoals vitamine C.

In vitro histologische en functionele veranderingen komen overeen met de veranderingen die in vivo worden gezien

Histologische verandering in CMS na bestraling waren vergelijkbaar met veranderingen in vivo. Ook in vivo worden met name veranderingen in de basale, proliferatieve laag van de epidermis gezien. Voorbeelden zijn picnotische kernen en het ontstaan van micronuclei, zoals deze ook in de CMS onstaan (39,41). Opnieuw is dit een blijk van het feit dat de CMS zich gedraagt als echte mucosa.

Het heden en de toekomst

Een van de conclusies van dit proefschrift is dat het CMS zijn plek heeft gevonden in experimentele, in vitro studies, en dat deze rol zich verder zal ontwikkelen. De kracht van een drie-dimensionaal model als het CMS is zijn fysiologische gedrag, zoals geïllustreerd door onze hypoxie en bestralingsstudies. Met name dit gedrag maakt het dat het zich goed leent voor beantwoording van klinisch relevante vraagstellingen en optimalisatie studies. Hoe lang kan een CMS overleven in hypoxie? Welke doses bestraling zijn optimaal voor overleving, eventueel vergeleken met CMS vervaardigd van tumorcellen. Toevoeging van andere celtypes zoals endotheel en onstekingscellen zal de klinische relevantie in de toekomst verder doen toenemen. Een ander belangrijke reden waarom dit soort modellen toenemend zullen worden gebruikt is de reductie van het aantal proefdieren. Een groot aantal vragen kan worden beantwoord met een veel lager aantal of zonder proefdieren.

From Lab Bench towards Bedside, van laboratoriumtafel op weg naar het ziekenbed; we zijn er nog niet. Er zijn mensen die CMS klinisch hebben gebruikt. Wij hebben aan een aantal vereisten voor succesvolle klinische implementatie voldaan, maar in onze ogen blijft

er echter een aantal drempels over (tabel 2). De resultaten van dit proefschrift hebben de CMS een stap dichter bij het ziekenbed gebracht. Er is een robuust kweekprotocol, er is een CMS en het CMS is in klinisch zeer relevante tests goed uitgekomen. De volgende stap zal dan ook de stap naar het proefdier en vervolgens de kliniek moeten zijn. Alleen dan kun je uitvinden hoe het CMS presteert in de moeilijke orale omgeving vol bacteriën en enzymen. Een van de transplantatie protocollen waar wij aan denken is het gebruik van een tweestappen plan met een geprelamineerde lap. Hierbij wordt het CMS eerst subcutaan op de onderarmsfascie (radial forearm fascie) geplaatst. Vervolgens kan het CMS twee weken ingroeien waarna het op de fascie gevascularisseerd, als vrije lap, naar de mondholte kan worden verplaatst.

Wij denken dat ons construct klaar is voor het testen in vivo en zullen bovenstaande techniek dan ook testen op zijn bruikbaarheid. Wij denken dat de CMS in de toekomst naast een plek in het laboratorium een toevoeging zal worden aan het gereedschap van de reconstructief chirurg.

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Curriculum vitae

Hinne Rakhorst was born in 1974 in Utrecht, the Netherlands. After moving from Apeldoorn to Groningen he graduated at the St Maartens College and started for medical school in Leuven, Belgium.

In 1993 he started medical school in Amsterdam at the VU University. During his studies he was a student assistant at the Department of Anatomy (prof.dr. H.J. Groenewegen, prof.dr. P.V.J.M. Hoogland).

In 2000 he finished medical school and started for a two-year period of MD research fellowship at the Center for Engineering in Medicine, Massachusetts General Hospital/ Shriners Burn Institute in Boston (http://cem.sbi.org). Here he performed research under supervision of J.R. Morgan Ph.D. and prof.dr. I.H.M. Borel Rinkes (General Surgery Utrecht, the Netherlands) in the field of skin substitutes and gene therapy.

After 1 year of clinical work in the department of Plastic and Reconstructive Surgery at the Erasmus MC, Rotterdam, the Netherlands he started research in cultured mucosal substitutes at the end of 2003 under supervision of Dr. S.O.P. Hofer M.D., Ph.D. and prof. Hovius M.D., Ph.D.

As of januari 2006 he started his residencies at the department of General Surgery, Reinier de Graaf Hospital, Delft, under supervision of Dr. L.P.S. Stassen M.D.,Ph.D. Currently he is doing residencies at the department of Plastic and Reconstructive Surgery at the Erasmus Medical Center in Rotterdam, the Netherlands (www.erasmusmc.nl).

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Gehonoreerde Fondsaanvragen:

- 2005: Stichting Vanderes wetenschappelijk fonds
- 2004: Stichting Nuts-Ohra wetenschappelijk fonds
- 2003: Revolving Fund Erasmus MC
  Stichting Nuts-Ohra wetenschappelijk fonds
  Stichting De Drie Lichten
- 2000: Persoonlijk onderzoeksfonds vanuit de *Nederlandse Brandwonden-stichting*

Congres organisatie:

- 2005-2007: Wondcongres Rotterdam (www.wondcongres.nl)
- 1-2 juni 2007: 12de Esser Course: Aesthetics in Facial Reconstruction; new perspectives (www.erasmusmc.nl/essercourse)
- 2006-heden: Bestuurslid en website design Kortjakje, Zondagsschool voor Plastische Chirurgie (www.kortjakje.info). Organiseert tweejaarlijks een ochtend-symposium over plastisch chirurgische onderwerpen.
- 8-11 Okt 2006; European Tissue Engineering and Regenerative Medicine Society Congres; Rotterdam (www.termis.org).