

**From the Department of Surgical Science
Clinic for Reconstructive Plastic Surgery
Karolinska Institutet
Stockholm, Sweden**

**IN VITRO AND IN VIVO STUDIES ON
BIODEGRADABLE MATRICES FOR
AUTOTRANSPLANTATION**

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Cover page: From left to right: Sections labelled using FISH to visualise all nuclei, H&E stained sections from reepithelialised wound beds grafted with MCK, Sections labelled using FISH to visualise all nuclei.

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O me! O life!.. of the questions of these recurring; of the endless trains of
the faithless—of cities filled with the foolish; what good amid these,
O me, O life? Answer. That you are here - that life exists, and identity; that
the powerful play goes on and you may contribute a verse.
That the powerful play goes on and you may contribute a verse.
(What will your verse be?)

(From *Leaves of Grass*, by Walt Whitman 1819-92)

*To Helena and
Jesper*

There must be some way out of here
Said the joker to the thief
There's too much confusion here
I can't get no relief
Businessmen they drink my wine
Ploughmen dig my earth
None of them know along the line
What any of this is worth

No reason to get excited
The thief, he kindly spoke
There are many here among us
Who think that life is but a joke
But you and I, we've been through that
And that is not our fate
So let us not talk falsely now
Because the hour is getting late

All I got is a red guitar
Three chords
And the truth

(from 'All along the watchtower', by Bob Dylan. Reprinted with the courtesy of
Columbia Records)

CARPE
DIEM...!

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LIST OF PUBLICATIONS

This thesis is based on the following articles, which will be referred to in the text by their Roman numerals:

- I** C-J. Gustafson, G. Kratz
Cultured autologous keratinocytes on a cell-free dermis in the treatment of full- thickness wounds
Burns 1999; 25: 331-335
- II** C-J. Gustafson, J. Eldh, G. Kratz
Culture of Human Urothelial Cells on a Cell-Free Dermis for Autotransplantation
Eur Urol 1998; 33:503-506
- III** M. Fossum, C-J. Gustafson, A. Nordenskjold, G. Kratz
Isolation and in vitro cultivation of human urothelial cells from bladder washings of adult patients and children.
Scand J Plast Reconstr Surg Hand Surg. 2003; 37(1):41-5.
- IV** F. Huss, E. Svensson, C-J. Gustafson, K. Gisselfält, E. Liljensten, G. Kratz
New degradable polymer scaffold for regeneration of the dermis: In vitro and in vivo studies
Submitted to Annals of Plastic Surgery
- V** C-J. Gustafson, A. Birgisson, J. Junker, F. Huss, L. Salemark, H. Johnson, G. Kratz
Employing human keratinocytes cultured on macroporous gelatine spheres to treat full thickness wounds: an *in vivo* study on athymic rats
Submitted to BURNS

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ABSTRACT

Tissue engineering (TE), one of the most rapidly growing fields of life science, is an interdisciplinary area in which technical, biological and medical expertise co-fertilize on another with the ultimate aim of restoring, maintaining or improving tissues and/or organs. This purpose links TE closely to research concerning reconstructive plastic surgery.

At present, TE utilizes two major approaches:

- I. Autologous cells are cultivated outside the body – *in vitro* – and returned as autotransplants to the patient.
- II. Autologous cells are stimulated to regenerate in the patient - *in vivo* – usually together with a suitable carrier structure or substances that regulates cell function. This approach is referred to as guided tissue regeneration

With regards to full thickness skin wounds, such as deep burn wounds, the belief today is that optimal treatment must achieve restoration of both the dermal and the epidermal regions of the skin. At present, the treatment considered to be the gold standards for treatment of full thickness wounds only restore the epidermis of the wound, with either split thickness skin grafts or cultured keratinocytes. This thesis focuses on approaches allowing restoration of both the dermis and the epidermis.

In order to achieve this, different dermal matrices have been characterised and evaluated. A suitable dermal matrix should fulfil a number of demands:

- I. It should be biodegradable
- II. Epithelial cells should be able to attach to this matrix
- III. Epithelial cells should be able to migrate and proliferate on the matrix

- IV. The dermal matrix should stimulate regeneration of an autologous neodermis (i.e. ingrowth of fibroblasts and angiogenesis)
- V. It should be possible to store the matrix for long period of time without loss of function
- VI. Production, use and storage of the matrix should be cost-effective

In Paper I and II cell-free dermis was used as a carrier for epithelial cells cultured *in vitro*. Cell-free dermis fulfils most of the demands enumerated above, and reliable techniques for the cultivation of both keratinocytes and urothelial cells for autotransplantation were developed. A disadvantage associated with the use cell-free dermis is its limited ability to be maintained as a ready-to-use product, primarily due to the complicated and, to date, costly process of production.

In Paper III the possibility to harvest urothelial cells with a non-invasive technique was investigated. The majority of patients suffering from hypospadias are children. To be able to harvest cells for transplantation through bladder-washing, without loss of proliferative capacity would be a great advantage.

In search for a more convenient product, a poly urethane urea scaffold (PUUR) was investigated in paper IV. An *in vitro* study followed by a pilot study- *in vivo*-on healthy volunteers, revealed that the PUUR scaffold possesses great potential as template for dermal regeneration. Unfortunately, this scaffold is not rapidly degraded *in vivo*.

In Paper V the suitability of a three-dimensional system consisting of biodegradable macroporous gelatine spheres as carriers for transplantation of keratinocytes into full-thickness wounds was examined in an *in vivo* study, involving nude rats. Gelatine is an autologous material and thus, biodegradable and the small size of the spheres allow them to be injected with a syringe at location where they are needed. In addition, the pores greatly increase the number of keratinocytes that can be cultured on these spheres.

In comparison to treatment with cultured keratinocytes in single-cell solution, macroporous gelatine spheres coated with keratinocytes promoted an earlier wound closure and better skin quality. Accordingly, such spheres represent a highly promising and

interesting matrix for dermal regeneration and for co-cultivation with epithelial cells.

ABBREVIATIONS

2-D	two-dimensional
3-D	three-dimensional
3T3	three times three/three days transfer, 300 000 cells/plate
CFD	acellular human dermis
Ch.	Charriere
CK	Cytokeratin
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
ECM	extracellular matrix
EDTA	ethylene diamine tetra-acetic acid
EGF	epidermal growth factor
FCS	fetal calf serum
FISH	fluorescence in situ hybridization
FITC	fluorescein isothiocyanate
GTR	guided tissue engineering
H & E	haematoxylin-eosin
HTX	hematoxylin
IHC	immunohistochemistry
J2	immortalized mouse fibroblasts
MCK	porous microcarriers
MEM	modified Eagle's medium
MGS	macroporous gelatine sphere
NCS	newborn calf serum
OrO	oil red O
PFA	paraformaldehyde
PBS	phosphate-buffered saline
PMN	polymorphonuclear cells
PUUR	poly(urethane urea)
RT	room temperature
SCK	single-cell suspension
SFM	serum-free medium
STSG	split-thickness skin grafts

TE

tissue engineering

TEMP

tissue engineering manufactured product

PREFACE

Man has long dreamt of being able to replace damaged and lost tissue through regeneration of new tissue. Examples of this ability can be found among animals and even in the human foetus. During the first two trimesters of pregnancy in humans lesions in the skin heal by regeneration of all the necessary components, rather than by scarring.

In recent years major advances in the treatment of full thickness wounds have been made. In 1981 transplantation of autologous keratinocytes was used in this context for the first time. However, it has become more and more obvious that lack of a dermal component yields suboptimal results. Thus, cultured keratinocytes alone provide a skin cover that is thin, fragile and cosmetically unsatisfactory.

Furthermore, in connection with reconstructive surgery of the genitourinary tract, the lack of a native urothelial mucosa for autotransplantation has proven to be a major obstacle. In the treatment of various malformations (e.g., hypospadias) and reconstructive surgery following, e.g., removal of a tumour, reliable procedures for auto-transplantation of native urothelium have not been available.

Contemporary tissue engineering is an interdisciplinary field involving application of the principles of engineering and life sciences to the development of biological substitutes designed to maintain, restore or improve tissue function (Atala & Nyberg, 2000).

If we manage to overcome certain obstacles such as the lack of appropriate scaffold materials and poor vascularization of the transplanted tissue and improve our understanding of various external factors that regulate tissue differentiation, proliferation and related phenomena, the potential usefulness of guided tissue regeneration in the field of reconstructive surgery is enormous. In the present thesis the development and evaluation of techniques for autotransplantation of epithelial cells onto different biodegradable matrices are described and discussed.

BACKGROUND

INTRODUCTION

Although regeneration of skin occurs in the human foetus (see above), after birth most of our wounds heal by scar formation rather than regeneration. This situation is glaringly obvious in the field of reconstructive surgery where the primary task is to reconstruct malformed and surgically or accidentally traumatized tissues and organs in such a way as to obtain optimal recovery of function and aesthetic appearance. Until recently this could only be achieved by transplanting tissues from an intact region of the body (donor site) to the affected region (recipient site). Procedures for such intraindividual tissue transfer were described in Eber's papyrus as early as 1600 B.C. and, even if the techniques have been developed further and refined since then, many of the fundamental principals remain the same.

Tissue engineering, which is one of the fastest growing areas in medicine today, involves application of the principles of biology and engineering to the development of functional substitutes for damaged tissues. The ultimate objective is to integrate the engineered tissue with the patient's own tissue. In layman terms, the essence of tissue engineering can be said to be the restoration of various tissues and even entire organs. The production, or engineering of, tissues tailor-made for this purpose, with respect to size, shape and other properties, is the common goal of all scientists working in this field.

At present, two major strategies are employed in attempts to regenerate tissues:

- I. Autologous cells cultured outside of the body, *in vitro*, and later replaced as autotransplants
- II. Tissues are stimulated to regenerate *in vivo*, usually with the help of some platform structure, an approach referred to as guided tissue regeneration

The work described here focuses on guided tissue regeneration, in which biodegradable scaffolds provide a platform or a template for the infiltration of host cells.

This scaffold functions as physical support that guides the cells to differentiate and proliferate in the matrix of the damaged area when the cells have infiltrated the scaffold and begun producing autologous extracellular matrix, the scaffold is degraded, as in the case of physiological tissue remodelling (Ma et. al., 2003).

Today, scientific efforts tend to concentrate more and more on guided tissue regeneration rather than *in vitro* techniques. This is simply because the final clinical result with engineered tissue, cultured outside the body, is inferior to that obtained with tissue engineered *in vivo*. In addition, guided tissue regeneration involves simpler laboratory procedures and shorter culture times and is generally more cost-effective.

For most research applications a two-dimensional (2-D) system for cell culturing are adequate. In recent years microcarriers representing 3-D systems, have been developed in order to increase the culture area many-fold and, thus, the cell yield in a bio-reactor or spinner flask. Moreover, to further increase the culture area porous microcarriers within which the cells can also grow have been developed. Furthermore, certain porous microcarriers are biodegradable allowing them to be utilized as vehicles for transplantation.

For more than two decades, cultivated autologous keratinocytes (Rheinwald & Greene, 1975) have been in clinical use (O'Connor et. al., 1981). Employed primarily in the treatment of extensive burn wounds, this procedure has saved many lives. However, the associated long-term post-operative outcome leaves a great deal to be desired, for instance;

1. Scar contraction, frequently necessitating in several additional surgical procedures over a period of, in many cases, several years
2. Poor skin quality, the thin epidermis blisters easily in response to mechanical stress, thereby giving rise to new wounds.

3. Poor cosmetic appearance, e.g., the presence of ‘fish-net’ patterns caused by meshing, poor surface texture and poor colour matching

All of these problems are due primarily to the lack of an underlying dermis. Every investigator presently working with burn wounds or tissue engineering will probably agree that optimal treatment of a full- thickness wound, of any kind, requires reconstruction of both the dermal and the epidermal layers. The dermis provides mechanical strength to the skin, making it much more resistant to mechanical stress and more pliable, as well as promoting a much more satisfactory cosmetic appearance.

Today, a number of dermal substitutes, both biological and synthetic are commercially available. At the beginning of our study, several years ago, the goal was to find the absolutely optimal dermal substitute. Gradually, this goal has shifted somewhat and now we believe that there are different, optimal dermal substitutes for different purposes, e.g., covering extensive burn wounds versus small burn wounds, with less loss of fluid. Thus, one particular dermal matrix will be best suited for regeneration of soft facial tissue while another will be the first choice in connection with, for example, guided tissue regeneration in the genito-urinary tract.

WOUNDS

The incidence of extensive, deep burn wounds in Sweden is low, but such injuries are nonetheless extremely costly to the health system. Furthermore, such wounds are a trying ordeal for the patients involved, who must be prepared to undergo years of corrective surgical procedures even after being discharged from the burn unit.

Another problem of great clinical and economic interest is chronic ulcers. In Sweden alone, the annual costs for the treatment of venous leg ulcers alone amount to 120.5 million dollars, or 964 million Swedish Crowns (Ragnarsson, Hjelmgren, 2005). When the costs for diabetic ulcers and other chronic wounds are also taken into

consideration, this sum must be increased by a factor of approximately 2.5 (Ragnarsson et.al. 2004). Moreover, since we are an aging population, it seems likely that these figures will increase rapidly in the near future.

Before a patient is referred to a plastic surgery clinic for treatment of a chronic wound, this wound has usually been cared for during some period of time at an outpatient clinic, and/or by another specialist. Standard treatment for such wounds usually involves surgical transplantation of a split-thickness skin graft. Postoperatively, the patient must remain in the hospital for an average of 5-10 days, which is highly costly, both in terms of direct costs and loss of income as well.

Use of tissue engineered skin in single cell solution would allow this treatment to be offered primarily at the outpatient clinic, thereby saving a significant sum of money per patient. The general practitioner (GP) can remove a punch biopsy from a suitable location on the patient under local anaesthesia and send this sample to the laboratory. Thereafter, the doctor applies a piece of cell-free dermis to the wound and the patient goes home. Approximately 10 days later the large number of keratinocytes obtained by culturing in the laboratory is sent back to the GP, together with a device for spraying these cells onto the cell-free dermis, which now has become vascularized to provide a granulated surface suitable for autotransplantation. The patient returns to the GP to allow these cells to be transplanted, and then, following a rest period of approximately 30-60 minutes returns home. This is a procedure that is not yet generally employed, but the appropriate clinical studies are presently ongoing.

The skin

The skin is the body's largest organ and composed of three distinct layers, the outer of which is called the epidermis. (The words epidermis and dermis is both derived "derma", the ancient Greek word for the skin.). The epidermis is translucent, allowing part of the incident light to pass through it, in a manner similar to frosted glass.

This outer layer does not contain any blood vessels but receives oxygen and nutrients from the deeper layers of the skin.

Below the epidermis is a very thin membrane, the basement membrane, which attaches this upper layer firmly, but not rigidly, to the layer below, referred to as the dermis. This second layer contains blood vessels, nerves, hair roots and sweat glands. Below the dermis lies a layer of fat, the subcutaneous fat. The depth of this layer varies from one individual to another. This fat contains larger blood vessels and nerves, and is composed of clusters of fat-filled cells called adipocytes. This subcutaneous fat lies in turn, on top of the muscles and bones, to which the entire structure of the skin is attached by connective tissues. This attachment is quite loose, so that the skin can move relatively freely.

The junction between the epidermis and the dermis is not straight but undulates like rolling hills - more markedly so in certain parts of the body than in others. A series of finger-like structures, called rete pegs, project up from the dermis, while similar structures project down from the epidermis. This arrangement enhances the area of contact between these outward two layers of the skin and helps prevent the epidermis from being sheared off. Rete pegs are not present in the skin of unborn babies but develop rapidly after birth, and can be clearly seen in a young person's skin upon examination under the microscope. As the skin ages these structures become smaller and flatter.

The networks of tiny blood vessels running through the rete pegs carry nutrition, vitamins and oxygen to the epidermis. In people with pale skin these vessels can be seen through the epidermis, particularly when the veins widen (so-called 'broken veins'). If the blood flowing through the dermis is saturated to a high degree with oxygen the skin will tend to have a rosy colour. In contrast, if this blood flows sluggishly and/or has lost most of its oxygen the skin will appear bluer. Furthermore, these blood vessels respond to temperature changes, dilating in hot weather, to bring a pink flush to the skin and contracting in the cold, often making the skin look blue.

The epidermis consists of five layers that are referred to, from innermost to outermost as the stratum basale, stratum spinosum, stratum granulosum, stratum lucidum, and stratum corneum. The cells of the bottom layer, the stratum basale, are shaped like columns and

divide continuously pushing cells already formed into the higher layers. As cells move into the higher layers, they flatten and eventually die, so that the top layer of the epidermis, the stratum corneum, is composed of dead, flat skin cells that are shed after approximately two weeks.

Three types of specialized cells are present in the epidermis. The melanocytes produce pigment (melanin) and the Langerhans' cells constitute the frontline of the immunological defences of the skin while the function of the Merkel cells remains unclear.

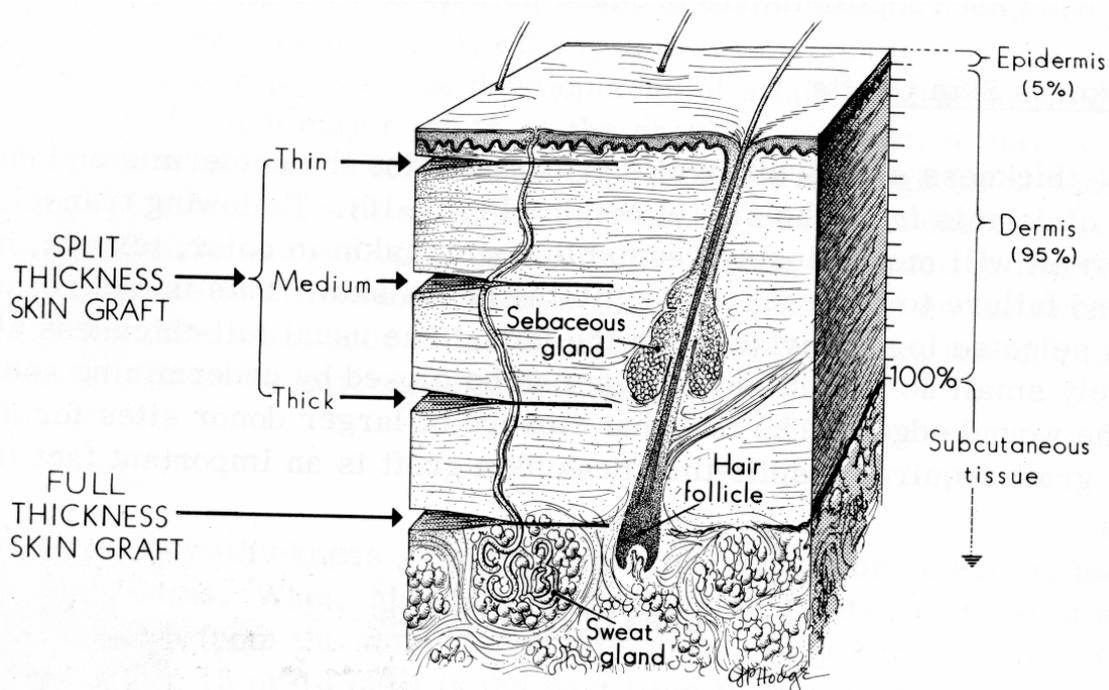


FIG. A schematic sketch of normal human skin (from Grabb & Smith's 'Plastic Surgery', 5th edition. © Lippincott Williams & Wilkins)

In addition, the epidermis participates in numerous physiological functions, such as regulating of body temperature and fluid loss, vitamin synthesis, barrier to the outer world and more.

Following damage/disruption, the dermis heals by scarring, whereas the epidermis heals by regeneration.

Fibroblasts

Fibroblasts produce the structural fibers (primarily collagen) and other components of the extracellular matrix of connective tissue.

These cells exhibit a branched cytoplasm surrounding an elliptical, speckled nucleus containing 1 or 2 nucleoli. Active fibroblasts can be recognized by their abundant rough endoplasmic reticulum whereas inactive fibroblasts, also referred to as fibrocytes are smaller; spindle-shaped and possesses less rough endoplasmic reticulum.

Since fibroblasts produce the collagens, glycosaminoglycans, reticular and elastic fibers, and glycoproteins found in the extracellular matrix, these cells divide and carry out synthesis of such compounds continuously in growing individuals. In addition, tissue damage stimulates fibrocytes and induces mitosis in fibroblasts, which can differentiate into other types of cells, including bone cells, fat cells, and smooth muscle cells. Note that all of these cells are of mesodermal origin(Wikipedia).

In our studies cultured normal human fibroblasts of the third generation (which had undergone three passages) were used (Paper IV).

HYPOSPADIAS

Among the numerous different kinds of malformations exhibited by newborn children, many of them involve the absence or the underdevelopment of an organ. In pediatric urology hypospadias is considered a common malformation. This diagnosis is reported in 0,3% of male newborn children. In this disorder the meatus of the urethra is dislocated ventrally or, in severe cases, the penile urethra is totally absent. These patients must often undergo repeated surgical procedures during their childhood and during growth. In adolescence they commonly experience psychosocial problems as well.

A major limitation in connection with reconstructive surgery of the genito-urinary is the inaccessibility of native urothelium. Urothelial cells obtained by culturing require a suitable carrier for autotransplantation.

The use of a cell-free dermis as a carrier renders the sensitive and work-intensive production of cultured urothelial sheets unnecessary. In addition, the possibility to transplant the cells in a nonconfluent state onto the dermis allows grafts to be obtained more rapidly and might also promote more extensive cell growth *in vivo*,

since the cells transplanted have not experienced contact inhibition of their growth. Moreover, cell-free dermis has been shown to stimulate both fibroblasts and endothelial cells in the surrounding tissue to grow out onto the matrix,.

Development of procedures for obtaining a large supply of urothelial cells by culturing *in vitro* and subsequent transplantation of these cells onto a cell-free dermis under conditions that stimulate the formation of a vascularized neo-dermis as the urothelium becomes confluent, would be a highly valuable contribution to the treatment of hypospadias. Another target group for such procedures are female patients who want to undergo surgery to become males (transsexual patients) since urethra is required to allow such a patient to urinate through the newly created penis. With this approach a groin flap containing a urethra produced by guided tissue engineering could be pre-fabricated and the anastomosis to the original urethral orifice established in a second step.

MATRICES

Several dermal substitutes, both biological (e.g., KaroDerm[®], Alloderm[®] and Euroskin[®]) and synthetic (e.g. Artelon[®] and Dermagraft[®]) are now commercially available. Both kinds of products have their pros and cons; for example, biological scaffolds evoke less inflammation and less pronounced rejection whereas synthetic scaffolds on the other hand, are easier to keep as an off-the-shelf product. These scaffolds, and others, have improved the treatment of deep skin wounds, but none of them possesses all the optimal characteristics required of a dermal substitute. Another disadvantage is that the price of these products is often very high, resulting in a reluctance to make wide-spread use of them. At the same time availability of synthetic scaffolds is increasing and the possibilities they offer to custom-design tissues with respect to; size, shape, degradation time, pore size and mechanical and biological properties allow new approaches to guided tissue regeneration.

The technology of engineering skin substitutes involves cultivation of human cells on matrices composed of either synthetic polymers or collagen fibers. These skin substitutes are single or multiple layered (usually two) in an attempt to act as a dermis and

epidermis and give the advantages of these layers (Cairns et. al., 1993, Demling et.al 1994). In the present thesis we have examined the ability of human cell-free dermis and two different types of synthetic scaffolds to act as carriers of autologous cells and scaffolds for guided tissue regeneration.

Cell-Free Dermis

Treatment of human dermis with Dispase and a strong detergent (Triton-X), removes all cells and cell remnants present, leaving an acellular dermis, containing components of the extracellular matrix in an intact and stable form (Takami et. al., 1996). Thus, this cell-free dermis contains of;

- Collagen skeleton, that provides stability and facilitates the ingrowth of autologous fibroblasts and endothelial cells, i.e. the formation of an autologous neo-dermis
- The basement membrane, which has proven to be a suitable site for attachment and proliferation of epithelial cells
- Other components of the extracellular matrix

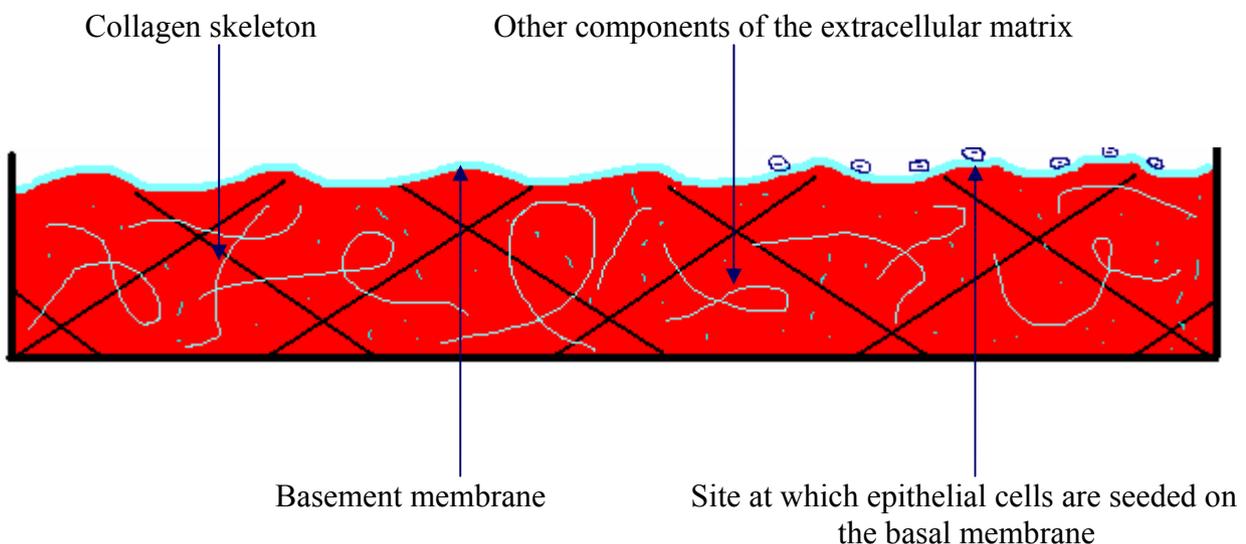


FIG. A schematic illustration of cell-free dermis

The cell-free dermis with its organized 3D collagen skeleton can be regarded as a template onto which the autologous fibroblasts migrate and deposit their ECM in an organized fashion. Cell-free dermis also

attracts endothelial cells and facilitates neo-angiogenesis, which is vital to the survival of the transplanted cells.

It is not possible to transplant dermis itself since this will create an immunogenic response directed primarily against fibroblasts and endothelial cells resulting in rejection by the host after approximately three weeks. Cell-free dermis on the other hand, is non-immunogenic since it contains no foreign major histocompatibility complex (MHC) antigens (Livesay et. al., 1995). In an animal model acellular dermis was placed subcutaneously in different geometries, including monolayers and rolled configurations. After two weeks revascularization was complete in the monolayer sheets, slower in the rolled configurations. The orientation of the basement membrane did not alter revascularization (Eppley, 2000).

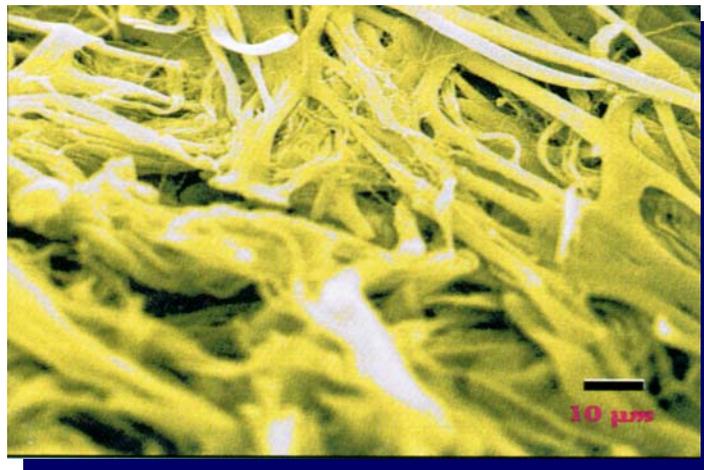


FIG. Electron micrograph of acellular dermis

Artelon[®]

Artelon[®] a poly(urethane urea) (PUUR) synthesized in a two-step polymerisation procedure (Gissselfält et al., 2002), is manufactured as fibers, films, and porous scaffolds, which have different applications. Artelon[®] is currently being used clinically to augment the anterior cruciate ligament in the knee, as well as a trapezoidal metacarpal spacer in the base joint of the thumb. Long-term clinical observations and analysis of biopsy specimens from patients have revealed that Artelon[®] is biocompatible in connection with both of those applications. In the present work scaffolds of Artelon[®] with two different macroscopic structures, i.e., porous and fibrous were tested on the basis of our findings *in vitro*, the porous scaffold was concluded to be more promising for clinical applications and was therefore employed for the *in vivo* studies.

Artelon[®] has been shown to have a degradation time *in vitro* of more than 4 (Gissselfält et al.2002) This observation raises an interesting question, i.e., should the rate of degradation of a scaffold mimic the normal turnover time for the tissue in question or is a prolonged degradation time beneficial for the clinical outcome?

Microcarriers

CultiSphere-S, a microcarrier containing large pores (70-170µm in diameter) (Percell Biolytica AB, Sweden) consists primarily of highly cross-linked gelatine (type A, porcine) with an average internal pore size of 10-20 µm following rehydration in PBS. These carriers are manufactured from an 8% solution of gelatine by a double emulsion technique, in sizes between 40 and 400µmØ.

An important feature of CultiSphere-S is that, as a porous carrier this matrix can support a larger number of cells, compared to a solid carrier. Moreover, cells are protected from damage by shearing forces and at air-liquid interfaces. A variety of human and animal cell lines, both primary and secondary have been successfully cultivated on macroporous gelatine spheres (Malda et al., Del Guerra et al., Liu et al., Ng et al., Shiragami et al.). This dermal scaffold can be described as an injectable 3D matrix. The fact that these scaffolds can be coated with, e.g., human fibroblasts and subsequently injected with

a syringe at the desired location makes them highly interesting in connection with clinical guided tissue regeneration of soft tissue.

AIMS OF THE PRESENT THESIS

The primary goals of the present investigation were:

- to evaluate various dermal matrices with respect to their suitability for use in guided regeneration of epithelial tissues.
- to establish a reproducible procedure for the successful cultivation of urothelial cells *in vitro*;
- to isolate urothelial cells suitable for such cultivation and subsequent autotransplantation in a non-traumatic manner that can be utilized with children;
- to develop a procedure for the cultivation of epithelial cells on a biodegradable dermal matrix suitable for autotransplantation *in vivo*; and

MATERIAL AND METHODS

BACKGROUND

Cell culturing

Modern cultivation of epithelial cells commenced in 1975, when Rheinwald and Greene described a reliable procedure for the cultivation of autologous keratinocytes *in vitro*. This procedure is based on culturing the keratinocytes on a layer of feeder cells with bovine serum in the culture medium. The feeder cells are mouse fibroblasts and are commonly known as 3T3-cells, which have been immortalized by irradiation or by treatment with mitomycin. Other cell lines known by the same name, e.g., the J2 cell line, do not originate from the original 3T3 cell line

Although the exact role of the feeder cells is still not known in full detail, it is known that these cells produce fibronectin and laminin that stimulate the adherence of epithelial cells to the surface of the culture flask. The presence of feeder cells also maintains the epithelial cells in a proliferative state. Furthermore, the feeder cells play another important role in suppressing the growth of fibroblasts, which would otherwise proliferate more rapidly than the keratinocytes or urothelial cells (Pellegrini et. al., 1999).



FIG.

Keratinocytes growing in a 75- cm² culture flask

Cells obtained by cultivation *in vitro* can be stored in liquid nitrogen (at -180°C) for long periods of time, without, after re-thawing, any loss of proliferative capacity or change in morphology. The maximal period for which live cells can be cryopreserved is not known and may very well vary for different types of cells.

Procedures for cell harvest techniques

Explants

The original technique utilized to initiate a cell culture involved placing a piece of tissue on the bottom of a culture vessel addition of culture medium and subsequent incubation of the sample. In time, cells migrate from the explant to the bottom of the vessel and adhere to the surface to form proliferating colonies, and, eventually, the explant will lose contact with this surface. This approach is time-consuming and furthermore, since the tissue specimen contains more than one type of cell, the culture easily becomes contaminated with unwanted cell types.

Single-cell suspension

In this approach the tissue specimen is first digested by mechanical and enzymatic treatment in order to obtain a single cell suspension (Alberts et. al., 1989), following which the cells are allowed to sediment to the surface of the culture flask where they adhere and form proliferating colonies. In the case of keratinocytes and urothelial cells, this procedure is by far the most commonly used. Fibroblasts, on the other hand, can be successfully cultured employing the explant technique.

Visualization of cells

Routine histological examination

The staining procedure most commonly used in connection with routine histological examination involves HTX/eosin, which stains the cell-nucleus blue and the surrounding cytoplasm pink. Such staining visualizes morphological features but is not adequate for examining chemical characteristics.

Immunohistochemistry

Mono- or polyclonal antibodies can be employed to stain cells selectively for a specific feature, component or structure. For example, endothelial cells can be visualized and identified by immunohistochemical staining with an antibody directed towards the von Willebrandt factor. In the case of direct immunohistochemical labelling, the antibody is conjugated to a fluorescent dye and can be observed in a fluorescence microscope. Alternatively, the antibody can be conjugated to an enzyme (e.g. peroxidase) which upon addition of the appropriate substrate, can be seen under the light microscope.

SPECIFIC METHODS

Isolation and cultivation of human cells:

Human keratinocytes (Paper I, V)

Paper I

Keratinocytes in full-thickness skin samples obtained from healthy volunteers (males 33-56 years of age) were separated from the dermis by a 12 hour treatment with Dispase (2,5 mg/ml). Subsequently these cells were placed in a spinner flask containing trypsin/EDTA and incubated at 37⁰ C for 30 minutes. Thereafter, the medium was

removed by centrifugation and the cells resuspended in culture medium (DMEM:F12 Ham's Medium, 3:1 (Gibco)) containing insulin (5 µg/ml), transferrin (5 µg/ml), triiodothyronine (2×10^{-9} M), hydrocortisone (0,4 µg/ml), cholera toxin (10^{-10} M), adenine (24 mg/ml), antibiotics (penicillin (50 µg/ml) and streptomycin (50 U/ml)) and 10% FCS (Gibco).

Paper V

In this case skin biopsies from healthy patients undergoing routine reduction mammoplasty were processed within 24 hours based on the protocol described earlier by Rheinwald and Green (1975). Briefly, tissues were washed repeatedly in sterile PBS and subcutaneous fat and as much of the dermal layer as possible were removed with sharp scissors. Next, the remaining tissue was cut into fragments approximately 1 mm^3 in size and these fragments placed in a spinner flask containing EDTA (0.02%)/ trypsin (0.1%, ICN Biomedicals Inc., USA) (1 volume of fragments/volume of medium) and incubated at 37°C for 30 minutes, the supernatant was collected, fresh EDTA/Trypsin added to remaining tissue fragments and incubation repeated in the same manner. Subsequently, this entire process was repeated a third time, after which remaining undigested tissue was discarded.

The supernatants from these three incubations were pooled and centrifuged for 5 minutes at 1500 rpm and thereafter the resulting supernatant was discarded and the cell pellet resuspended in serum-free keratinocyte medium containing bovine pituitary extract (25 µg/ml) and recombinant epidermal growth factor (EGF) (0.2 ng/ml; GIBCO™, Invitrogen Corp., Sweden). This cell suspension was then seeded into conventional 75-cm^2 culture flasks and incubated at 37°C under 5.0% CO_2 , and with a humidity of 95 %. The culture medium was changed 3 times each week. Cells were harvested for seeding into new flasks or experimental use by first washing them twice with 2 ml EDTA (0.02%) and thereafter detaching them enzymatically from the surface by incubation with 4 ml trypsin (0.1%)/EDTA (0.02%) solution (1 volume per volume cells) at 37°C for approximately 15

minutes. For the experiment, cells of the 2nd to 4th generation were used.

Urothelial cells (Paper II and III)

Paper II

Samples of urothelial tissue were obtained from nephrectomies of benign kidneys and from the urethra of male transsexual patients, five individuals in all. These biopsies (5 x 5 mm) were minced with a pair of scissors and then treated repeatedly with trypsin/EDTA in the same manner as described above. After harvesting by centrifugation at 1500 rpm for 5 minutes, the cells were resuspended in 15 ml culture medium and added to a layer of mitomycin-treated (4 g/ml, 2h) feeder cells (Swiss 3T3-cells) in a 75-cm² culture flask.

The urothelial cells were cultured in DMEM: F12 Ham's Medium (3:1)(Gibco) containing; insulin (5µg/ml), transferrin (5µg/ml), triiodothyronine (2×10^{-9} M), hydrocortisone (0,4 µg/ml), cholera toxin (10^{-10} M), antibiotics (penicillin 50 µg/ml and streptomycin 50 U/ml), adenine (24mg/ml) and 10% FCS (Gibco) at 37⁰ C and 95% humidity under 5% CO₂. After 48 hours of incubation epidermal growth factor (EGF) (10 ng/ml) was also added to this culture medium. The medium was changed every second day.

Paper III

In this case the urothelial cells were obtained from pediatric patients and adults. The children (n=9) included were undergoing surgery that required a urinary catheter (10 Ch) or cystoscopy. The urinary bladder was washed 6-8 times with 50 ml isotonic saline at body temperature, which was aspirated immediately, resulting in a total volume of 270-370 ml.

In the case of the adult patients (n=8) the same procedure, although with a 14 Ch catheter was applied to obtain 270 – 400 ml of aspirated fluid containing cells.

None of these patients had an ongoing urinary infection or a known or suspected malignancy.

The catheter had been in place for a maximum of six hours prior to rinsing the bladder. The resulting aspirate was transported immediately to the laboratory in 50 ml tubes under sterile conditions. Following centrifugation (at 1500 rpm for 10 minutes), the supernatant was discarded and the cells resuspended and washed in Dulbecco's Modified Eagle Media (DMEM), pooled and transferred to 15 ml disposable centrifuge tubes and subsequently harvested by centrifugation (1500 rpm for 5 minutes) again. The resulting cell-pellet was resuspended in 3 ml of keratinocyte culture medium, i.e., DMEM and F12 Ham's Medium (4:1, Gibco, Scotland) containing insulin (5 µg/ml), transferrin (5 µg/ml), triiodothyronine (2×10^{-6} M), hydrocortisone (0.4 µg/ml), cholera toxin (10^{-7} M), antibiotics (penicillin (50 µg/ml) and streptomycin (50 U/ml)), adenine (24 mg/ml) and 10% FCS (Gibco).

Fibroblasts (Paper IV)

Autologous fibroblasts were obtained from skin-punch biopsies and employed after three passages in culture.

Feeder cells

The J2 feeder cells used here are mouse fibroblasts immortalized by treatment with mitomycin (4 µg/ml) for 2 hours, and similar to the original 3T3 cells. These feeder cells were grown in DMEM supplemented with 10% newborn calf serum and antibiotics (as above).

Morphology

The cells in culture flasks (75-cm² or 25- cm²) were examined under the phase-contrast microscope regularly, i.e., at least three times each week. If there was any indication of contamination by micro-organisms, the culture was immediately discarded.

Routine histology

The tissues were fixed in 4% formaldehyde, washed in PBS overnight and dehydrated through a series of immersions in ethanol-xylene. These dehydrated samples were embedded in paraffin, cut into blocks and cooled. Finally these samples were cut into 6-10 µm-thick sections using a microtome, mounted onto glass slides and subjected to routine staining with HTX/eosin for light microscopy analysis.

Immunohistochemistry

Immunohistochemical analyses were performed on tissue sections following deparaffination. In the case of epithelial cells, a mouse monoclonal antibody directed against an epitope, present in a wide range of cytokeratins including keratins 5, 6, 8, 17 and 19 (Dako, Denmark) was employed (Paper I – III and V). With endothelial cells an affinity-purified polyclonal antibody directed against the human von Willebrand factor (Santa Cruz Biotechnology, CA, USA) was utilized (Paper I and V). In addition, a monoclonal rat antibody directed towards pro-collagen I (MAB 1912, Chemical International Incorporated, Temecula, CA, USA) proved useful in these studies. Fibroblasts that are actively secreting collagen synthesize pro-collagen and could thus be distinguished from fibroblasts that are simply migrating or dividing with the help of this antibody (the *in vitro* study in Paper IV). In all cases biotinylated anti-IgG antibodies (Vector Laboratories, Burlingame, CA, USA) were utilised as the secondary antibody. Endogenous peroxidase activity was blocked by a 30-minute incubation in 1% hydrogen peroxide and non-specific protein binding was blocked with 2% normal goat serum mixed with PBS.

Subsequently, the cells were incubated with primary antibodies directed towards cytokeratin (Paper I-III and V) or pro-collagen or the von Willebrand factor, at a final concentration of 10 µg/ml, for one hour at room temperature. Thereafter the cells were rinsed in PBS and subsequently incubated with the biotinylated secondary antibodies (2 µg/ml) for 30 minutes. After washing, bound antibody was visualized with the avidine-peroxidase Vectastain kit (Vector Laboratories, Burlingame, CA, USA) involving a 3-amino-9-ethylcarbazole and hydrogen peroxide as the peroxidase substrate. Controls for the immunohistochemical analyses included both omission of the primary antibody (saline controls) and replacement of these primary antibodies by non-immune immunoglobulins at identical dilutions.

Assays for cell viability

Two different procedures for the assessment of cell viability were employed:

- Trypan blue (0.4% (w/v) in water; Biovision, Mountain View, CA, USA) was added (1:1) to single-cell suspensions obtained by trypsinization and the ratio between viable (unstained nucleus) and non-viable cells (blue nucleus) determined by counting in a Bürker chamber.
- 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) is a tetrazolium salt that enters cells with intact membranes and is subsequently reduced by mitochondrial succinate dehydrogenase and NADH and/or NADPH. The reduced form of MTT forms a water-insoluble, brightly coloured (reddish-purple-blue) precipitate, which is easily seen under the light microscope. Analysis of cell growth and viability with MTT was performed on all cell cultures 1, 2, 4, 6, 8, and 10 days after initiation of culturing. For this purpose, a 400 µl aliquot was removed from the culture vessel containing the cell/microcarrier suspension, mixed on a 24-well plate with MTT (40 µl, 5 mg/ml) dissolved in Ca²⁺ and Mg²⁺-free PBS and thereafter incubated at 37° C for 45 minutes. The pattern of

staining was then evaluated using an Olympus IX51 inverted light microscope, at a magnification of 40/0.55 and photographs taken with an Olympus DP70 CCD camera (Paper V and VI).

Cell counting

Cell counting was performed using two different procedures;

- by the addition of 0.4 % (w/v) aqueous trypan blue to a single-cell suspension (1:1) after trypsinization, whereafter viable cells were counted in a Bürker chamber.
- by first detaching the cells with a solution containing 0,1% trypsin and 0.02% EDTA (1:1; both from ICN Biomedical, Aurora, OH, USA). A standardized triturating procedure involving the addition of dilution fluid (Diluid, J.T. Baker, Deventer, Holland) was applied to the single-cell suspension thus obtained prior to determination of cell number in a semi-automatic cell counter (VDA 140, Analys Instruments AB, Bromma, Sweden).

Fluorescence *in situ* hybridization (FISH)

FISH was performed with the commercially available Vysis Inc. Spectrum Green Female Total Human Genomic DNA probe and kit (Abbott Scandinavia AB, Solna, Sweden). In brief, transverse sections (4-6 μm in thickness) were prepared using a microtome (Historange Microtome, LKB, Bromma, Sweden), thawed onto a Superfrost[®] Plus slide (Menzel GmBH & Co KG, Braunschweig, Germany) and baked overnight on a hot plate at 58° C. These slides were subsequently deparaffinized in xylene and re-hydrated in ethanol (99.5%) after which they were transferred to a solution containing 0.2 M HCl for 20 minutes and then rinsed in dH₂O and neutral washing buffer (from the kit) followed by incubation with the pre-treatment solution for 30 minutes at 82 ° C.

After washing with dH₂O and neutral washing buffer, the sections were incubated in a solution of protease at pH 2.0 for 15 minutes at 37° C and again rinsed in washing buffer, followed by dehydration in a series of ethanol solutions (70%, 95%, and 99.5% EtOH). Thereafter, a mixture of 1.5 µl probe, 7 µl hybridization buffer, and 1.5 µl dH₂O was spread onto the sections and a cover-slip applied using DPX mounting agent (KEBO Lab, Spånga, Sweden). Hybridization process was then carried out for 16 hours in a Vysis HYBrite (Abbott Scandinavia AB, Solna, Sweden).

Following removal of the cover slips the sections were transferred to a washing solution containing 2 x SSc and 0.3% NP40 (Abbott Scandinavia AB, Solna, Sweden) maintained at 82° C. The sections were then allowed to air-dry and finally mounted using fluorescent mounting medium (DakoCytomation, Solna, Sweden) containing 0.75 nM DAPI (Sigma, Stockholm, Sweden).

All specimens were examined under an Olympus BX41 epifluorescence microscope equipped with proper filter settings for FITC (filter cube U-MWIB2; dichroic mirror at 505 nm; excitation filter at 460-490 nm and barrier filter at 510 nm) and photographs taken with an Olympus DP70 CCD camera.

Measurement of epithelial thickness

The epithelial thickness in sections stained with H&E was determined employing the analySIS® Pro image analysis system (Soft Imaging System, GmbH, Germany). These measurements were performed by a blinded evaluator at four standardised points equally distributed along the wound in 10 slides for each type of grafting and animal.

The mean thickness was calculated for each group and statistical evaluation was carried out using unvaried analysis of variance with Turkey HSD as post hoc test (SPSS software, SPSS inc, US), with a *p* value of < 0.05 being considered statistically significant.

MATRICES

Cell-free dermis

Skin grafts obtained from healthy volunteers (Paper I) or in connection with routine operations for breast reduction (Paper II) were treated with Dispase II (2.5 U/ml, Boehringer-Mannheim, Indianapolis, Ind., USA) for 48 h, following which the epidermis could be peeled off. The dermis was then incubated with Triton X-100 (0.5%, United States Chemical Corporation, Cleveland, Ohio, USA) in a spinner flask at room temperature for 24 h. Finally, the remaining dermal matrix was rinsed three times in phosphate-buffer saline.

PUUR (Artelon[®])

Poly(urethane urea) was synthesised in solution employing the two-step polymerisation procedure described by Gisselält et al. Scaffolds of the various PUURs were prepared either combining three layers of packed fibers or by a solvent casting/particle leaching process. The fibers were formed by extrusion of a 30% (w/w) polymer solution through a spinneret (120 holes, diameter 80 μm) submerged in a coagulating bath containing hot water (80° C). These fibers were subsequently packed in a cylindrical mould (diameter 12 mm) and a solution of N, Ndimethylformamide (DMF): water (92%: 8%, v/v) passed through the layer of fibres to cross-link them together. The fibrous scaffold thus obtained was then washed with water, dried and sliced into discs 2 mm thick.

A macroporous microcarrier of gelatin

The CultiSphere-S macroporous microcarrier (diameter 133-321 μm) (Percell Biolytica AB, Sweden) is composed of an extensively crosslinked matrix of type-A porcine gelatin following rehydration in phosphate buffered saline (PBS) the average internal pore size of this microcarrier is 20 μm . All microcarriers were

prepared for use according to the instructions supplied by the manufacturer.

Briefly, dry microcarriers were rehydrated in Ca^{2+} - and Mg^{2+} -free PBS (50ml/g dry carrier), for a minimum of one hour at room temperature (RT). While still immersed in this PBS, the microcarriers were sterilized by autoclaving (121° C for 20 minutes, with cooling at 80° C and a support pressure of 2.8 bar) and thereafter stored at 4° C until use. Sterilized microcarriers were washed twice with fresh PBS and once with the appropriate culture medium immediately prior to inoculation with a cell culture.

RESULTS

Paper I

The purpose of this study was to develop a procedure for transplantation of autologous keratinocytes onto an allogenic cell-free dermis. Two dermal biopsies from each of four healthy volunteers were treated with Dispase and Triton X-100 (see above) and the dermal sections thus obtained shown to be completely free from cells and cell remnants, and were therefore designated as *cell-free dermis*. Half of these cell-free dermis preparations were seeded with autologous keratinocytes whereas the other half were not. Each volunteer received a kerato-dermal graft on one of the wounds left by the biopsy procedure, while the other wound was covered with un-seeded cell-free dermis. The surgeon performing this procedure did not know which graft was which. Application of the cell-free dermis that had been seeded with autologous keratinocytes resulted in a complete epidermal coverage within two weeks after transplantation; whereas, in contrast with the un-seeded dermis only regions at the edges of the wound were covered with keratinocytes after this period. In this latter case, after 3, 4 and 6 weeks, the keratinocytes had migrated further in, but never completely covered the wound. The neo-epidermis covering the seeded dermis became stratified, increased in thickness and exhibited a much more mature appearance by 6 weeks after transplantation.

The in-growth of fibroblasts was examined using routine staining with HTX/eosin. In both the seeded and un-seeded transplants fibroblasts were present through-out the entire dermis, predominantly in the deeper regions after 2 weeks. With respect to the more superficial regions the fibroblast in-growth was more pronounced in the seeded dermis at this same timepoint, but this difference had disappeared four weeks after transplantation.

In-growth of new vessels, i.e., neo-angiogenesis, was examined employing immunohistochemical staining for the von Willebrand factor. In this respect there was a pronounced difference between the seeded and un-seeded dermal transplants, with a larger

number of vessels being observed in the seeded samples at all time points.

Paper II

This investigation was designed to facilitate transplantation of cultured urothelium through the development of a procedure for culturing urothelial cells on an immunologically inert and biodegradable matrix. Utilizing a modified version of the Rheinwald and Greene procedure we were able to isolate normal human urothelial cells and establish a reliable technique for their cultivation *in vitro*. These cells were cultured through 9 passages, at 8-day intervals, without any loss in proliferation potential. They reached confluence in 75-cm² flasks after 7 – 10 days of culturing and cells were shown to be of urothelial origin by immunohistochemical staining against cytokeratin.

The components of the extracellular matrix in the cell-free dermis were shown to be intact, resulting in a stable and durable layer. When urothelial cells seeded onto this layer, the urothelial cells adhered and began to proliferate. After 7 days of incubation viable urothelial cells could be detected on the basal membrane of the cell-free dermis and the number of these cells was significantly higher after 14 days of incubation, indicating active cell proliferation. Twenty-one days after seeding of these cells onto the cell-free dermis, a confluent, stratified layer of urothelial cells was observed. The cells in this stratified layer stained intensely for cytokeratin, confirming their urothelial origin, whereas no such immunostaining was seen in the control.

Paper III

Here, we examined whether rinsing the urinary bladder of children and adults could provide a sufficient number of proliferative and colony-forming urothelial cells to generate a urothelial mucosa *in*

vitro and if so, whether these cells could be stored frozen without any loss of viability. For this purpose, urothelial cells were harvested by rinsing the bladders of 17 catheterized patients, including nine children (1 to 4 years of age) and eight adults. Urothelial cell colonies had been established in all 17 samples following 3-8 days of culturing, and these primary cultures were confluent by day 13-21. After an additional 4-9 days these colonies became confluent in 25-cm² flasks as well. A second generation of confluent monolayers could be obtained 3 – 4 weeks later.

Following storage in liquid nitrogen for 1 – 12 months and subsequent thawing, these cells were all able to start growing again and to proliferate through at least 3 or 4 generations after thawing. In this case, confluence in 75-cm² flask was reached within 1 week with maintenance of normal morphology and immunohistochemical staining for cytokeratin.

In no case was there any difference in growing patterns demonstrated by cells from children and adults.

Paper IV

In this investigation the suitability of biodegradable poly (urethane urea)(PUUR) as a scaffold for dermal transplants was characterized. Normal human fibroblasts were found to attach to the surface of the porous PUUR matrix within one week in culture. With time, these cells migrated deeper into the scaffold with and, after 2 weeks, were seen halfway to the center of the matrix and after 4 weeks they populated the entire depth of the scaffold. The considerable increase in cell number and frequent cell divisions observed reflected a proliferative activity.

On the other hand, all parts of the fibrous PUUR scaffold were populated by fibroblasts after only one week. Gradually, the number of fibroblasts increased and aggregates of cells connected the fibers within 4 weeks in this case. In both matrices the fibroblasts were actively producing collagen following 6 weeks of culture, as indicated by immunohistochemical staining for pro-collagen.

The porous scaffold was considered to be more suitable for the clinical application and was therefore introduced surgically (without seeding) into the dermis of four healthy volunteers. All four exhibited normal values for laboratory parameters at both the beginning and end of the study and no adverse effects were reported. A slight redness was noticed around all incisions, including control sites. All but one subject exhibited minor inflammation, with minor secretion at the study sites, with the B scaffold (9% Artelon[®]) eliciting a slightly more pronounced inflammation. At all sites as well, the skin covering the scaffolds contracted partially exposing segments of the scaffolds.

Histological examination two weeks after implantation revealed the presence of a large number of cells in the scaffolds, predominantly neutrophils and lymphocytes. The numbers of these inflammatory cells decreased in all subjects after 8 weeks at which time thick bundles of fibroblasts occupied the entire scaffold material. Immunohistochemical examination showed that all of these fibroblasts were synthesizing pro-collagen.

Immunohistochemical staining for von Willebrandt factor revealed capillary formation in two of the subjects after 8 weeks, indicating neo-angiogenesis. The other two subjects demonstrated endothelial budding, but this process was not extensive enough to be considered capillaries.

Paper V

Next, we explored in more detail the potential usefulness of the Cultisphere-S microcarrier as a biodegradable scaffold for guided regeneration of soft tissues and epithelia in connection with the wound-healing process, employing nude rats subjected to full-thickness skin injury.

Culturing of cells on these microcarriers

Staining with MTT revealed that human keratinocytes cultured in serum-free medium adhered to the surface of these microcarriers after approximately 48 hours of incubation. Furthermore, cells exhibiting positive immunohistochemical staining for cytokeratin were clearly visible at the surface of these microcarriers at this same time-point. Analysis of cell-growth on these microcarriers showed that the rate was maximal on day 16.

Epithelial thickness

In all wounds, except the ones treated with spheres alone epithelial thickness 23 days after transplantation was greater than on day 16. However, the mean epidermal thickness in the different groups varied markedly at this time point. The MCK and STSG wounds had significantly thicker epidermis than all other groups. Most interestingly, there was no statistical difference in epithelial thickness between the MCK and STSG group, either at day 16 or 23, indicating that transplantation of cultivated keratinocytes on macroporous gelatine spheres is as effective in providing a protective epidermis as is transplantation of split thickness skin graft when measured later than 16 days post operatively.

Furthermore, growing and transplanting the cultured keratinocytes with macroporous gelatine spheres significantly increased the thickness of the newly formed epidermis as compared to cultured keratinocytes transplanted as single cells.

FISH

Using the FISH-technique, labelled cells of human origin could be visualized in sections from all transplanted full-thickness wounds at all time-points examined. Positively labelled cells were present in both the dermal and epidermal layers covering these wounds, providing strong evidence that the cells remained viable following transplantation.

DISCUSSION

Paper I

Optimal treatment of full- thickness wounds, such as deep burn wounds, would of course be to achieve reconstruction of both the epidermal and the dermal layers of the skin. Unfortunately, for the last two decades reconstruction of the epidermis has been the only option available clinically, due to the lack of a suitable dermal matrix. Transplantation of keratinocytes cultured *in vitro* has provided the only life-saving clinical treatment for extensive burn wounds. This approach was first described by Rheinwald and Greene in 1975 and was shown to be applicable with success in the clinic by O'Connor et al in 1981.

Such treatment does, however, have disadvantages including the following;

- The regenerated skin is fragile and blisters easily. Surfaces subject to mechanical stress, such as at the elbows, ankles and knees, often develop new wounds.
- There are problems associated with wound contraction, which very often necessitate further surgical procedures over a period of years.
- The cosmetic appearance obtained is far from optimal

These problems are, due primarily to the lack of a dermal layer beneath the cultured keratinocytes. The dermis provides the skin with strength to endure mechanical stress, functions as a shock absorber and also provides the suppleness necessary for an optimal cosmetic and functional outcome.

Another disadvantage connected with the transplantation of cultured keratinocytes is the prolonged period required in the laboratory (approximately 3 weeks) to obtain a sufficient number of cells, which is negative in at least two respects;

- A high percentage of the cells have already differentiated.
- The cost in term of money and labour is high.

In the study described in Paper I we have shown that a cell-free dermis seeded with autologous keratinocytes stimulates the formation of a neo-dermis at the same time as the wound is re-epithelialised by the transplanted keratinocytes *in vivo*. Accordingly, cell-free dermis is a suitable template for both dermal and epidermal regeneration, at least in part because the basal membrane remains intact. Histologically and immunohistochemically, the cell-free dermis is also repopulated with autologous fibroblasts and endothelial cells, indicating an active formation of both a neo-dermis and neo-angiogenesis. Furthermore, the cell-free dermis is immunologically inert, since no HLA-expressing cells remain. We propose that this approach employing a matrix which stimulates the regeneration of dermis, in combination with non-confluent autologous keratinocytes, will both shorten the time required obtaining appropriate cultured grafts and improving the final outcome in terms of biomechanical strength as well as appearance.

Paper II

Application of reconstructive surgery in the genito-urinary tract has, been limited primarily by the lack of ready availability of urothelium. Attempts made to circumvent this problem involve; the use of other tissue instead, e.g., placental membrane (Fishman et al., 1987), omentum (Goldstein et al., 1966), de-epithelialized bowel segments (Motley et al., 1990), local pedicled and micro vascular free flaps (Perovic et al., 1992, Morrison et al., 1996,). Full-and split-thickness skin grafts and buccal mucosa have also been used. All of these techniques are associated with complications of various sorts and, indeed, the large number of alternative techniques in itself indicates that none of them are really successful.

Our purpose with the investigation documented in Paper II was to determine whether the approach to guided tissue regeneration provided by Paper I, i.e., the use of cell-free dermis as a matrix for the cultivation of epithelial cells, could also be applied to urothelial cells. In this context, we first needed to establish a reproducible procedure for the cultivation of normal human urothelial cells. Thereafter we

examined whether these urothelial cells could be cultured on cell-free dermis as a carrier for autotransplantation. Earlier studies had shown that an acellular allodermis is repopulated with cells from the tissue surrounding the wound bed and provides a vascularized base for cultured epithelium, suggesting that the use of cell-free dermis could facilitate the transplantation of cultured urothelial cells and also stabilize the fragile sheets of neo-urothelium.

We did manage to develop a reproducible procedure for the cultivation of urothelial cells. In addition, we demonstrated that normal, human urothelial cells can be cultured and divide, on a cell-free dermis *in vitro*. These findings suggest that addition of a cell-free dermis to cultured urothelium could facilitate autotransplantation as well as promote the formation of a more stable urothelium *in vivo*. Although this proposal remains to be tested in the clinic, we can think of several possible medical applications of this approach;

- Reconstruction of the genito-urinary tract following cancer surgery
- Surgical reconstruction of malformations, such as hypospadias
- Construction of an urethra in connection with transsexual surgery

It is our hope that this approach will provide an autologous urothelium of high quality suitable for reconstructive surgery in the genito-urinary tract.

Paper III

These experiments were performed in order to find out whether rinsing human bladders *in situ* can provide a sufficient number of proliferative and colony-forming cells to regenerate urethral mucosa *in vitro*. In addition, we wanted to test whether these cells could be stored in a frozen state without losing their viability. Indeed, rinsing the bladder of both from children and adults is a reproducible procedure for harvesting significant numbers of urothelial cells and the cells obtained in this manner can be stored in liquid nitrogen at -150° C for at least 12 months without any loss of proliferative capacity.

The most common need for urethral reconstruction is, of course, experienced by patients with hypospadias, who are almost always children. The ability to harvest urothelial cells with such a non-invasive technique is highly advantageous. Furthermore, this procedure can be performed at an out patient clinic, using topical lignocaine gel without any premedication or sedation.

In comparison to harvesting cells by urethral biopsy the procedure we have developed possesses certain obvious advantages:

- The patient does not have to be subjected to general anaesthesia or sedation, since local anaesthesia is sufficient.
- Our procedure can be performed at an out-patient clinic
- Risks for complications such as stricture formation and infection are minimized
- The procedure can be repeated easily if necessary
- This procedure is equally applicable to both children and adults
- There is minimal damage to the donor site.

Another bonus is that our cultures are not contaminated with fibroblasts, although this is not a major problem in connection with the biopsy-technique. Finally, there were no side-effects, such as infections, bleeding or discomfort, reported in our study.

The fact that the urothelial cells cultured *in vitro* can be stored frozen allows the surgeon to plan the urethral reconstruction in two stages. In connection with the patient's first visit to the outpatient clinic the cells are harvested. Then the patient goes home, while the urothelial cells are expanded in numbers at the laboratory. When the desired numbers of cells have been obtained, these can be stored until the patient's scheduled return for the surgical procedure. If the culture should become infected or if the cells for any reason do not proliferate, the procedure can easily be repeated.

Paper IV

This study was designed to characterize the potential usefulness of scaffolds made of biodegradable poly (urethane urea) (PUUR) as dermal templates in connection with guided tissue

regeneration. As described above, cell-free dermis has been shown to provide a suitable dermal matrix for autotransplantation of cultured epithelial cells. An optimal dermal matrix should fulfil the following demands:

1. Biodegradability
2. Suitability for adherence and proliferation of epithelial cells
3. Immunologically inertness
4. Ease of handling
5. Cost efficiency
6. Ability to be stored as long as possible without loss of function

Cell-free dermis meets the first four of these demands. However, synthetic dermal matrices are less expensive to produce and, in particular suitable for storage for long periods of time.

For these reasons we became interested in this PUUR scaffolding. Moreover, one of the most interesting aspects of synthetic polymers is that they can be ‘tailor-made’ to the specific size, shape, pore size etc required. Furthermore, properties such as degradation time and mechanical characteristics can be altered as well.

Also worth mentioning is the fact that the use of synthetic scaffolds circumvents the special handling and regulations connected with donation of tissues, banking of skin/tissue samples and the like.

The *in vitro* portion of the study demonstrated that dermal fibroblasts can attach to, proliferate on and migrate into this porous scaffold, eventually occupying the entire structure, whereas with the fibrous scaffold there is no growth into the fibers. Cells did however proliferate on and between the fibrous strands, eventually forming thick cellular connections between them. However, since, in addition to its more clinically desirable properties, the porous scaffold is easier to handle, *in vivo* experiments focused on this structure.

In all four subjects semicircular pockets were made in the dermis in order to harvest the implants. In retrospect, we now know that these pockets were too shallow, resulting in lid contraction and protrusion of the scaffolds. This in turn led to an inflammatory reaction around the pockets. In a future study these skin pockets will be made larger and deeper to avoid these problems.

Nonetheless, dermal fibroblasts clearly grew into the scaffolds which also promote production of collagen and neo-angiogenesis.

It has been reported on the basis of *in vitro* studies (Gissselfält et al., 2002) that the Artelon[®] material may exhibit a degradation time *in vivo* of more than four years. Although the common opinion has been that a matrix employed in tissue engineering should have a degradation time similar to the turnover time of the target tissue, this is not necessarily the case. There are certain indications that biodegradable dermal templates with a longer degradation time may produce a more favourable clinical outcome, including a neo-dermis with more natural properties and less scarring.

Although these initial findings are promising and give rise to ideas concerning new approaches, clinical studies to evaluate the Artelon[®] material as a dermal template must be performed.

Paper V

In the present investigation we examined the possibility of using biodegradable macroporous spheres as a matrix for culturing these cells *in vitro*, as well as for their transportation and transplantation. Our hypothesis was that this approach would not only facilitate cell culturing, but also transportation of the cells without prior trypsination, as well as transplantation of a larger number of viable cells than is possible with single-cell suspensions.

The study was performed in an *in vivo* model in athymic rats. This model allowed us to study the effect of transplanted human keratinocytes in a large number of standardised wounds. However, due to the rapid spontaneous reepithelialisation of cutaneous wounds in these animals we did not expect to be able to see any effects on the time until the wounds were covered with at least a single layer of keratinocytes. The aim of the study was to investigate if keratinocytes transplanted on macroporous gelatine spheres had any positive effects on the stratification of the newly formed epidermis and thereby decreased the time needed until a functional, stratified, epidermis had formed.

We demonstrate here that human keratinocytes adhere well to and proliferate actively on the gelatine spheres. This observation indicates that culturing autologous keratinocytes on microcarriers could simplify their expansion *in vitro*, in agreement with what has been reported for other types of cells, and thus represents a promising approach to the treatment of a number of clinical conditions in addition to extensive burns. Not only may culturing cells on macroporous gelatine spheres facilitate this process and decrease the cost involved, but it has also been shown that cells cultured in this manner are more metabolically active than are cells grown as a monolayer in standard culture flasks.

One of the most critical steps involved in using cultured autologous keratinocytes in clinical practice is the transfer of these cells from the laboratory, where they are grown, to the patient's bedside, where they are transplanted. Although the development of procedures involving single-cell suspensions rather than sheet grafts has facilitated this transfer considerably, the requirement for trypsinization and transportation in a non-adherent state exert significant negative effects on the cells. By culturing the cells on macroporous spheres, they can be subsequently transported and transplanted without being detached from this matrix. In this fashion trypsinization is eliminated and the cells can be transported in a more natural state, growing on a biological matrix.

The key question posed in the present study was whether transplantation of cells cultured on macroporous spheres into wounds can decrease the amount of time required to establish a stratified and protective epidermis. Split-thickness skin grafts, which are expected to promote rapid formation of an adequate epidermis, were used as the positive control. We found that transportation of the cells on the same biodegradable spheres that served as their growth substrate *in vitro*, followed by transplantation in this same form resulted in accelerated stratification of the new epidermis in comparison to transplantation of single-cell suspensions.

One major question raised by our findings concerns to what extent the keratinocytes cultured on microcarriers actually contributes directly to the formation of a new epidermis. In our model system re-epithelialization occurs rapidly even in untreated wounds, so that the presence of transplanted human cells in the regenerated rat epidermis

must be demonstrated. The FISH technique allowed us to selectively visualise transplanted human keratinocytes, which were clearly present in all wounds that had been transplanted with these cells in any form. Unfortunately, it is not possible to make a quantitative comparison between the different forms of transplantation in this respect employing FISH.

Our results provide new insight to the use of Cultisphere-S microcarrier as a biodegradable scaffold for guided soft tissue and epithelial regeneration in connection with the wound healing process. Clinical trial may prove that these biodegradable microcarriers represent an interesting option to a non-toxic skin substitute in connection with reconstructive surgery, as well as an important tool for soft tissue regeneration (“filler”) in connection with both reconstructive and cosmetic surgery. Furthermore, a clinical study has been initiated, where cultured autologous keratinocytes are being transplanted to chronic leg ulcers in an out-patient setting.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

In order for tissue engineering to achieve major clinical advances our understanding of several biological processes must to be improved. These processes are interdisciplinary in nature, e.g., cell-to-cell interactions, cell differentiation and formation of extracellular matrix to mention a few.

Improved preservation and cryopreservation of cells in order to facilitate the storage of designed tissues as ready-to-use products, is another important issue. We must put serious effort into making these products as easy to use as possible. Moreover, the search for better scaffolds, which are crucial to achieving a successful clinical outcome, must continue. Most likely, both synthetic and biological matrices will have roles to play in this context. Our present initial findings concerning an injectable 3D matrix (the gelatin microcarriers) are highly promising and might, with future clinical studies, open a new door into the field of guided tissue regeneration.

One of the central problems that need to be resolved is how to provide a circulatory system for the transplanted tissue/organ. During the first few days the transplanted cells receive nourishment from the surrounding extra-cellular fluid, but thereafter a capillary network must be established in order for the cells to survive.

Clearly, guided tissue regeneration is a field of life science that shows tremendous potential for the future. Today, we are often dependent on donated organs, e.g. a heart, liver, kidney, lungs, cornea, pancreas etc., to help our patients. The demand for organs vastly exceeds the supply, a situation that is not likely to change in the foreseeable future. Even patients lucky enough to receive an organ in time, have to deal with lifelong immunotherapy and the risk of rejection and organ failure. With a tissue engineered organ these risks could probably be eliminated. Such an engineered organ would also solve a special problem encountered by pediatric patients who receive a mechanical prosthesis, i.e., that the mechanical device does not grow along with its host.

Recently the possibility of transplanting Langerhans' cells into diabetic patients has become a reality. However, to extract enough cells to treat one patient 7 or 8 donors are required. It is easy to imagine how wide spread this, at present highly exclusive treatment, could become with the help of tissue engineering. This is a situation where a patient suffering from a chronic and incurable disease associated with several severe complications, can be cured.

There are numerous other areas, in the future, where tissue engineering and guided tissue regeneration, possess a real potential to amaze us and drastically change the way we look at a disease or the loss of an organ.

Let us hope we will soon be amazed!

The future looks very promising...



(Jesper 6 years, my more immediate future...)

SAMMANFATTNING PÅ SVENSKA

Inom den medicinska forskningen har man länge drömt om att kunna ersätta förlorad vävnad och organ med patienteget (autologt) material. Det första stora steget mot att uppnå denna dröm var när Rheinwald och Greene 1975 publicerade en metod för att kunna odla hudceller (keratinocyter) i en flaska (in vitro). Denna metod visade sig vara kliniskt tillämpbar och blev relativt snart globalt spridd för behandling av hudskador som omfattade hela hudens tjocklek, tex djupa brännskador.

Nästa stora steg togs 1988, då man vid en ”workshop” i USA definierade den unga men snabbt växande forskningsgrenen Tissue Engineering som:

metoder som syftar till att bibehålla, återskapa eller förbättra vävnaders och organs funktioner

Alltså är Tissue Engineering en synnerligen multidisciplinär vetenskap där experter inom bl.a. cellodling, stamcellsbiologi, biomedicin, kemi, fysiologi, biomaterial, immunologi och molekylärbiologi samverkar för att föra vetenskapen framåt.

Sedan drygt två decennier tillbaka har man kunnat odla patientens egna (autologa) keratinocyter *in vitro* vid bl.a. djupa brännskador. Dock innebär den metoden att man endast ersätter den epidermala (överhud) delen av huden, ej dermisdelen (läderhud), vilket medför att den transplanterade huden blir oföljsam, skör och ömtålig. Hud som utsätts för mekanisk påverkan som nötning, slag e.dyl. bildar lätt blåsor, spricker och ger upphov till sår som måste behandlas. Det är dermis som bär huvudansvaret för hudens hållfasthet och följsamhet. Det finns idag både biologiska och syntetiska ersättningsmaterial (matrix) för dermis, som kan användas till att behandla sår med riktad vävnadsåterväxt (guided tissue regeneration). Biologiska matrix ger mindre inflammationsreaktioner, är mindre benägna att stötas bort av kroppen medan syntetiska alternativ kan

”skräddarsys” med avseende på storlek, form, porstorlek, mekaniska egenskaper, nedbrytningstid osv.

Vid rekonstruktiv kirurgi i urinvägarna har man länge begränsats av brist på autologt urothelium för transplantation, t.ex. vid behandling av patienter med missbildningar, t.ex. hypospadiier. Syftet med den här avhandlingen har varit att utveckla tillförlitliga laborativa tekniker för odling av epiteliala celler (keratinocyter, urotelceller) för autotransplantation. Vidare har syftet varit att utvärdera lämpligheten hos olika biologiska och syntetiska, dermala matrix i samband med guide tissue regeneration.

Avhandlingen bygger på sex delarbeten som hänvisas till med hjälp av deras romerska siffror:

I

Den optimala behandlingen av fullhudsbrännskador innebär rekonstruktion av både epidermis samt dermis. Transplantation med delhudstransplantat är 1: a handsalternativ, men begränsas av tillgången på oskadad hud vid stora brännskador. Odlade, autologa keratinocyter har i många fall visat sig vara livräddande men har svagheter i form av; ärr-kontraktion, skör hud som lätt spricker samt dåligt kosmetiskt resultat.

I den här studien etablerade vi en pålitlig teknik för att odla keratinocyter *in vitro*, samt låta dem expandera till konfluens, dvs. till läkning, på en cell-fri dermis, *in vivo*.

Vi kunde konstatera att cell-fri dermis stimulerar till inväxt av autologa fibroblaster samt endotelceller, dvs. bildandet av en autolog, vaskulariserad neodermis. Samtidigt expanderas keratinocyterna, *in vivo*, till konfluens.

Sammanfattningsvis har vi visat att autologa keratinocyter som utsätts på cell-fri dermis, stimulerar till bildandet av en neodermis, samtidigt som såret re-epitelialiseras av de transplanterade keratinocyterna.

Detta innebär en rekonstruerad vävnad med högre kvalitet, bättre kosmetiskt resultat samt, inte minst, betydligt förkortad odlingstid av keratinocyter. Dels medför det naturligtvis lägre kostnader, dels att

andelen redan differentierade celler i transplantaten minskar, med högre proliferativ förmåga som följd.

II

I nästa arbete ville vi undersöka om de nyvunna kunskaperna från arbete I, kunde tillämpas på urotelceller. Först etablerade vi en pålitlig teknik för odling av urotelceller, *in vitro*. Nästa steg var att undersöka om de kunde expanderas på cell-fri dermis, vilket visade sig fungera utmärkt. Detta innebär att vi har en modell för att rekonstruera t.ex. ett urinrör med patientegen vävnad med hjälp av guided tissue regeneration.

Den tänkta patientgruppen primärt var hypospadi-patienter, dvs. patienter som fötts med för kort urinrör. Urinröret kan hos dessa mynna någonstans utmed penisskafet eller i grava fall proximalt om penis.

Denna teknik med patientegna urotelceller på cell-fri dermis skulle kunna vara mycket användbar vid rekonstruktiv kirurgi i urogenital-apparaten.

III

Absoluta majoriteten av hypospadipatienterna är barn. I följande arbete ville vi undersöka om man med en icke-invasiv metod kunde skörda celler med bibehållen proliferationsförmåga.

I det förra arbetet erhöles celler via en vävnadsbiopsi från distala delen av urinröret. Genom att skörda celler via sköljning av urinblåsan skulle man slippa att söva barnen.

Resultaten visade att man kunde utvinna urotelceller från blåssköljvätska samt expandera dessa till sammanhängande transplantat. Själva blåssköljningen genomförs i lokalbedövning med en tappningskateter.

I studien ingick både barn och vuxna försökspersoner.

Denna metod kommer att underlätta förfarandet vid auto-transplantation inom urinvägarna.

IV

Hittills har vi använt oss enbart av cell-fri dermis som dermal matrix. Den har många kvalitéer, men är svårare att hålla som lagervara än en

syntetisk dermal mall. I det här arbetet undersökte vi ett syntetiskt 3-dimensionellt matrix. Materialet heter Artelon[®] och har använts i kliniska studier för reparation av rupturerade främre korsband i knän. I en initial *in vitro* studie utvärderades två makroskopiskt olika former av materialet. Den ena var fibrös, den andra porös. I det porösa materialet växte fibroblaster på ytan samt migrerade in i matrix, medan de endast beklädde ytan på det fibrösa materialet. I bägge fallen producerade fibroblasterna kollagen, vilket visar att de är fungerande fibroblaster.

Det porösa materialet bedömdes kliniskt mera användbart, varför det valdes till den följande pilotstudien på friska försökspersoner. Här implanterades bitar av materialet under huden och undersöktes sedan histologiskt. Efter 2 veckor sågs huvudsakligen inflammatoriska celler i det implanterade materialet. Efter 8 veckor var dessa dock ersatta av fibroblaster. Cellbilden var mycket lik normal human dermis och även nu producerade fibroblasterna kollagen. Man såg även kärlinväxt i materialet.

Sammanfattningsvis bör Artelon[®] kunna vara ett användbart alternativ vid dermal regeneration. Om den långa nedbrytningstiden är av godo eller av ondo, är det för tidigt att svara på.

V

Sökandet efter optimala, dermala substitut vid guided tissue regeneration gick vidare. Intresset riktades nu mot en makroporös microcarrier, tillverkad av gelatin.

Utvecklandet av microcarriers (kolor) innebar att den odlingsbara ytan mångfaldigt ökades och med den även expansionsgraden.

Att använda en makroporös kula ökar den odlingsbara ytan ännu mer, eftersom celler kan adherera och expandera även i kulans porer. Dessa makroporösa gelatinkulor är dessutom bio-degraderbara *in vivo*, vilket innebär det att de kan användas som transportvehiklar för odlade celler vid transplantation.

Då storleken på dessa gelatinkulor möjliggör att de kan injiceras med en spruta till önskad lokalisation, öppnar sig nya möjligheter för regeneration av mjukdelsvävnad *in vivo*.

Detta arbete visade att de porösa gelatinkulorna gav ett betydligt bättre resultat avseende sårhäkning och bildning av ett neodermis jämfört med odlade keratinocyter i "single-cell suspension".

Sammanfattningsvis visar dessa makroporösa gelatin-carriers potential som ett icke-toxiskt alternativ till hudsubstitut inom rekonstruktiv kirurgi. De kan även visa sig användbara vid rekonstruktion av mjukvävnad både vid rekonstruktiv samt vid kosmetisk kirurgi.

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