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Short communication

Cryopreserved tissue engineered mucosa

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Abstract: Single cells suspensions used for grafting in the clinical setting may be reliably cryopreserved by established protocols. However, for tissue engineered constructs which now also get used as grafts in the clinic such established protocols and assays which indicate graft viability and their function as graft do not exist. a) The purpose was to develop a cryoprotocol and an animal model to test the efficacy of tissue engineered to act as graft after cryopreservation. b) Therefore, tissue engineered mucosa grafts consisting of keratinocytes and fibroblasts grown in a collagen sponge were cryopreserved and grafted in the nude rat to test its efficacy to function as mucosa graft. At different points after cryopreservation the mucosa was grafted into the nude rats. Healing of grafts was allowed for one or three weeks. c) Sufficient cells survived the cryopreservation allowing for the development of epithelial-fibroblast tissue in the collagen sponge. After three weeks of healing the formation of mucosa tissue was more complete and more collagen sponge had disappeared. d) The nude rat model is suitable to assess the efficacy of tissue engineered mucosa to function as graft after cryopreservation. The formation of human epithelial-fibroblast tissue *in vivo* has to be interpreted as proof of principle that the approach of cryopreservation of tissue engineered grafts is working.

Key words: tissue engineering, mucosa, cryopreservation

Introduction

Clinical applications of engineered tissue become more and more popular. For example tissue engineered (TE) mucosa is used to improve the soft tissue around dental implants. This mucosa which consists of gingival keratinocytes, gingival fibroblasts and a carrier material is used as a transplant after open vestibuloplasty [9]. After healing of the transplant, there is fixed immobile mucosa on the alveolar crest where the dental implants pierce the mucosa.

In order to tissue engineer such grafts enough tissue must be cultured from a biopsy also from elder patients using autogenous serum during the culture period *e.g.* over three weeks [3,4]. Therefore, cells must multiply and not dedifferentiate. As specific markers for differentiation keratinocytes express a certain cytokeratin pattern.

The tissue engineered transplants are prepared for each patient individually and can not be stored because cell proliferation and cell viability are limited. After a culture period of approximately 60 days proliferation of the cells ceases [3]. However, what happens when the patient for whom a graft is just in preparation becomes sick and the operation has to be postponed. Knowing that there is only a certain time frame for the cells in the transplant being proliferative, viable and differentiated, the transplant must be preserved. The only way to achieve this goal is cryopreservation.

Cryopreservation of single cell suspensions are well established, but protocols and procedures to cryopreserve complete tissues are not available. Hence, the aim of this investigation was as to see whether a cryopreserved tissue engineered human mucosa transplant is viable and leads after healing to a specific tissue formation at the recipient site when grafted in an animal model.

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Materials and methods

For the culture of gingival keratinocytes and fibroblasts and for the tissue engineering of mucosa transplants, human gingival biopsies (obtained from patients undergoing wisdom tooth removal – Approval of the Ethic Committee Dresden University of Nr. 15022002) were micro dissected into connective tissue and epithelium. Using the enzyme collagenase, the fibroblasts were dissolved from the connective tissue and seeded as single cells. The fibroblasts adhered and formed a monolayer. The epithelium was cut into explants which were placed on the floor of the culture dishes. From the explants gingival keratinocytes grew out. Fibroblasts were cultured in DMEM, keratinocytes in DMEM and Ham F12 (1:1) both supplemented with 10% FCS. After primary culture for approximately three weeks fibroblasts and gingival keratinocytes were seeded in a collagen sponge to tissue engineer the human mucosa transplants. The mucosa transplants were cultured for another week in DMEM and Ham F 12 before cryopreservation was applied.

Prior to cryopreservation the culture medium of the transplant was successively changed to a self made cryoprotective medium (based on DMEM +5% HES and 2.5% DMSO) and incubated for 10 minutes at each medium removal. Next the transplants were cooled down stepwise to -80°C: First cooling down to 0°C with 5 K per minute and waiting for thermodynamic equilibrium up to 20 minutes. Next cooling with the same gradient to -5°C and waiting for thermodynamic equilibrium up to 5 minutes. During this time additional cold is applied for rapid freezing with defined ice crystal structure. Afterwards further cooling with 1 K per minute to -80°C. After rapid thawing the transplants were twice washed by successively adding normal cell culture medium. Then respectively four transplants were either used directly or cultured for another 24, 36 to 48 hrs. Overall 24 transplants were evaluated and four animals were operated on. Cell counting in a transplant is not possible, therefore the cell survival is checked by fluorescence methods. The transplants were stained with a non-toxic and cell-friendly fluorescence dye (DIO) marking the lipid membrane of the cells without causing cell death.

The animal model of the nude rat used here shows no immunologic response against allogenous or xenogenous transplants. The TE mucosa transplant consisting of human cells will therefore not be rejected and heals well. Because host and donor tissue originate from different species the transplanted cells can be identified using human tissue specific antibodies. The TE mucosa was transplanted subcutaneously in a dissected pouch on the back of the rats (Fig. 1). Silicon foil is used to fix the tissue engineered mucosa grafts and to simplify transplantation and localization of the transplanted tissue after sacrifice of the animals. After wound closure there were healing periods of either one week or three weeks.

Histology and immunohistology were used to assess the success of the transplantation and the viability and differentiation of the cells. In particular the morphology of the formed tissue was looked at. To identify the human cells to methods were used.

Prior to grafting the nuclei of cells (more correct the DNA) was labelled with BrdU. After healing and immunhistologic preparation Anti BrdU (Becton Dickinson) was applied to identify the labelled cells. Further, human keratinocytes were identified with antibodies to different cytokeratins in the rat tissue.

Results and discussion

Immediately 36 hrs after cryopreservation the TE mucosa transplants maintain some tissue formation with fibroblasts and keratinocytes in the collagen sponge (Fig. 2). According to the preparation technique of the TE transplant the keratinocytes are on

Fig. 1. The tissue engineered mucosa (fortified with a silicone sheet) is transplanted subcutaneously in a dissected pouch on the back of the rat.

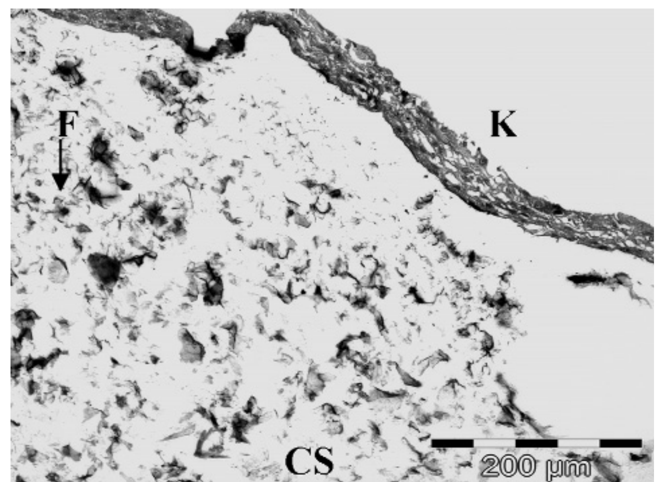

Fig. 2. Tissue engineered mucosa transplants maintain some tissue formation with fibroblasts (F) and keratinocytes (K) in the collagen sponge (CS) immediately after cryopreservation

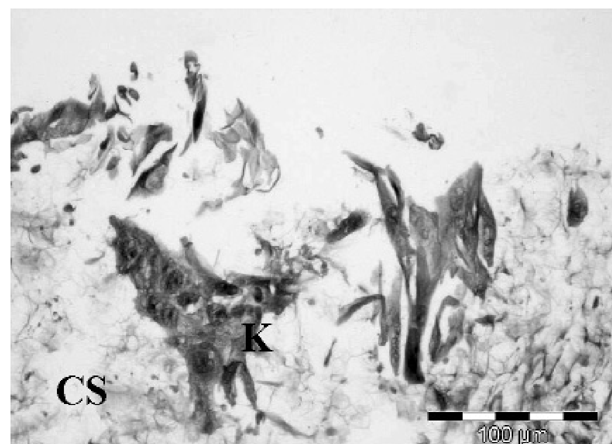

Fig. 3. One week after transplantation and healing no proper tissue formation is seen, however clusters of keratinocytes (K).

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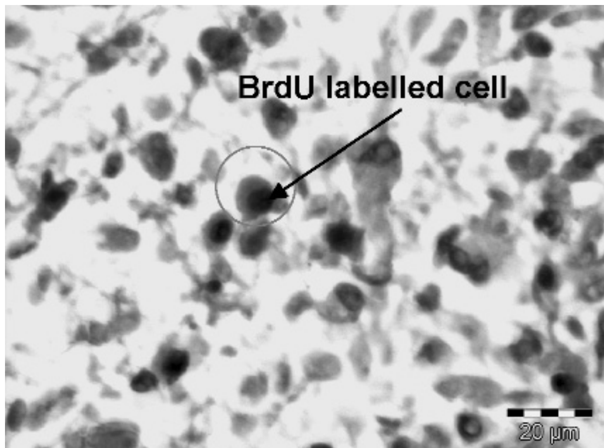

Fig. 4. One week after transplantation cells within the collagen sponge BrdU labelled prior to cryopreservation – evidence that cells survive the procedure are taken at the recipient site.

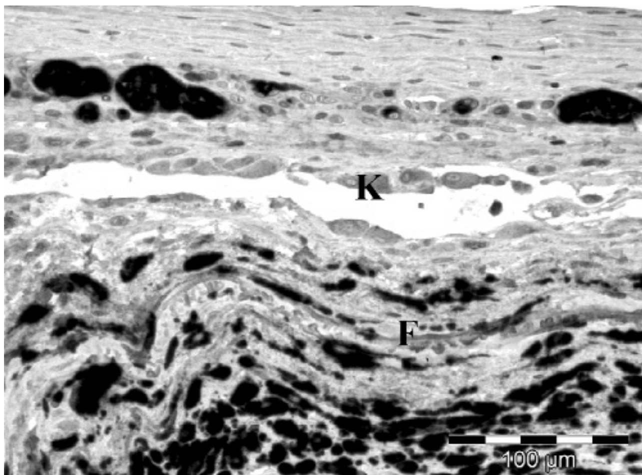

Fig. 5. Three weeks after transplantation a proper epithelial fibroblast tissue is formed (K – keratinocytes; F – fibroblasts, collagen sponge CS).

top and the fibroblasts mainly within the collagen sponge.

One week after transplantation and healing no proper tissue formation is seen any more, only some clusters of keratinocytes (Fig. 3). The collagen sponges are not collapsed and contain also fibroblasts. There are BrdU labelled cells within the collagen sponge (Fig. 4) providing evidence that cells labelled prior to cryopreservation survive the procedure and are integrated at the recipient site.

Three weeks after transplantation a proper epithelial fibroblast tissue had formed (Fig. 5). The collagen sponge is condensed, keratinocytes started to form cell layers. In the sections investigated no BrdU labelled cells could be detected. The epithelial fibroblasts tissue that has formed after a healing period of three weeks is however not in such a mature state as seen when TE grafts are used for covering epithelial defects in human after a similar period of time [5].

There are several reports about cryopreservation of keratinocytes cultured as cell layers or on starch or collagen beats [1,8]. Other cryopreserved tissue engineered constructs are pancreatic and a dermal substitutes [2,6]. However, for all these cells viability and function which ranges around approx. 60 % were assessed only in vitro after direct thawing. The transplantation of cryopreserved tissue is only described for heart valves. They may be seeded with endothelial cells or be without [10,11]. However, between the two groups there were no differences in respect to change in formation of the valve collagen matrix after a healing period of up to 42 days. To our knowledge this is the first description that for cryopreserved tissue engineered mucosa transplants – consisting of gingival keratinocytes and fibroblasts in a collagen sponge – the *in vivo* proof of function by forming a human epithelial-fibroblast tissue after grafting was reported.

Conclusion

In conclusion the results presented underline that the nude rat model is suitable for the assessment of cryopreserved tissue to function as transplant. This means that there is a proof of principle that a cryopreserved tissue engineered mucosa transplant (with vital cells after cryopreservation) can function to induce specific tissue formation at the host site. This model will be available to assess the effects of optimizing cryoprotocols and cryofacilities in respect of their in vivo outcome.

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