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Establishment and initial characterization of a simple 3D wound healing model

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Abstract:
Poor wound healing as consequence of malfunctions in the regulation of the healthy tissue repair response affects millions of people worldwide. The number of therapies available to successfully treat chronic wound is still very limited and their development is costly and time consuming. Therefore simple to use 3D systems, reflecting the *in vivo* tissue complexity, are urgently needed. We introduce a novel 3D organotypic model (OTC) containing the major cell components active during wound healing i.e. keratinocytes, fibroblasts and inflammatory cells that allows to determine the effects of different therapeutic approaches on wound closure, cell differentiation and cytokine secretion in chronic wounds. There are first reports on irradiation with visible light of different wave length (Low Level Light Therapy) as a means to enhance wound closure. However the mechanisms underlying this therapy as well as optimized irradiation wavelength and dose are not clear and were therefore analyzed using our 3D organotypic model.
In the standardized OTC model we could demonstrate epithelial closure under control conditions as well as differential effects of red and blue light irradiation with respect to stability of the newly formed epithelium and time until epithelial closure. First results show differential cytokine profiles upon different wavelength irradiation e.g. high expression of TGF beta and IL-1 beta in red light irradiated cultures and increased GM-CSF expression in blue light irradiated and control cultures.

Keywords: in vitro, wound healing assay, wounding technique

1 Introduction

Poor wound healing after surgery or trauma leading to chronic wounds affects millions of people worldwide. It results from malfunctions in the regulation of the healthy tissue repair response, including inflammation, angiogenesis, matrix deposition, and cell recruitment [1]. While the complex biological repair process in healing wounds is well understood and is known to encompass an extensively regulated interaction between inflammatory cells, fibroblasts and vascular cells in the dermal and keratinocytes in the epithelial compartment [2], the number of therapies available to successfully treat chronic wound is still very limited. There are first successful approaches employing irradiation with visible light of different wave length to enhance the closure of chronic wounds during „Low Level Light Therapy“(LLLT), yet the functional effects of this therapy are not known so far. To better understand the molecular mechanisms of wound healing therapies and to develop novel therapeutic strategies a number of different assay systems have been developed. E. g. 2D monolayers and wounding techniques have been used to study cell migration [3, 4]. However, they do not represent a coordinated multi-cellular system and are thus insufficient for a successful testing of new therapies in an *in vivo* like setting.
More advanced models, include explant models which suffer from high variabilities between donors and explant procedures [4] and are thus of limited use for standardized experiments. Consequently defined 3D organotypic systems are urgently needed. A 3D organotypic *in vitro* skin model based on a collagen type 1 gel with normal human dermal fibroblasts on which primary keratinocytes or immortalized HaCaT-keratinocytes grow air exposed was previously published [5]. This model was extended to analyze tumor stroma interaction of epithelial tumor cells with fibroblasts and inflammatory cells (i.e. neutrophils and macrophages) in the gel. Using the system the crucial influence of cytokine driven interaction between macrophages and fibroblasts on invasion and M2 differentiation of macrophages and [6] the invasion promoting effect of neutrophils was demonstrated (Hensler S and Mueller MM. unpublished data).
The 3D model was modified growing primary human keratinocytes and /or immortal HaCaT keratinocytes air exposed for the epithelial compartment and a standardized wounding protocol to analyze the complex mechanisms during wound healing. Using the model system the effect of different wave length „Low Level Light Therapy“(LLLT) on wound closure, cell differentiation and cytokine interaction between the cells involved was determined allowing the identification of an optimal regimen of light intensity and wave length for the treatment of chronic wounds.

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2 Materials and Methods

2.1 Cell lines

Human immortalized HaCaT keratinocytes [7] and human dermal fibroblasts were maintained in Dulbecco's modified Eagle's medium, 10% fetal calf serum (FCS) and 1% penicillin/streptomycin (10.000 U/10.000 µg/ml) (DMEM 10) at 37°C, 5% CO_2. Cells were passaged at a split ratio of 1:3. U937 cells and the HL60 cells were maintained in RPMI 1640 Medium with 10% FCS and 1% penicillin/streptomycin (10.000 U/10000 µg / ml) (RPMI 10) and subcultured twice a week.

2.2. Differentiation of U937 macrophages and HL 60 neutrophils

Differentiation of U937 cells to macrophages was induced by a 48 h incubation with 50nM phorbol 12-myristate-13-acetate (PMA) in RPMI 10 as described. Differentiation of HL-60 neutrophils was achieved by incubating 3×10^6 cells per 25 cm dish for four days in 30 ml RPMI 10 with 1.25% DMSO. Medium was changed after 2 days. Success of differentiation was determined based on cell morphology of the adherently growing cells. Nonadherent cells were removed by washing with PBS and only differentiated cells were used for establishing the OTC.

2.3. Preparation of Organotypic co-culture (OTCs) and wounding

OTCs were established as described [8, 9]. 1.25 ml dermal equivalent from type I rat collagen (3.59 mg/ml), containing 0.4 x 10^5 human fibroblast, U937 macrophages and HL60 neutrophils each were added to 12 well filter inserts (Falcon, Becton Dickinsion, Heidelberg). After 24h primary keratinocytes (data not shown) or HaCaT cells (0.75 x 10^6) were seeded on the dermal equivalent and lifted to be cultivated air-exposed 24h later. Medium (DMEM 10 with 50 mg/ml L-ascorbic acid (Sigma- Aldrich, Steinheim) was exchanged three times a week.

After 14 days, OTCs were wounded by 2-mm circular biopsy punch. The hole in the dermal equivalent was closed using a 15 µl plug of 50% collagen (3.59 mg/ml) and 50% fibrin (2 µg/ml), to better adapt the model to the in vivo wound situation. Wounded OTCs were cultivated for 17 days and irradiated daily for 2 hours and 50 minutes with maximum intensity of blue (460 nm) or red (640 nm) light. Samples and conditioned were taken at day 7, 11 and 17. Conditioned media was frozen at -80°C, tissue samples were processed for histology and cryostat sectioning.

2.4. Histology

Cryosections (10 µm) were mounted on glass slides and air dried. Histology was assessed by standard haematoxylin and eosin (H&E) staining and Elastica van Gieson staining with Resorcin Fuchsin (Roth, Karlsruhe) for 60 min at 60°C, followed by ddH_20 wash and differentiation in 96% ethanol. Nuclei were stained with Kernechtrot (Roth, Karlsruhe) for 30 min at RT and washed in ddH_20. Staining was concluded by 5 sec in van Gieson (Roth, Karlsruhe) solution and ddH_20 wash followed by dehydration and mounting.

2.5. Immunofluorescence

Cryosections (8 µm) were fixed in 80% methanol for 5 min at 4°C and 100% acetone for 4 min at -20°C and re-hydrated in PBS. Slides were blocked with 12 % bovine serum albumin for 30 min. Primary antibodies were incubated over night at 4°C, washed in PBS, incubated with the fluorescent secondary antibody for 1h at RT, washed and mounted. Sections were photographed using a Leica microscope with epifluorescence. Primary antibodies: mouse pankeratin (guinea pig-polyclonal, Progen, Heidelberg), collagen IV (rabbit-polyclonal, Progen, Heidelberg); secondary antibodies: donkey anti-guinea pig Cy2 and donkey anti-rabbit Cy3 (Dianova, Hamburg). Nuclei were stained with 10 µg/ml Hoechst 33258/bisbenzimide.

2.6. ELISA

Secretion of IL-1ß; GM-CSF and TGF-ß into the conditioned media was measured by ELISA using Quantikine Immunoassay kits (R&D Systems, Darmstadt). Samples were tested in duplicate. Data shown are mean values ± SD.

3 Results

Human immortalized HaCaT keratinocyte [7] or immortal HaCaT keratinocytes were cultured as a multi-layered epithelium on a dermal equivalent of collagen I with primary dermal fibroblasts, U937 macrophages and HL60 neutrophils. Standardized and reproducible wound defects were generated by punch biopsy. The model allows an optimal analysis of the interactions between dermal and epidermal cells and of potential therapeutic effects, e.g. of low-level light therapy with different wavelengths in an in vivo-like tissue context. After wounding OTCs were cultured for 17 days and subjected to different treatment protocols to enhance wound closure. Histological analysis of probes taken at day 5 and 10 revealed an increase in epithelial thickness in the wounded cultures irradiated with blue light after 5 days (figure 1C) compared to non irradiated OTCs at the same time point (figure 1A). Epithelial thickness in these cultures normalizes to the level of non irradiated controls at day 10 (figure 1G and data not shown). In non-wounded cultures, this effect of blue light does not occur (figure 1 E, H).

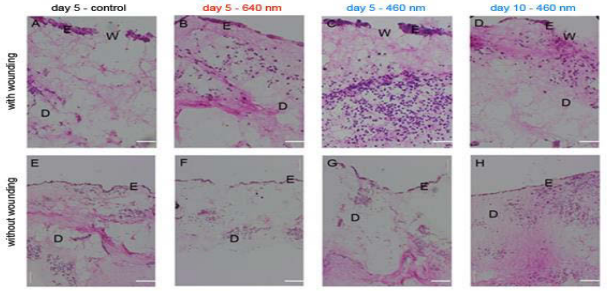

Figure 1: Histological analysis of OTCs after wounding: H&E staining of OTC at day 5 and 10 after wounding show a multilayered epithelium. W wound area E epithelium D dermal equivalent, bar 0,2 mm.

Initial experiments where the punch biopsy resulted in a deep hole in the dermal equivalent made complete wound closure difficult. Therefore, the hole in the dermis was closed by a collagen/fibrin plug that resembles closely the fibrin clot in wounded tissue in vivo. As result complete wound/epithelial closure could be observed in control as well as blue irradiated samples. In samples irradiated with red light epithelial formation was initially observed, however the intact epithelial layer disappeared again at later times (Fig. 2).

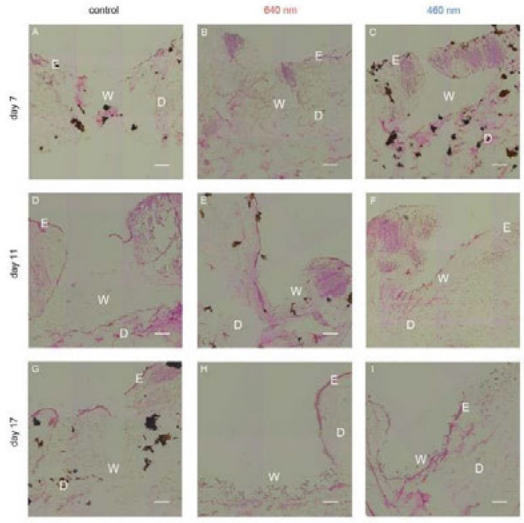

Figure 2: Histological analysis of OTCs after wounding: H&E staining of OTC at day 7, 10 and 17 after wounding show a multilayered epithelium. W wound area E epithelium D dermal equivalent, bar 0,2 mm

To determine the cell types involved in the differential response of the wounded tissues to treatment with light of different wavelengths cryosections of the 3D tissues were characterized by immunofluorescent staining against cell type specific marker proteins. Figure 3 - 5 displays staining for CD15 (Neutrophils) and CD68 (macrophages) (red) and epithelial keratin (green). Cell nuclei are stained with Hoechst/bisbenzimide (blue). In addition to the increased epithelial thickness in blue light irradiated cultures on day 7, a higher number of CD15 positive neutrophils was observed in the dermal equivalent in non irradiated controls and after blue irradiation as compared to red light irradiated cultures (figure 3).

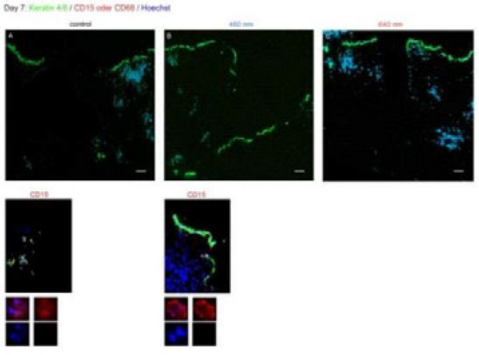

Figure 3: Immunofluorescent staining of cryosections of wounded OTC day 7 with (B and C) and without irradiation (A)., for CD15 (red, Neutrophiles) keratin (green, epithelial cells) and Hoechst/ bisbenzimide (blue, nuclei). bar 50 µm.

On day 7 after blue light irradiation CD15 positive cells were recruited into the epithelium.
An increase in CD15 positive neutrophils in the dermal equivalent was observed in red and blue light irradiated cultures after 11 days, control cultures exhibited CD15 positive cells in the epithelial layer (figure 4).

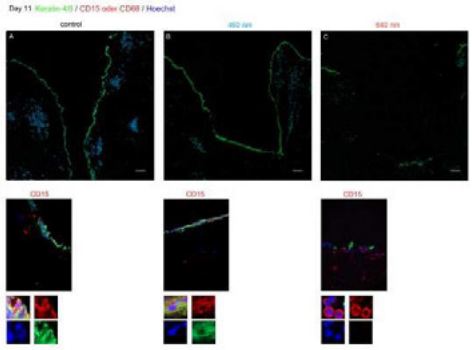

Figure 4: Immunofluorescent staining of cryosections of wounded OTC day 11 with (B and C) and without irradiation (A)., for CD15 (red, Neutrophiles) keratin (green, epithelial cells) and Hoechst/ bisbenzimide (blue, nuclei). bar 50 µm.

At day 17, CD15 positive cells appear in the epithelial layer of controls, red light and blue light irradiated cultures. In addition numerous CD68 positive cells were observed in the dermal equivalent in controls and red light irradiated samples (figure 5).

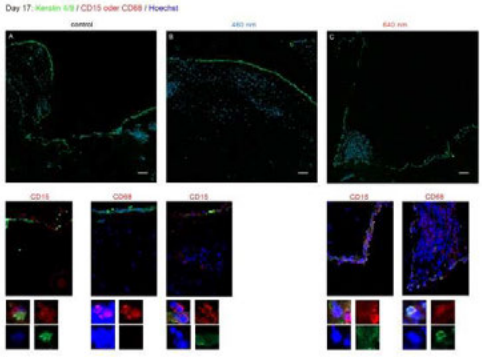

Figure 5: Immunofluorescent staining of cryosections of wounded OTC day 17 with (B and C) and without irradiation (A)., for CD15 (red, Neutrophiles) keratin (green, epithelial cells) and Hoechst/ bisbenzimide (blue, nuclei). bar 50 µm.

Irradiation of the wounded 3D OTC cultures with red or blue light clearly influenced epithelial closure and inflammatory cell recruitment suggesting potential alterations in the cytokine expression profiles of the cultures. Therefore, we analyzed the expression of TGF-ß-, IL-1ß- and GM-CSF- by ELISA of conditioned media (figure 7).

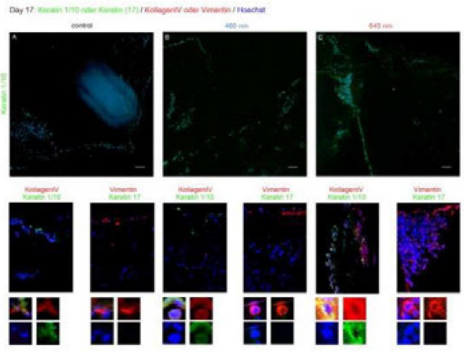

Figure 6: Immunofluorescent staining of cryosections of wunded OTC day 17 with (B and C) and without irradiation (A)., for Vimentin and Collagen IV (red, Neutrophiles) keratin (green, epithelial cells) and Hoechst/ bisbenizmide (blue, nuclei). bar 50 µm.

First results indicate an upregulation of TGF beta and IL1 beta in red light irradiated cultures and an increase of GM-CSF expressions in controls and samples irradiated with blue light.

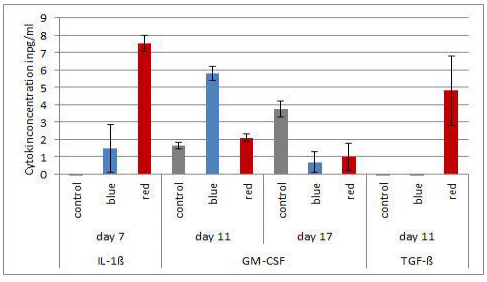

Figure 7: Quantification of IL-1β, GM-CSF and TGF-ß **(C)** in conditioned media of OTCs between day 7and day 21 after wounding. Cytokins are measured in duplicate samples by ELISA.

4 Discussion

A cost efficient, reproducible and simple to use in vivo like 3D organotypic tissue model (OTC) for wound healing was established and validated by determining the mechanisms underlying the therapeutic efficacy „Low Level Light Therapy“(LLLT) used in the management of chronic wounds. The model consists of a collagen type 1 gel as dermal equivalent containing the major cellular components active during wound healing i.e. normal human dermal fibroblasts, human primary or immortalized (HaCaT) keratinocytes as well as macrophages and neutrophils differentiated form human U937 cells and HL60 cells respectively. Primary keratinocytes or immortalized HaCaT cells are grown air exposed on the dermal equivalent. The model can be wounded by punch biopsy and was subsequently maintained for 3 weeks to analyze the wound healing process. As such it provides a prolonged period of observation that is frequently not achieved by other organotypic skin models using commercially available dermal equivalents or explant cultures [13 and ref there in]. A similar observation period has so far only been achieved by Boehnke [14] et al using a 3D scaffold that is activated by the secretion of authentic fibroblast derived matrix and allows the establishment of tissue homeostasis in skin *in vitro*. However, this model is very complex and costly and as such not optimally suited for the analysis of therapeutic efficacy *in vitro* that requires a high number of samples to provide a basis for statistical evaluation. In addition the model published by Boehnke et al as well as other known models [13 and ref therein] did not include inflammatory cells thus lacking one of the major cellular component involved in wound healing [1,2] . The use of inflammatory cells together with normal human dermal fibroblasts instead of foreskin fibroblasts that would represent a different tissue context allows the observation of in vivo like cellular interactions. Further studies will help to evaluate whether the neutrophils that are unique for this 3D OTC model and are known to secrete inflammatory mediators such as TNFα and IL-1 may be responsible for the biphasic response to LLLT of different wave length that was observed in this study.

5 Conclusion

The work presented here introduces a cost efficient simple to use and yet sufficiently complex 3D model for studying therapies to enhance wound healing. First characterization of the model with respect to molecular mediators of wound healing suggest an in vivo like kinetics of secretion for these molecules providing an excellent basis to better understand the molecular effects of therapeutic regimen like „Low Level Light Therapy“ (LLLT) and others.

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Author's Statement

Authors state no conflict of interest. Informed consent has been obtained from all individuals included in this study.

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