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Effects of hydrogen peroxide in a keratinocyte-fibroblast co-culture model of wound healing

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ABSTRACT

Recently, there has been renewed interest in the role of reactive oxygen species (ROS), especially H_2O_2, in wound healing. We previously showed that H_2O_2 stimulates healing in a keratinocyte scratch wound model. In this paper, we used a more complex and physiologically relevant model that involves co-culturing primary keratinocytes and fibroblasts. We found that the two main cell types within the skin have different sensitivities to H_2O_2 and to the widely used "antioxidant" *N*-acetyl-L-cysteine (NAC).

Keratinocytes were very resistant to the toxicity of H_2O_2 (250 and 500 μM) or NAC (5 mM). However, the viability of fibroblasts was decreased by both compounds. Using the co-culture model, we also found that H_2O_2 increases re-epithelialization while NAC retards it. Our data further illustrate the possible role of ROS in wound healing and the co-culture model should be useful for screening agents that may influence the wound healing process.

1. Introduction

One of the most important repair processes after dermal wounding is the restoration of the epidermal barrier over the wound site so as to prevent dehydration and opportunistic infections. This is also known as re-epithelialization and is largely achieved by a combination of keratinocyte migration and proliferation [1,2].

Various models of different complexity have been developed to study the re-epithelialization process. The simplest of these models is the monolayer keratinocyte scratch wound in which a layer of confluent keratinocytes is mechanically injured with a pipette tip and the resurfacing of the denuded area is used as an indicator of re-epithelialization [3]. This model has already been used widely by us and others to examine the effect of soluble compounds on wound healing [4,5]. H_2O_2 has been shown to play an important role in the events following dermal wounding [6–8] and we have previously found that H_2O_2 can induce cell proliferation and migration in a keratinocyte scratch wound model by inducing a sustained activation of the ERK pathway [5]. Nevertheless, the scratch wound model is a considerable over-simplification of the re-epithelialization process, which involves multiple cell types [9].

Three-dimensional organotypic cultures of keratinocytes and fibroblasts as well as *ex vivo* culture of skin explants have emerged as additional models to study the re-epithelialization process. 3D organotypic cultures involve the culturing of keratinocytes on dermis equivalents, which are created by mixing fibroblasts with type I collagen and allowing the collagen to solidify [10]. Keratinocytes are seeded onto the dermis equivalent, allowed to attach and exposed to the air–liquid interface. The organotypic culture can then be mechanically wounded by complete bisection and the re-epithelialization process studied using histological techniques [11].

In the *ex vivo* explant model, skin biopsies of mice or human subjects are allowed to attach onto a tissue culture dish and over the following days, keratinocytes emerge from the edge of the explants, migrating steadily as a cell sheet [12]. The rate of re-epithelialization can be conveniently monitored using a normal phase contrast microscope. However the effect of any treatment on the fibroblasts in the explants would still need to be evaluated by histological techniques.

Two-chamber type co-culture models involve culturing fibroblasts and keratinocytes in two separate chambers separated by a semi-permeable membrane. Such models have long been used to study paracrine signaling between keratinocytes and fibroblasts [13]. In this model, keratinocytes are usually grown on a semi-permeable transwell membrane insert while fibroblasts are grown in a tissue culture dish. The keratinocytes are exposed to

Abbreviations: AUC, area under the curve; HKGS, human keratinocyte growth supplement; NAC, *N*-acetyl-L-cysteine; ROS, reactive oxygen species.

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the air–liquid interface and allowed to stratify and differentiate. Soluble factors secreted by the fibroblasts are able to pass through the membrane and affect the growth and differentiation of keratinocytes and vice versa. However such models have never been used for studying re-epithelialization.

In the present paper, we used a co-culture model to explore if our previous studies on the mitogenic effect of H_2O_2 in keratinocytes would still be relevant in a more complex and perhaps more physiologically relevant system. We developed a simple model based on the two-chamber type co-culture model which can be used to evaluate re-epithelialization rate and effects of added compounds much more easily compared to the 3D organotypic culture models and *ex vivo* explant models used by others previously.

2. Materials and methods

2.1. Materials

Epilife medium with 60 μM calcium, human keratinocyte growth supplement (HKGS), primary human dermal fibroblasts and primary human keratinocytes (both derived from neonatal foreskin) were purchased from Invitrogen, Eugene, OR. Radioimmunoprecipitation assay (RIPA) buffer was purchased from Cell Signaling Technology (Danvers, MA, USA). Mouse monoclonal anti-involucrin (Cat# SC 21748) and mouse monoclonal anti-GAPDH (Cat# SC 47724) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horseradish peroxidase-conjugated goat anti mouse secondary antibody (Cat 0031430) was purchased from Pierce Chemicals (Rockford, IL, USA). 0.4 μm Pore size Transwell transparent polyester membrane inserts and their corresponding 6-well plates were purchased from Corning (USA). Thirty-five millimeter μ-dish inserts, purchased from Ibidi (München, Germany), were used as the wound assay inserts in the co-culture model. High glucose Dulbecco's Modified Eagle's Medium (DMEM) with added glutamine and fetal bovine serum was purchased from PAA (Linz, Austria). *N*-acetyl-L-cysteine (NAC), H_2O_2, phenylmethanesulfonylfluoride (PMSF), dimethyl sulfoxide and thiazolyl Blue Tetrazolium Bromide (MTT) were purchased from Sigma–Aldrich (USA).

2.2. Cell culture

Primary fibroblasts were cultured in a humidified 95% air, 5% CO_2 incubator at 37 °C and maintained in DMEM supplemented with 10% FBS. Primary keratinocytes were cultured in EpiLife medium supplemented with HKGS at 100:1 ratio. Keratinocytes from passage 4–5 and fibroblasts from passage 6–8 were used for the experiments.

2.3. Co-culture wound model

An adhesive wound assay insert was stuck onto the centre of a 6-well transwell to occlude the centre of the transwell from the surroundings. Keratinocytes were trypsinized, re-suspended in Epilife medium supplemented with HKGS and seeded into the area outside the wound assay insert at 3×10^5 cells, 2 ml per transwell. Fibroblasts were trypsinized, re-suspended and seeded into 6-well plates at 3×10^5 cells, 2 ml per well. Keratinocytes and fibroblasts were grown separately for 48 h until confluent. The transwells containing the keratinocytes were then placed above the fibroblasts. Medium for both cell type was changed to the co-culture medium, which was basal Epilife medium:basal DMEM:FBS at a ratio of 49:49:2. The volume of medium was 2 ml per well and 1 ml per transwell insert (Refer to Fig. 1). After 2 days, the keratinocytes were exposed to the air–liquid interface by removing the medium in the transwells inserts while the medium at the bottom fibroblasts layer was replaced with fresh medium. Two days later, the wound assay insert was removed using a pair of sterile forceps. A sterile scalpel was also used to lightly dislodge the wound assay insert from the keratinocytes around it. With the wound assay insert removed, the keratinocytes could grow into the denuded area.

The co-cultures were treated with the cell permeable thiol compound, NAC or with H_2O_2 to evaluate their effects on re-epithelialization. As NAC is acidic, it was dissolved in the co-culture medium and the pH was adjusted to pH 7.4 with NaOH. Both NAC and H_2O_2 were filter-sterilized before use. 2 ml of co-culture medium containing H_2O_2 or NAC was added into the fibroblasts layer and 1 ml into the keratinocytes. After 30 min, the medium in the keratinocytes layer was aspirated leaving only the medium in the fibroblasts layer. Thirty minutes was chosen because we have previously shown that cells were able to decompose almost all the H_2O_2 present in the medium by 30 min [5].

The treatment was repeated every 24 h, over a period of 96 h. The same treatment was carried out for control experiments except that no test compounds were added. Images of the closure were monitored with a dissection microscope (Leica Microsystems, Wetzlar, Germany) for 4 days at 24 h intervals. The size of the denuded area was determined using the polygon tool in the image analysis software, ImageJ (NIH, USA) and expressed as percentage closure of the original wound.

2.4. Cell viability assay

Cells were grown in the co-culture system but without the wound assay inserts. Samples were treated with H_2O_2 and NAC every 24 h over a period of 96 h, using the same method as described for the co-culture wound model. At 96 h, the keratinocyte layer and the fibroblast layer were separated. Cell viability was determined using the (Thiazolyl Blue Tetrazolium Bromide) MTT reduction assay. At 96 h, the transwell inserts (keratinocytes) were separated from the tissue culture dish (fibroblasts). 5 mg/ml MTT dissolved in the co-culture medium was added to the keratinocytes (1 ml) and fibroblasts (2 ml) respectively. The cells were incubated at 37 °C for 15 min before the medium was aspirated and DMSO was added to each well to dissolve the purple formazan product. 1 ml of DMSO was added to the keratinocyte layer and 2 ml of DMSO was added to the fibroblast layer. The extracted formazan dye from each well was then further diluted 5 times in a 96-well plate and the absorbance was read at 570 nm against a reference wavelength of 690 nm on a microplate reader (Tecan, Switzerland). Cell viability was computed by comparing absorbance of different treatments against that of untreated cells and expressed as a fraction.

2.5. Western blot analysis

Cells were washed twice with ice-cold PBS and 150 μL RIPA buffer supplemented with 1 mM PMSF was added per transwell membrane insert. Cell lysates were briefly ultrasonicated in a sonicator bath before centrifuging for 10 min at 10,000*g*, 4 °C. The remaining cell pellet was further extracted with 6 M urea to dissolve insoluble proteins that might be present in the suprabasal layers of keratinocytes. Depending on the size of the insoluble cell pellet, different volumes of 6 M urea were added. Five micro liters of 6 M urea was added to day 0 samples and 50 μl was added to day 4 and day 10 samples. The extracted insoluble proteins were then pooled together with the RIPA buffer extract.

Samples were separated on a 10% SDS–polyacrylamide gel and wet-transferred onto nitrocellulose membranes. Membranes were blocked with 5% skim milk in tris-buffered saline (TBS) with 0.1% Tween-20 for 1 h at room temperature and washed with TBS containing 0.1% Tween-20 prior to incubation with the antibodies