EXTERNAL ELECTRIC FIELDS INDUCE MORPHOLOGICAL CHANGES ON HUMAN SKIN CELLS CULTURED *IN VITRO*.

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Introduction

Normal skin contains a physiological battery originating from the living epidermis. The trans-epithelial potential is around 10 to 60 mV and varies in different part of body. In the event of skin injury, an electrical field (EF) is induced in the wound epidermis by sodium gradient and this is essential to regeneration of tissue [1,2]. This endogenous electric field could be implicated in wound healing by attracting cells and altering their phenotypes. Some studies carried out *in vivo* with a guinea pig burn wound model showed that an EF stimulation improves wound recovery [3]. Enhancements of epithelial cell proliferation were also observed and a faster revascularization a better takes of implant in autologous skin graft as well as scar processes. Furthermore *In vitro* studies showed that EF stimulation also increases cell migration, proliferation and collagen synthesis [4,5,6]. Although benefic effects of EF stimulation on wound healing were noted, their mechanism is still unclear. Skin wound healing is a complex and multifactor processing and more explorations are needed in order for better clinical protocols to be established. Advances in tissue engineering now permit new approaches to try defining the mechanism implicated in wound healing. The present research investigated the effect of an external electrical field (EF) on human skin (HS) fibroblasts and keratinocytes cultured in monolayer or on the wound healing of reconstructed human skin (RHS) produced *in vitro* by tissue engineering.

Materials & Methods

Cell Isolation and Culture

Human fibroblasts and keratinocytes were extracted from the same skin sample. The isolation procedure was initiated within 3 hours following the surgery according to previously described methods established in our laboratory [7,8]**.** Briefly, the specimens were washed five times with a phosphate buffer saline (PBS; 0.14M NaCl; 2.68 mM KCl; 8.1 mM Na_2HPO_4; 1.47 mM; KH_2PO_4) supplemented with antibiotics. Skin fragments (10mm x 2mm) were put overnight at 4°C in 500 μg/ml thermolysin (Sigma Chemicals, St-Louis, MO, USA) in N-[2-Hydroxyethyl]piperazine-N'-[2-ethane-sulfonic acid] (HEPES) buffer (1mM $CaCl_2$). The epidermis was then separated mechanically from the dermis with forceps and incubated under agitation for 30 min with 0.05% trypsin-0.1% EDTA in PBS at 37°C. After centrifugation and trypsin inhibition by the addition of medium containing 10% serum, cells were expanded in T75 flasks and subsequently frozen for further use. The remaining dermal parts were cut in smaller pieces and put under agitation with collagenase 0.2% in complete DME medium with 10% fetal bovine serum (FBS; Gibco BRL, Grand Island, NY, USA) for 3-4 h at 37°C. Cells were then plated in T75 flasks for expansion and subsequently frozen for further use.

Keratinocytes between passages 1 and 3 were plated at 2 x $10^4/cm^2$ on coverslips (18mm x 18mm) with 2 x $10^4/cm^2$ irradiated (60 Gy rad) mouse 3T3 fibroblasts, acting as a feeding layer. Since keratinocytes grow in round colonies, this cell density was found to be optimal to have a nice distribution of the colonies. For both cell types (keratinocytes and 3T3 fibroblasts), cell viability was always superior to 95% prior to seeding. The culture medium used is a combination of the Dulbecco-Vogt modification of Eagles medium (DME) with Ham's F-12 in a 3:1 proportion, supplemented with 5% fetal calf serum

(FCS; HyClone, Flow Labs) , 5 μg/ml bovine crystallized insulin, 5 μg/ml human transferrin, 2 x 10^{-9} M 3,3',5'triiodo-L-thyronine (Sigma Chemicals), 0.4 μg/ml hydrocortisone (Calbiochem, La Jolla, CA, USA), 1 x 10^{-10} M cholera toxin (Scharz/Mann, Cleveland, OH, USA) and 10 ng/ml human epidermal growth factor (EGF, Chiron Corp., Emeryville, CA, USA)and antibiotics.

Fibroblasts between passages 1 and 5 were plated at 1 x $10^4/cm^2$ on coverslips (18mm x 18mm) in Dulbecco-Vogt modification of Eagles medium (DME) supplemented with 10% fetal bovine serum, and antibiotics. Fibroblasts grow individually and this cell density permits the fibroblasts to attach, spread and grow without touching themselves too much, allowing a nice distribution of the cells. Cell viability, measured prior to seeding, was always superior to 95%.

Wound-healing model

Our completely biological wound-healing model was elaborated as described previously [9]**.** Briefly, human fibroblasts grow for 28 days in the presence of 50 μg/ml of ascorbate secreted an abundant extracellular matrix and the formation of manipulatable sheets [10]. Two fibroblast sheets were superimposed and left to adhere together for 7 days. Human keratinocytes were then seeded on the double layer of fibroblasts and cultured in submerged conditions for 7 days. Differentiation of the keratinocytes was induced by elevating the tissue construct to the air-liquid interface for 14 days. The mature RHS was wounded with a 6 to 10 mm diameter punch biopsy (Laboratoire Stiefel, Nanterre, France) and placed over a third fibroblast sheet (obtained as stated above) to allow reepithelialization by keratinocytes onto a natural matrix. The wounded RHS was further cultured at the air-liquid interface for 3, 7, or 14 days. The reepithelialized surface was then photographed at the time of biopsy. Biopsies were taken to investigate reepithelialization of the wound processed for histology, and immunofluorescence staining.

Results and Discussion

Skin fibroblasts (dermal cells) exposed to an electric field responded with a reorientation of their cell bodies in a manner perpendicular to the electric field. The non-exposed controls clearly showed a random orientation of the fibroblasts. The reorientation of the fibroblasts occurred for an electrical stimulation of a duration as low as 3 hours at an EF above physiological values (Fig. 1a-b)**.** A physiological electric field also reoriented fibroblasts when used over a period of 24 hours, as was made evident by labeling of tubulin filaments, a component of the cytoskeleton that reorients itself in a direction perpendicular to the electric field (Fig. 1c-d)**.** An exposure of 3 hours to a physiological EF however did not result in a reorientation of these cells as striking as that obtained after a period of 24 hours. Actin and tubulin filaments are cytoskeletal proteins that reorganize continuously as the cell changes shape, divides and responds to its environment. Among other things, these filaments are important for maintaining cell shape, keeping organelles well distributed in the cytoplasm and are essential for attachment and cell movement. Like tubulin, actin filaments tend to be perpendicular to the EF as the cells reorient when they are subjected to an external EF (Fig. 1e-f).

In contrast to fibroblasts that normally present a bipolar cell shape, skin keratinocytes (epidermal cells) in monolayer culture normally grow in round colonies (Fig. 2a) and have a polygonal shape without any preferential orientation (Fig. 2b). Under our culture conditions, keratinocytes are joined to their cell neighbors by desmosomes. When subjected to an external EF, keratinocyte colonies adopt a more elongated shape in the direction perpendicular to the EF (Fig. 2c). Moreover, following an electrical stimulation, keratinocyte spreading is altered at the periphery of the colonies toward a more bipolar shape (Fig. 2d). The irradiated mouse 3T3 fibroblasts, essential for the culture of keratinocytes, also reorient in the same direction (Fig. 2c). We also observed that the width of the keratinocyte colonies in the direction parallel to the EF was often lessened more on one side, noted by the lack of 3T3 fibroblasts normally occupying the space between the colonies (Fig. 2c). Keratinocytes reacted similarly for both intensities of electric field tested. Actin filaments were also studied for keratinocytes and showed no preferential orientation when exposed to an electric field (Fig. 2e-f). A similar result was found for tubulin filaments (not shown), although the distribution pattern is more cytoplasmic when compared with actin, which has a peripheral labelling [11].

The development of a new stimulator is in progress in order to expose our RHS wounded model to a perpendicular EF. Our system allows work in a sterile environment and controlled temperature for long term experiments. The evaluation of the effect of EF on healing taking advantage of the EF exposition apparatus that we developed together with the RHS wounded model is now under way.

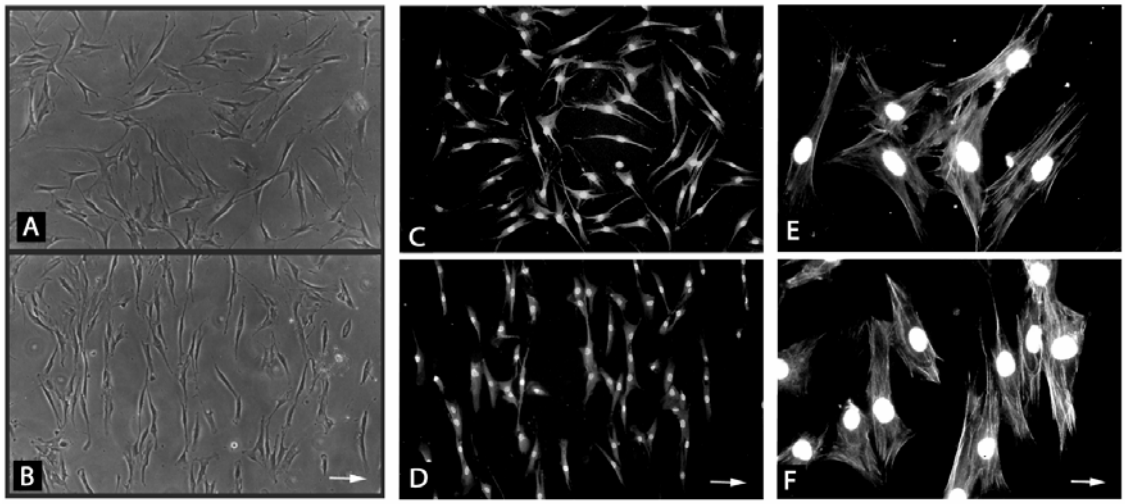

Figure 1: Fibroblasts reorientation after an exposure to an EF above physiological intensity. Phase contrast micrographs representative of the results obtained in the triplicatas of 3 separate experiments.
A) Control fibroblasts not exposed to an EF have a random orientation. B) Fibroblasts exposed to a 0.4 V/mm EF for 3 hours reorient in a manner perpendicular to the electric field. The arrow indicates the direction of the EF. C) Immunofluorescence staining of tubulin filaments and Hoechst staining of nuclei representative of the results obtained in the triplicatas of 3 separate experiments. Control fibroblasts not exposed to an EF have a random orientation. D) Fibroblasts exposed to a 0.1 V/mm EF for 24 hours reorient in a manner perpendicular to the electric field. E) Control fibroblasts not exposed to an EF have a random orientation. F) Fibroblasts exposed to a 0.1 V/mm EF for 24 hours reorient in a manner perpendicular to the electric field. Actin filaments also reorient in the same direction.

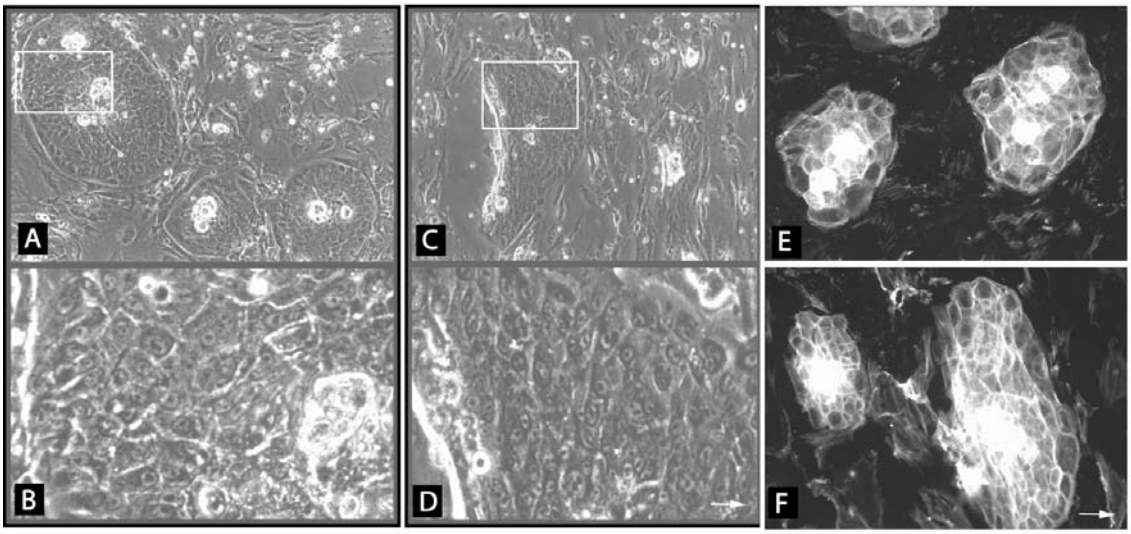

Figure 2: Fibroblasts reorientation after an exposure to an EF of physiological intensity. Immunofluorescence staining of actin filaments and Hoechst staining of nuclei representative of the results obtained in the triplicatas of 3 separate experiments. A) Control fibroblasts not exposed to an EF have a random orientation. B) Fibroblasts exposed to a 0.1 V/mm EF for 24 hours reorient in a manner perpendicular to the electric field. Actin filaments also reorient in the same direction. The arrow indicates

the direction of the EF. C) Keratinocytes exposed to a 0.4 V/mm EF for 3 hours. The colonies tend to be more elongated in a manner perpendicular to the electric field. We can also observe reoriented mouse 3T3 fibroblasts, acting as a feeder layer for keratinocytes. D) Keratinocytes stimulated with higher magnification of EF shows a tendency of the cells toward a bipolar shape, resembling the one of fibroblasts. The arrow indicates the direction of the EF for both pictures. E) Immunofluorescence staining of actin filaments. Control keratinocyte colonies not exposed to an EF show a peripheral labeling of actin filaments. Keratinocyte colonies exposed to an EF. E) Keratinocyte colonies exposed to a 0.4 V/mm EF for 3 hours. The actin filaments do not show a preferential orientation.

References

[1] A.M. Altizer, S.G. Stewart, B.K. Albertson, R.B. Borgens, Skin flaps inhibit both the current of injury at the amputation surface and regeneration of that limb in newts. J Exp Zool. 1; 293(5):467-77, October 2002

[2] L.S. Jenkins, B.S. Duerstock, R.B. Borgens. Reduction of the current of injury leaving the amputation inhibits limb regeneration in the red spotted newt. Dev Biol. 15; 178(2):251-62, September 1996

[3] C.S. Chu., A.T McManus, C.V. Okerberg, A.D. Jr Mason, B.A. Jr. Pruitt. Weak direct current accelerates split-thickness graft healing on tangentially excised second-degree burns. J Burn Care Rehabil. ; 12(4):285-93, July-August 1991

[4] K. Cheng., R.J. Goldman. Electric fields and proliferation in a dermal wound model: cell cycle kinetics. Bioelectromagnetics.; 19(2):68-74, 1998

[5] M. Zhao., A. Agius-Fernandez., J.V. Forrester, C.D. McCaig. Directed migration of corneal epithelial sheets in physiological electric fields. Invest Ophthalmol Vis Sci.; 37(13):2548-58, December 1996

[6] A.K. Harris, N.K. Pryer, D. Paydarfar. Effects of electric fields on fibroblast contractility and cytoskeleton. J. Exp. Zool.; 253(2):163-76, February 1990

[7] F.A. Auger, C.A. Lopez Valle, R. Guignard, N. Tremblay, B. Noel, F. Goulet, L. Germain. Skin equivalent produced with human collagen. In Vitro Cell Dev Biol Anim.; 31(6):432-9, June 1995

[8] L. Germain, M. Rouabhia, R. Guignard, L. Carrier, V. Bouvard, F.A. Auger. Improvement of human keratinocyte isolation and culture using thermolysin. Burns.; 19(2):99-104, April 1993

[9] A.F. Laplante, L. Germain, F.A. Auger, V. Moulin. Mechanisms of wound reepithelialization: hints from a tissue-engineered reconstructed skin to long-standing questions. FASEB J.;15(13):2377-89, November 2001

[10] N. L'Heureux, S. Paquet, R. Labbe, L. Germain, F.A. Auger. A completely biological tissue-engineered human blood vessel. FASEB J.; 12(1):47-56, January 1998

[11] Methot S., Moulin V., Rancourt D., Bourdages M., Goulet D. Plante M. Auger F.A and Germain L. Morphological Changes of Human Skin Cells Exposed to a DC Electric Field In Vitro Using a New Exposure System. Can.J.Chem.Eng.,79 (4): 668-677.