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Effects of THz Exposure on Human Primary Keratinocyte Differentiation and Viability

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Abstract. Primary human keratinocytes can be driven, *in vitro,* to differentiate, via activation of transglutaminases, by raising the culture medium calcium concentration above 1 mM. This results in transglutaminase regulated cross linking of specific amino acids with resultant cornified envelope formation. The differentiation was monitored via the incorporation of fluorescein cadaverine into the cornified envelops. This differentiation assay was combined with assessment of reductive capacity of resazurin, as a measure of cell activity/viability. One primary aim is to assess the effects of THz radiation on human skin, since medical imaging of the body through the skin is envisaged. Human keratinocytes, at passage 2 from isolation, were grown to confluence, and transported in a buffered salt solution at 22 °C. The exposure to the THz source was for 10, 20 or 30 minutes at room temperature. No donor specific inhibition or stimulation of cell activity, compared with non-exposed cells, was noted following exposure in the range 1 to 3 THz, at up to 0.45J/cm^2. The differentiation also occurred in a normal way, for exposed and non-exposed cells, with the FC incorporation increasing between day 3 and day 8, as previously noted.

Key words: Differentiation, human primary keratinocytes, *in vitro*, resazurin assay, THz effects

1. Introduction

Primary human keratinocytes can be driven, *in vitro,* to differentiate via activation of transglutaminases, by raising the culture medium calcium concentration above 1 mM. This results in transglutaminase regulated cross-linking of specific amino acids with resultant cornified envelope formation. The differentiation can be monitored via the incorporation of fluorescein cadaverine, as a substitute for L-Lysine [1]. This differentiation endpoint has been co-assayed with the keratinocytes reductive capacity for converting resazurin to resorufin, as a measure of cell activity/viability [2–5], in studies on the adverse effects of chemicals to the epidermal cells capacity to differentiate.

One primary aim of the THz Bridge project is to assess the effects of THz radiation on human beings, the effects on the basal keratinocytes ability to continue growth and normal patterns of differentiation. These are relevant since new medical imaging modalities of the body through the skin are being developed in the THz region [6, 7]. For imaging, a range of 1–3 THz is applied to human tissue [6–8], to

donor keratinocytes isolated from human skin grown in culture in defined medium [1, 9].

The human keratinocytes, at passage 2 from isolation, are grown to confluence, since once confluent the cells divide only slowly. Dividing cells are more sensitive to chemicals and light induced damage. Keratinocytes can be transported in a buffered salt solution at 22 °C, as confluent cultures in 96 well tissue culture plates. They were exposed to the THz source for up to 30 minutes at room temperature.

The resazurin reduction assay [2–4], based on the Alamar Blue assay, was performed to assess the cells redox potential, since cell death is frequently linked with excess free radical production. The enhancement of fluorescein cadaverine uptake over 6 and 8 days was employed to detect changes in differentiation capacity [1].

This paper presents the effects of 1–3 THz exposure at up to 0.45 J/cm^2 on human primary keratinocytes, their activity/viability and capacity to subsequently differentiate.

2. Materials and Methods

2.1. PRIMARY HUMAN KERATINOCYTES

These were isolated from human skin, donated with patient consent, using the standard protocol as described by Khammo et al. [9, 10].

Primary keratinocytes derived from different donor male skin samples, were passaged once, then the cells were seeded (passage 2) from a 5×10^4/ml suspension, at 100μl per well into a 96 well plate. The cells were cultured for 48 hours in Keratinocyte Growth Medium with 0.06 mM calcium (Clonetic; BioWittaker, Wokingham, Berks.) to form a confluent layer. Cells from donors were independently tested.

3. THz Exposure

The THz system at the University of Leeds (Institute of Microwaves & Photonics), School of Electronics & Electrical Engineering, University of Leeds, Leeds LS2 9JT) uses a Ti:Sapphire laser impacting on an electro-optic photoconverter to generate THz power. The total pulse duration is 20–30 ps, although approximately 90% of the THz power is delivered within the first two ps. The average output power for this (unamplified) system is approximately 1 μW within the frequency range 0.2–3.0 THz. The repetition rate of the THz pulse is approximately 80MHz. An overview of this type of system is provided by Zhao et al. [11].

The THz generation at Teraview (Cambridge) was achieved by optical excitation of a gallium arsenide wide aperture antenna [12]. A large DC-bias applied across the device which was excited using a Ti:Sapphire laser (RegA 9000, Coherent Inc, CA) emitting 250 fs pulses centred at a wavelength of 800 nm, with a 250 kHz repetition rate. This gave a usable frequency range of 0.1 THz to 2.7 THz with an average power of approximately 1 mW [8]. The THz-radiation was collected

and collimated by an f/1 off-axis paraobla and then focused by another paraobla on to the sample with a spot size of 130 μm to 3.7 mm. The THz spot was raster scanned over sample for the duration of the exposure.

A resazurin (Sigma, Poole, Dorset) assay was performed prior to the exposure to the THz. The resazurin/resorufin solution was removed and replaced with 100 μl of calcium containing HBSS (Hanks Buffered Salt Solution; Sigma). The plates were taped to keep the lids on, placed into a thermal box at room temperature (approx. 22 °C) and transported to the THz source at either TeraView Ltd, Cambridge, or the Institute of Microwaves and Photonics, University of Leeds. The keratinocytes were exposed to the THz through the base of the 96 well plate. A square block of 4 wells on the 96 well plate were exposed each time. Cells were exposed for periods of 10, 20 or 30 minutes, giving a total exposure of 0.15, 0.3 or 0.45 mJ/cm^2 or 0.15, 0.3 or 0.45 J/cm^2 per 4 wells, approximately a 2 cm bye 2 cm area.

Two sets of 4 control wells were exposed to the same conditions but not the THz beam. The plates were returned to the thermal box and transported back (2 hours). A resarzurin assay was then performed, post THz exposure and again at 3, 6 and 8 days. Following the initial post exposure resazurin assay the keratinocytes were returned to Greens Medium [1] containing 20 μM/ml fluorescein cadaverine (Sigma) [1, 5] to stimulate and quantify differentiation.

Assays

The resazurin (Sigma) was diluted in HBSS to 15 μg/ml. This was pre-warmed to 37 °C and added to the keratinocytes (100 μl) in place of the removed culture medium. The cells were incubated, for 1 hour, at 37 °C in 95% Air/5% CO_2, as per routine culture. The exposed, non-exposed controls and four no-cell wells were then assayed for resorufin formation in a FluoroStar Galaxy spectrofluorimeter (BGM, Aylesbury, Bucks), with an excitation filter of 530 $\pm$ 12.5 nm and emission of 590 $\pm$ 12.5 nm. The optical density of the exposed cells minus the no-cells blanks being finally expressed as a percentage of the non-exposed control minus the blank no-cells value (i.e. the level of resorufin in the resazurin prior to cell exposure).

The uptake of 20 μM fluorescein cadaverine (FC; Molecular Probes, The Netherlands), in medium, into the cells was measured at the same time as the resazurin reduction, but at an excitation of 485 $\pm$ 10 nm and emission of 530 $\pm$ 12.5 nm. The resazurin solution was removed and the cells washed once with 100 μl of HBSS prior to measuring the FC uptake. The uptake was expressed as a percentage of that for the non-THz exposed cells, minus the background in the no-cell wells.

4. Statistical Analysis

The data for cell activity and differentiation were compared using Repeat measurement ANOVA and the Dunnetts post hoc test in PRISM 3 software (Graph Pad

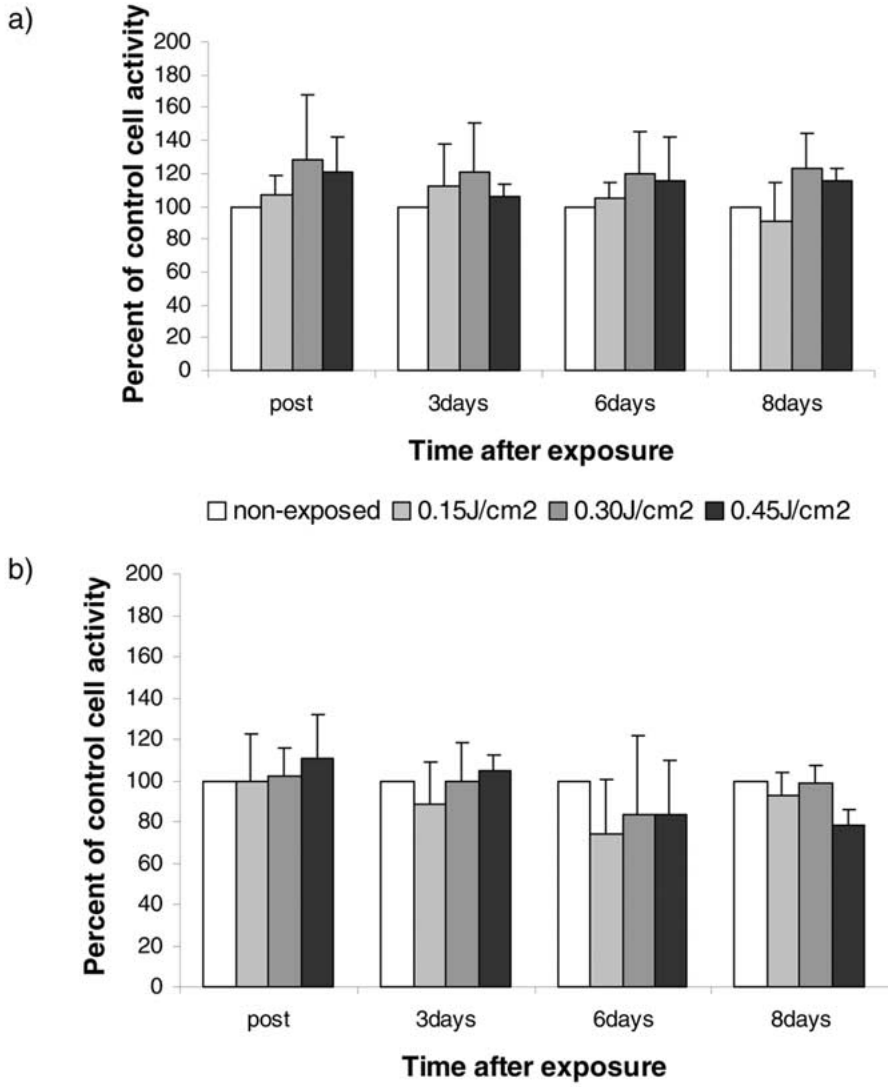

Figure 1. The effects on resazurin reduction in passage 2 [primary human keratinocytes of exposure to 1–3 THz radiation through the base of a tissue culture plate of 0, 0.15, 0.3 and 0.45 Jcm^2. Mean of three donors 1a (Teravision), or two 1b (University of Leeds).

Software, Inc,) on the original optical density data. Difference of $p < 0.05$ were considered significant.

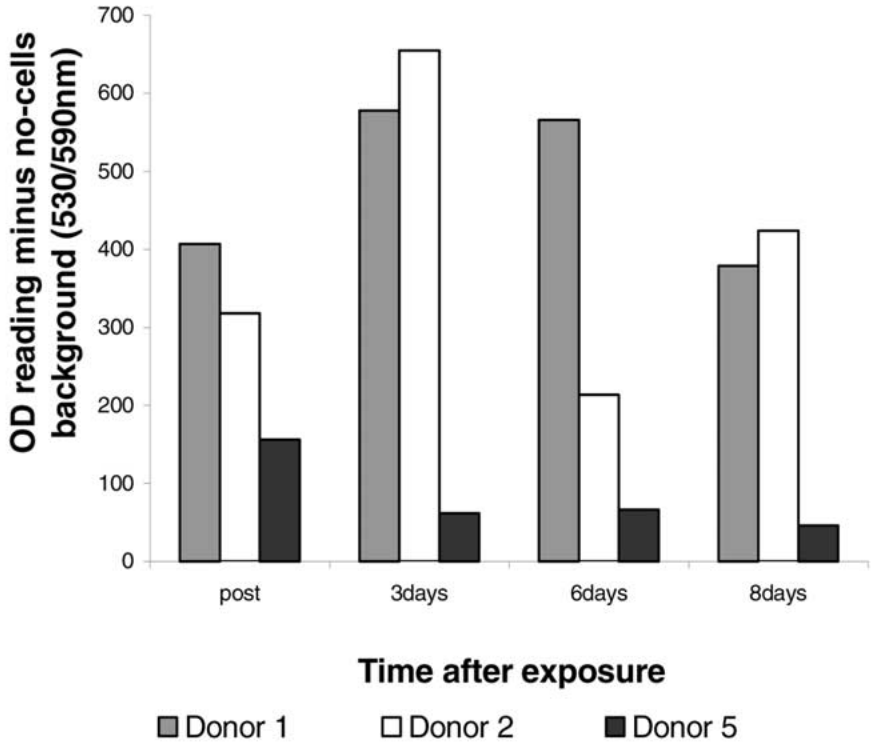

Figure 2. The differential resazurin reduction capacity in different donor keratinocytes at post exposure, day 3, 6 and 8. Non-THz exposure control cultures minus background values.

5. Results

Over the 8 days in culture there were no significant effects following THz exposure on the human primary keratinocytes activity, compared with the control. This was observed with both of the two THz sources employed (Figure 1a, 1b). This was despite the expected variation in the activity of the primary cells isolated from different donors (Figure 2).

The results reported are for those cells exposed to 0.15, 0.3 and 0.45 J/cm^2. Whilst the activity of the keratinocytes declined as they differentiated the exposure to THz, at any energy level tested, did not inhibit the capacity to differentiate in a dose dependent fashion overall (Figure 3). The same pattern of cell activity and differentiation was noted for the keratinocytes exposed to 0.15 to 0.45 mJ/cm^2 at Leeds University, THz generating laser (results not shown).

6. Discussion

The fluorescein cadaverine assay demonstrated that there was a time dependency for the cornification process via the formation of cornified envelops [1]. From

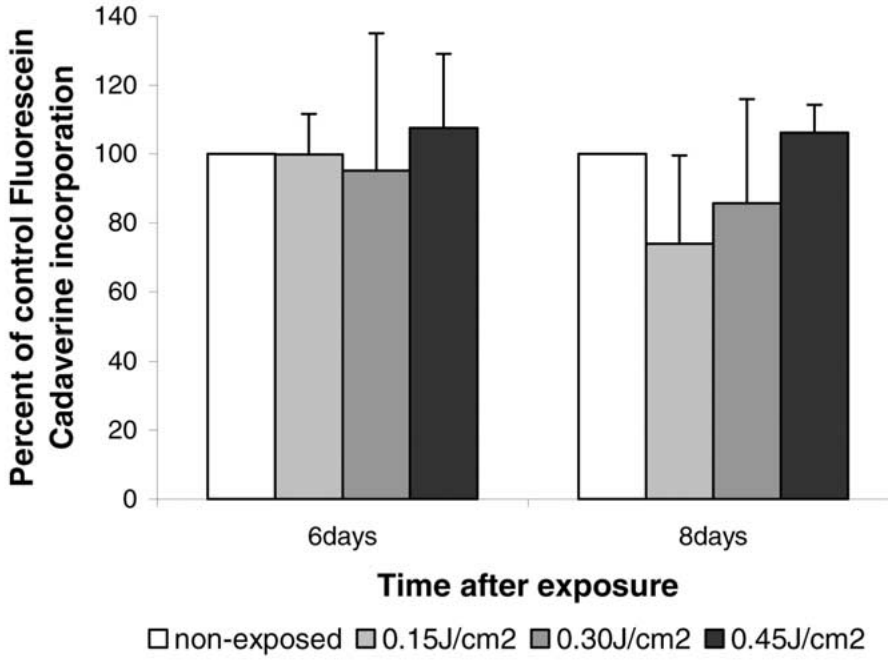

Figure 3. The effects of 1–3 THz radiation at 0–0.45 J/cm^2 upon the differentiation of primary human keratinocytes via capacity to incorporate fluorescein cadaverine (Mean ± SD, n = 3).

previous results and the database on FC incorporation rates, it has been shown that there is a rise in FC uptake in normal keratinocytes from <20 pg per well to 111 ± 38 pg per well over the period of day 3 to day 9. The results presented, with an average of 184 ± 85 pg per well, in these additional donors, were in line with these results.

The THz from the two sources gave comparable results with no signs of induced stress to the cells, over the subsequent culture period. The resazurin assay depends on the reduction of resazurin to resorufin [3, 4], via a range of enzyme pathways that are involved in the protection of normal cells from free radical induced damage [2, 4]. It has been shown previously that resazurin reduction was greatest in dividing cells [12], and that it declines as the cells differentiate and cease division. This was noted with these donors.

The differentiation capacity was normal for all three donors. Therefore, normal keratinocytes do not appear to be adversely affected by THz radiation, with no stimulatory of the activity of non-differentiating or stimulation with Greens medium [13] of differentiation. Investigations are continuing into dividing keratinocytes sensitivity, and those were the differentiation is modulated.

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References

1. Gray, A.C., Garle, M. and Clothier, R.H.: Fluorescein Cadaverine Incorporation as a Novel Technique for the Characterisation of Terminal Differentiation in Keratinocytes, *Tox. in Vitro* **13** (1999), 773–778.
2. Andrews, M.J., Garle, M.J. and Clothier, R.H.: Reduction of the Newtetrazolium Dye Alamar Blue, in Cultured Rat Hepatocytes and Liver Fractions, *ATLA* **25** (1997), 641–653.
3. Rasmussen, E.S.: Use of Fluorescent Redox Indicators to Evaluate Cell Proliferation and Viability, In: A. Goldberg (ed.), *In vitro and Molecular Toxicology 12*, Mary Ann Liebert, New York, 1999, pp. 47–58.
4. O'Brien, J., Wilson, I., Orton, T. and Pognan, F.: Investigation of the Alamar Blue (resazurin) Fluorescent Dye for the Assessment of Mammalian Cell Cytotoxicity, *Eur. J. Biochem.* **267** (2000), 5421–5426.
5. Gray, A.C. and Clothier, R.H.: The Use of an *in vitro* Submerged Keratinocyte Model to Predict Induction of Squamous Metaplasia, *Tox. in Vitro* **15** (2001), 427–431.
6. Arnone, D.D., Ciesla, C.M., Corchia, A., Egusa, S., Pepper, M., Chamberlain, J.M., Bezant, C., Linfield, E.H., Clothier, R.H. and Khammo, N.: Applications of Terahertz Technology to Medical Imaging. Terahertz Spectroscopy & Applications – II, *SPIE.* **3828** (1999), 209–219.
7. Taday, P.F., Wallace, V., Woodward, R.M. and Arnone, D.: Terahertz Spectroscopy of Skin, (this volume).
8. Cole, B.E., Woodward, R.M., Crawley, D., Wallace, V.P., Arnone, D.D. and Pepper, M.: Terahertz Imaging and Spectroscopy of Human Skin, *In-vivo*, *Proceedings of SPIE* **4276** (2001), 1–10.
9. Khammo, N., Bartlett, A., Clothier, R.H. and Whitfield, P.: The Attachment of *Schistosoma mansoni* Cercariae to Human Skin Cells, *Parasitology* **124** (2002), 25–30.
10. Khammo, N., McPhie, P., Settle, J.A.D., Ingham, E. and Kearney, J.N.: Effect of Burn Patient Serum on Fibroblast and Keratinocyte Cell Morphology, *Burns* **23** (1997), 212–217.
11. Zhao, G., Schouten, R.N., van der Valk, N., Wenckebach, W.Th. and Planken, P.C.M.: Design and Performance of a THz Emission and Detection Set Up based on a Semi-Insulating GaAs Emitter, *Rev. Sci. Instr.* **73**(4) (2002), 1715–1719.
12. Van Exter, M. and Grischkowsky, D.R.: Characterization of an Optpelectronic Terahertz Beam System, *IEEE Transactions on Microwave Theory and Techniques* **38**(11) (1990), 1684–1691.
13. Garside, J.R., Clothier, R.H., Somekh, M.G. and Chung, W.S.: The Use of Simultaneous Fluorescence and Differential Phase Confocal Microscopy to study Alamar Blue Reduction in an Epithelial Cell Line, *ATLA* **26** (1998), 505–522.