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Depilatory chemical thioglycolate affects hair cuticle and cortex, degrades epidermal cornified envelopes and induces proliferation and differentiation responses in keratinocytes

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Supplementary material: Supplementary Methods, 1 Supplementary Table, 5 Supplementary Figures and their legends.

Abstract

Thioglycolate is a potent depilatory agent. In addition, it has been proposed to be useful as a penetration enhancer for transepidermal drug delivery. However, the effects on hair structure and stress responses it elicits in epidermal keratinocytes have not been fully characterized. We have used label-free confocal and fluorescence lifetime imaging supported by electron microscopy to demonstrate how thioglycolate damages hair

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cuticle cells by generating breakages along the endocuticle and leading to swelling of cortex cells. Maleimide staining of free SH-groups and a decrease in the average fluorescence lifetime of endogenous fluorophores demonstrate a specific change in protein structure in both hair cuticle and cortex. We found that the thioglycolate damages cornified envelopes isolated from the stratum corneum of the epidermis. However, thioglycolate-treated epidermal equivalent cultures recover within 48 hours, which highlights the reversibility of the damage. HaCaT keratinocytes respond to thioglycolate by increased proliferation, onset of differentiation and expression of the chaperone protein Hsp 70, but not Hsp 27. Up-regulation of involucrin can be blocked by an application of c-Jun N–terminal kinase (JNK) inhibitor, but the up-regulation of Hsp 70 takes place regardless of the presence of the JNK inhibitor.

Keywords: hair, label-free imaging, fluorescence lifetime imaging, keratinocyte differentiation, stress signaling

Running title: Thioglycolate stress in hair and keratinocytes

Background

Depilatory agents change the molecular organisation and morphology of the hair. Hair removal may be required for a range of purposes from a cosmetic benefit to easing symptoms of hair disorders [1,2]. Many depilatories are based on thioglycolate that reduces disulphide bonds in hair proteins [3]. Formation of disulphide bridges is important for keratin assembly resulting in an alignment and compaction of the filaments [4, 5], but these *in vitro* studies have not investigated the effect of thioglycolate. An X-ray diffraction study suggested that thioglycolate is localised in intrafibrillar areas in hair [3] but the method does not yield the spatial information that can be gained using advanced light microscopy. It has also been proposed that the cell-membrane complex is partly solubilised by thioglycolate [6]. Overall, the relationship between the changes in the keratin assembly and the subsequent alterations in the hair structure and integrity has not been fully resolved.

Importantly, thioglycolate can enhance epidermal drug penetration via impairment of the barrier function [7-9]. However, the use of thioglycolate can irritate the skin causing stinging and itching [10]. Thus, there is also a need to understand the mechanisms involved in the epidermal response to thioglycolate regardless whether the chemical is used for hair modification or assisting transepidermal penetration.

Questions addressed

We investigated how thioglycolate affects hair and skin addressing three questions:

(i) Where in the hair the morphological and biochemical changes that explain weakening of hair shaft are localized?

(ii) Given potential for enhancement of transepidermal drug delivery, how does thioglycolate affect corneocytes and epidermal equivalent cultures?

(iii) What cellular responses are elicited by the thioglycolate stress to facilitate recovery?

Experimental Procedures

Structural changes in human female head hair treated with thioglycolate were investigated using label-free confocal microscopy (Figure S1) and Fluorescence Lifetime Imaging (FLIM) [11] of endogenous fluorophores. These methods allow imaging without fixatives and dyes and can reveal changes in the chemical environment within hair. The observed changes were confirmed with electron microscopy.

To investigate the effect on epidermis, isolated corneocytes [12] and 3-D epidermal equivalents [13] were treated with thioglycolate and analysed with histochemistry and immunofluorescence. Reponses in HaCaT keratinocytes were studied by quantifying proliferation by Ki67 immunostaining, differentiation by involucrin immunoblotting, and stress responses by Hsp70 and Hsp27 immunostaining and by applying a JNK inhibitor (Calbiochem).

Results

Real-time imaging of hairs treated with a thioglycolate solution demonstrated a progressive damage to cuticle and swelling of the cortex. Hair surface deformed with invaginations and cellular (or sub-cellular) layers flaked away while the cortical cell-membrane complexes remained intact but slightly blurred and the organelle remnants remained streak-like. (Fig 1Ai – Aiv). On average, hair width increased by 136 ± 13% (n=6) compared to an 11±1% increase by hydration alone. Imaging of a thin optical section of the cuticle surface confirmed that the cuticle layers flake, moving to the focal plane (Figure 1Bi-ii) with uneven surfaces, degraded edges and gaps that indicate missing cuticle layers (Figure 1Biii). No changes were seen in control hairs from the same batches (Figure 1C).

Electron microscopy confirmed these results. To maintain the integrity of the treated hairs during the EM processing, extensive swelling was prevented by treating the hairs with a thioglycolate containing cream resulting only a limited change in their width (Figure 1D and 1Ei). SEM of the hair surface showed cuticle degradation (Figure 1E) or a complete loss in some areas (Figure 1E ii). TEM of the damaged cuticle (Figure 1G) compared to a control hair (Figure 1 F) revealed lost cuticle cells that can leave behind remnants of the endocuticle layer.

Breakage of disulphide bonds was visualised in hair cortex by staining free SH-groups with conjugated maleimide [14], which showed a 10 μm deep band indicating a possible boundary for the penetration of thioglycolate (Figure S2). FLIM imaging of the hair (Figure S2) revealed similarly localised and statistically significant decrease in fluorescence lifetimes (Supplementary Table 1). This further indicates alteration in chemical environment of endogenous fluorophores in both cuticle and cortex after thioglycolate treatment. Finally, TEM images showed a change in staining intensity and loss of detail in cell-membrane complexes in the same region of outer cortex that was strongly stained with maleimide (Figure S2).

The damage to the hair cuticle prompted us to investigate whether changes take place also in the stratum corneum. Nile Red-stained control corneocytes (Figure 2A) showed hexagonal morphology whereas those treated with thioglycolate were irregularly degraded with brightly stained aggregates on their surfaces (Figure 2B).

Because the irritation caused by depilation is temporary, we investigated responses of cultured keratinocytes to thioglycolate. We used 3-dimensional epidermal equivalents [13, 15], which is an established approach to study skin irritants [16]. Four minutes long exposure thioglycolate followed by 48 h recovery did not result in a permanent damage to the epidermis. (Figure 2C, D). Histology of the epidermal equivalents was unaffected, confirmed by immunofluorescence staining of keratin-14 and involucrin and measuring epidermal thickness of the cultures (Figure S3). Thus, cultured epidermal models can recover from thioglycolate exposure.

To elucidate the cellular responses that contribute to the recovery, we used cultured HaCaT keratinocytes. A statistically significant increase in Ki67 positive cells after thioglycolate application indicated a proliferative response (Figure 2E, F). No difference was seen in the number of apoptotic cells (Figure S4). Stress responses have been also shown to affect epidermal differentiation [17, 18]. Thioglycolate treatment resulted in an up-regulation of involucrin expression (Figure 2G, H). Thus, both proliferation and differentiation are accelerated as a response to thioglycolate. We next tested if inhibition of Jun N-terminal kinase signaling influences the differentiation after thioglycolate treatment. Use of JNK inhibitor that inhibits all three JNK isoforms, although with some off-target activity against other kinases [19], prevented the up-regulation of involucrin expression both in the control cells and in thioglycolate-treated cells (Figure 2G, H).

Finally, we investigated if an early response to thioglycolate involves induction of heat-shock proteins Hsp70 and Hsp27 that have previously been implicated in stress responses of keratinocytes [20]. We found a significant increase in the number of Hsp70 positive cells but no difference in the number of Hsp27 positive cells (Figure S5). Interestingly, use of the JNK inhibitor did not prevent Hsp70 up-regulation.

Conclusions

We show that cuticle cells are degraded by thioglycolate and that the breakage can take place in the endocuticle layer. Endocuticle is less cysteine-rich compared to exocuticle and but low in cross-linking [21] which makes it susceptible to swelling if the more rigid layers above are loosened.

Using both FLIM and imaging of free SH groups, we have visualised the changes in the cortex that relate to breaking of the disulphide bridges. Application of thioglycolate cream results in a distinct change in the outer cortex to 10 μm depth. The decrease in FLIM lifetimes confirms that hair protein denaturation alters the behavior of endogenous fluorophores and supports localisation of the damage to both cuticle and cortex. .

Thioglycolate treatment damages both stratum corneum and hair cuticle. Damage to corneocytes will potentially compromise the epidermal barrier. Increased fragility of corneocytes can result in a recruitment of CD4+ T-Cells [22] providing a connection between stratum corneum damage and skin irritation.

Various stresses influence keratinocyte differentiation. Manipulation of JNK pathway affects differentiation, but both acceleration [23] and inhibition [18] have been reported. We found that up-regulation of involucrin after thioglycolate treatment is prevented by a JNK signalling inhibitor supporting the results in [18] and indicating a role for JNK signalling in response to thioglycolate, although partial inhibition of other kinases can not be excluded [19].

Our results indicate that Hsp70 is a chaperone responding to thioglycolate stress whereas Hsp27 is not induced. Hsp70 induction has been implicated in wound healing, UV-B response and psoriasis [20] while Hsp27 is a target for p38 MAPK signaling and required for normal epidermal differentiation [24]. We show that the induction of involucrin can be uncoupled from up-regulation of Hsp27 expression, which supports

involvement of JNK rather than p38MAPK signaling in the thioglycolate response. Thus, thioglycolate causes two different stress responses: one leading accelerates differentiation that can be prevented by a JNK inhibitor and, secondly, an up-regulation of the chaperone protein Hsp70 that is not sensitive to the JNK inhibitor.

Epidermal equivalent cultures demonstrate that even though corneocytes are damaged by thioglycolate, the epidermis recovers, which is consistent with the induction of proliferation and differentiation in HaCaT cells. The transient effect supports findings of [7] on the feasibility of depilatory agents to help transepidermal drug delivery. No single enhancer is optimal for all types drugs and actives [25]. The accelerated turnover of keratinocytes after thioglycolate exposure indicates that this compound could be suited in assisting temporary and localised drug delivery.

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Figure legends

Figure 1. Thioglycolate disrupts hair cuticle cells. Ai – Aiii) Time lapse optical sections showing the outer cortex of a hair incubated in 450 mM thioglycolate solution up to ten minutes (n=6). Label-free imaging of 405 nm excitation at 0 min, 3 min and 9 min are shown. Scale bar = 25 μm. Aiv) Higher magnification showing cortical cells and organelle remnants (arrow). Scale bar = 10 μm. Bi – Biii) Optical sections of surface of the same hair at 1 min, 4 min and 10 min time points with a corresponding detailed image in Biv). Scale bar = 25 μm in Bi-iii, and 10 μm in Biv. C) Cuticle of untreated control hair. Scale bar = 10 μm. D) SEM of the surface of a control hair. Light microscopy image of a control hair shown in the insert in bottom left hand corner, scale bar fro the insert = 10 μm. Ei) SEM of hair treated with a cream formulation containing 450 mM thioglycolate for 4 minutes (n =3). Light microscopy image of a hair treated with thioglycolate cream is shown as an insert in the bottom left hand corner. Eii) Higher magnification detail showing loss of cuticle layers in thioglycolate treated hair. F) TEM of cuticle layers of a control hair with exocuticle (labelled Ex) and endocuticle (En) of a layer indicated. G) TEM of thioglycolate treated hair, scale bar= 2 μm.

Figure 2. Cornified envelopes are damaged by thioglycolate but keratinocytes recover after the treatment. A) Nile red stained control cornified envelopes. Scale bar = 10 μm. B) Thioglycolate treated cornified envelopes. C) H&E stained control epidermal equivalent culture. D) H&E staining of a thioglycolate treated epidermal equivalent culture after 48 hour recovery. Scale bar = 20 μm. E) Ki67 immunofluorescence (Green)

and DAPI nuclear counterstaining (blue) for control and thioglycolate treated HaCaT keratinocytes. T 45 mM= treatment with 45 mM thioglycolate; T 450 mM = treatment with 450 mM thioglycolate. F) Percentage of Ki67 positive cells, including both M phase and more diffuse S/G2 phase cells (n=6). G) Immunoblot of involucrin and keratin-14 expression in HaCaT cells. 1= Control; 2 = JNK inhibitor; 3= 45 mM thioglycolate; 4= 45 mM thioglycolate and JNK inhibitor; 5= 450 mM thioglycolate; 6= 450 mM thioglycolate and JNK inhibitor. H) Quantification of involucrin immunoblots (n=3), abbreviations as above and JNKi= cells treated with JNK inhibitor; T + J = cells treated thioglycolate and JNK inhibitor.

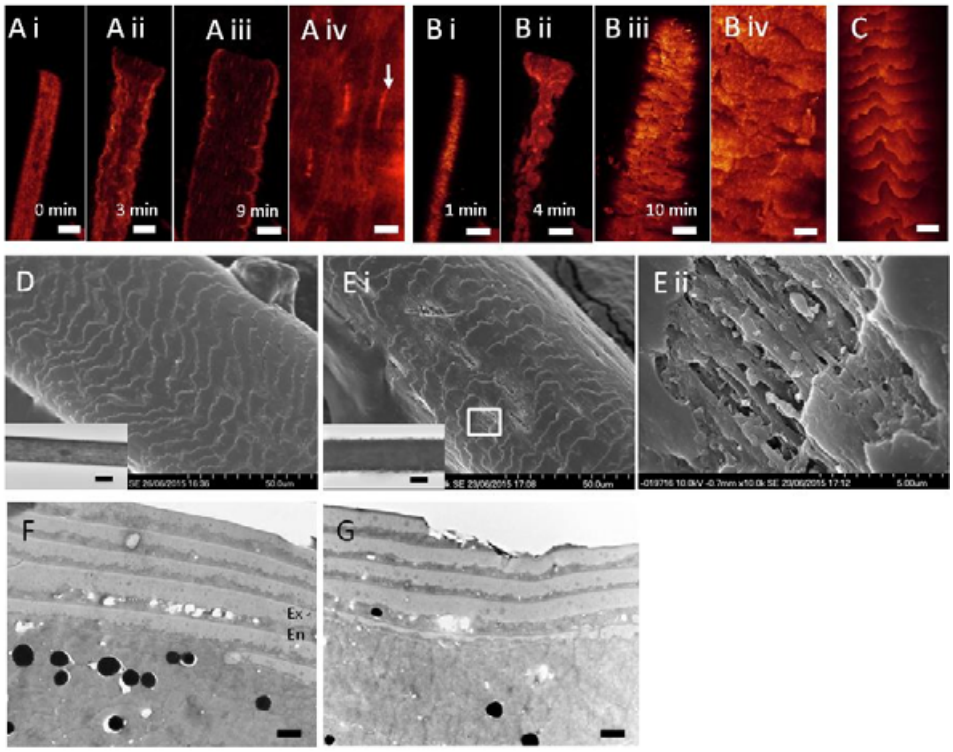

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Figure 1

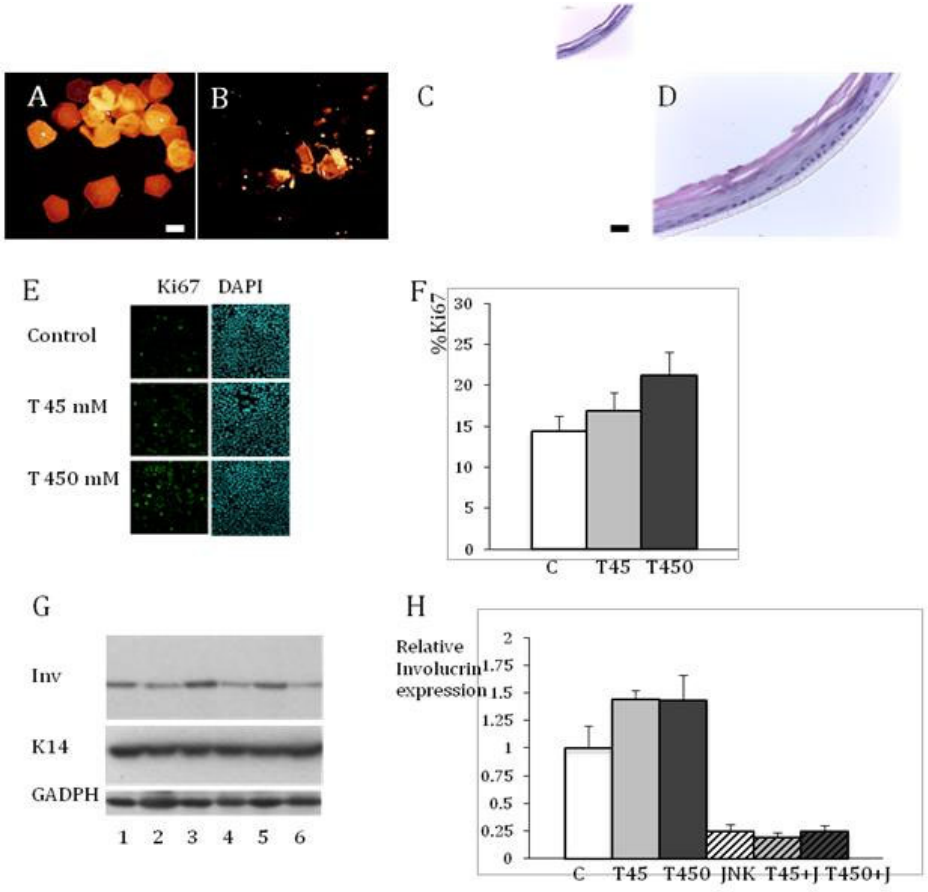

Figure 2