Characterisation of the inflammatory response triggered by topical ingenol mebutate 0.05% gel in basal cell carcinoma

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ABSTRACT

Background/Objective: Ingenol mebutate gel is approved for actinic keratosis field therapy, but little has been published as a treatment of basal cell carcinoma (BCC). Our objective is to characterise the histopathological changes and the infiltrating cell populations to better understand its mechanism of action.

Methods: Sixteen patients with various BCC subtypes were prospectively evaluated and treated once daily for two consecutive days with ingenol mebutate gel 0.05% under occlusion. Patients were randomised to two arms: the first arm was biopsied between the third and the tenth day after treatment initiation (‘early immune response’), and the second arm was biopsied at day 30 after treatment initiation (‘late immune response’). The immunopathology was evaluated by immunohistochemistry: anti-CD3, anti-CD4, anti-CD8, anti-CD20, anti-CD56, anti-CD68, anti-Bcl-2, anti-CASP3, anti-FoxP3, anti-GrzB and anti-TIA-1.

Results: Ten BCCs were in complete remission after 2 years of follow-up. The early immune response was characterised by a quick recruitment of T lymphocytes, macrophages and natural killer cells. At later time-points, T-regulatory cells and some pro-apoptotic markers were detected. Treatment-related adverse events were described.

Conclusion: Ingenol mebutate gel produces a transient immuno-inflammatory response and an important necrosis reaction in BCCs. Larger studies will be required to determine the maximum effective tolerated dose of ingenol mebutate gel for BCC.

Key words: basal cell carcinoma, CD4+, CD68+, immune response, ingenol mebutate gel, natural killer (NK) cells.

INTRODUCTION

Basal cell carcinoma (BCC) accounts for around 80% of all non-melanoma skin cancers (NMSC). It is typically slow growing and rarely metastasises but its location, tendency to relapse, multiplicity and possibility to invade and destroy local tissues increases disease morbidity. Surgical approaches are the standard strategy for well-defined BCC. Mohs micrographic surgery is generally used for high-risk tumours or those tumours in cosmetically sensitive areas, whereas cryotherapy or electrodesiccation and curettage are acceptable treatment options for small superficial BCCs on the trunk and limbs. Patients who are poor candidates for surgery, or have low-risk BCCs, as is the case of superficial BCCs, non-surgical methods such as topical treatment or photodynamic therapy may be alternative treatment options.

Topical ingenol mebutate gel (PEP005), a diterpene ester extracted from the plant Euphorbia peplus, is approved for actinic keratosis treatment. In actinic keratosis, ingenol mebutate gel 0.05% is applied once daily for two consecutive days to the affected areas of up to 25 cm². Although...
Ingenol mebutate gel is not considered a first-line option for the treatment of BCC, there is some evidence supporting its efficacy and safety.\textsuperscript{4,11}

Ingenol mebutate gel induces mitochondria swelling of dysplastic keratinocytes and cell death by primary necrosis. Topical application generates neutrophilic infiltration due to the activation of protein kinase C, causing effective wound healing. Its dual mechanism of action is characterised by a rapid necrosis lesion beginning 1–2 h after application (causing an increase of intracellular calcium, mitochondrial swelling and loss of cells membrane integrity) followed by tumour cell apoptosis via neutrophil-mediated cellular cytotoxicity occurring within days. At this time, there is also an increase of TNF-\(\alpha\) and IL-8, which recruits and subsequently activates neutrophils towards the inflammatory infiltrate.\textsuperscript{12} However, the exact mechanism of action of ingenol mebutate gel has not been fully elucidated.

We describe our experience using topical treatment ingenol mebutate gel 0.05\% under occlusion in a group of patients with BCC in low-risk locations (trunk and limbs).

**MATERIALS AND METHODS**

Between May and September 2015, a prospective, parallel, interventional and randomised clinical study on the efficacy of ingenol mebutate gel in BCC was conducted. Twenty-one patients were chosen for the screening visit of which five were excluded due to incorrect treatment compliance: a total of 16 BCC patients were included. Inclusion criteria were: Caucasian adults above 18 years of age with a histopathologically confirmed primary BCC (superficial, nodular or infiltrating histopathological subtypes defined in accordance with published criteria\textsuperscript{2}), located in low-risk zones (trunk and limbs) with an extension up to 1 cm\(^2\). Exclusion criteria were as follows: pregnant and breast feeding women, immunocompromised patients, genetic predisposition to BCC or recurrent BCCs. Informed consents and samples from patients were obtained with support from IRB Lleida Biobank (B.0000682) and PLATAFORMA BIOBANCOS (PT15/0010/0014). The study was approved by the Independent Ethics Committee at the Hospital Universitari Arnau de Vilanova de Lleida, Spain. The ingenol mebutate gel was applied on the tumour and in 1 cm\(^2\) area around the tumour under occlusion by 2.6–5 cm diameter aluminium disc made from foam paper.

Lesions were observed in the following days after treatment according to randomisation groups: biopsies were taken from the middle of each BCC between the third and the tenth day after treatment initiation in the first group as an ‘early immune response model’, coinciding with the maximum clinical inflammation (10 patients, Group 1) and the at day 50 after treatment initiation in the second group as a ‘late immune response model’ (six patients, Group 2; See Figure S1).

Control samples consisted of five biopsies obtained from both groups before starting ingenol mebutate gel application (untreated samples). The ingenol mebutate gel vehicle (isopropyl alcohol; hydroxyethylcellulose; citric acid monohydrate; sodium citrate; benzyl alcohol) does not produce a reaction as described in the literature,\textsuperscript{15} for this reason we did not use vehicle in the controls samples.

Treatment efficacy was assessed in terms of clinical and histological complete response. We used the local skin reaction grading scale to describe side effects related to therapy.\textsuperscript{13} This scale is based on a 0–4 numerical index (being 4 the highest grade of severity) related to six specific clinical parameters (erythema, scaling, crusting, swelling, and vesiculation/pustulation, erosion/ulceration) accompanied by a characteristic photographic image for each rating. Total local skin reaction score ranges from 0 to 24 points.\textsuperscript{14} The local skin reaction test was evaluated one week after treatment initiation for every patient. Pictures were also taken in every patient at the screening visit, before treatment initiation, and during the follow-up visits depending on each arm. Patients were randomised into group 1 and group 2 according to whether the inclusion visit was an even or odd day. An additional follow-up after 5 months, 6 months, 1 year and 2 years after treatment was conducted in every case. Patients with no tumour clearance in the follow-up biopsies discontinued the study and any remaining tumour was treated by surgery short time later.

**Immunohistochemical study**

Histopathological changes including necrosis and type and degree of the inflammatory infiltrate were analysed by a semi-quantitative measurements.

All post-treatment biopsies and the 5 screening biopsies (control) were analysed by immunohistochemistry with different antibodies: anti-CD3, anti-CD4, anti-CD8, anti-CD20, anti-CD56, anti-CD68, anti-Bcl-2, anti-CASP3, anti-FoxP3, anti-GrzB and anti-TIA1. See Table S1.

Tissue blocks were sectioned at 5 \(\mu\)m thickness, dried for 1 h at 65°C before pre-treatment procedure of deparaffinization, rehydration and epitope retrieval in the pretreatment module, PT-LINK (DAKO) (at 95°C for 20 min in 50 \(\times\) Tris/EDTA buffer and pH 9). Before staining, endogenous peroxidase activity was blocked with peroxidase blocking solution (Dako, Glostrup, Denmark). After incubation with primary antibodies, the reaction was visualised with the EnVision\textsuperscript{TM} FLEX Detection Kit (DAKO, Glostrup, Denmark) using diaminobenzidine chromogen as a substrate according to manufacturer’s instructions. Sections were counterstained with haematoxylin.

The histopathology of all tissue samples were reviewed by 2 blinded members of the team. For the staining scoring, an automated imaging system, the ACIS\textsuperscript{®} III Instrument (DAKO, Denmark, Glostrup), was used. The mean percentage of positive cells was obtained after evaluating regions of interest coincident with areas of higher infiltration.

Immunohistochemical post-treatment results were normalised by the mean value of the control group and were analysed by ANOVA statistical test.

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RESULTS

Efficacy and safety of ingenol mebutate gel 0.05% under occlusion in BCC

Five patients were excluded from analysis: four had not reapplied ingenol mebutate gel 0.05% the next day and one had not used ingenol mebutate gel 0.05% under occlusion. A total of sixteen patients were included in the study. Clinical and tumour baseline variables are shown in Table S2. The majority of patients were women (62.5%) with a mean age of 66, and more than half of the lesions were located in the trunk (56.25%). There were ten superficial BCCs, five nodular BCCs and one infiltrative BCC. The average tumour diameter was 8.9 mm (range 0.6 to 1 cm); 0.47 g of ingenol mebutate gel 0.05% was applied on each occasion.

After treatment, complete tumour clearance was observed in 8/10 (80%) superficial BCC, 2/5 (40%) nodular BCCs and 0/1 (0%) infiltrative BCC. All the failures were detected at the second biopsy. In total 10/16 tumours were cleared and remained so at the 2-year follow-up.

Most patients 10/16 (62.5%) experienced significant local inflammatory reactions with marked tumour erosion; the local skin reaction grading scale was over 20. Four out of sixteen patients had medium local skin reaction score (values between 12 and 18) and two patients had low local skin reaction score (score < 8). Interestingly, patients with medium or low values in local skin reaction score did not show clinical nor histological clearance.

From the first day of treatment, some patients experienced severe pain and flaking/blistering/erythema extending beyond the application site (Figs 1 and 2). From the third day, pain gradually decreased (with all symptoms disappearing after 1 week) and an erosive patch developed after blister rupture (Figs 1 and 2). The local skin reactions had almost resolved completely by 5 weeks. Distant BCC reactions beyond the area of ingenol mebutate gel application was not detected. Most of the tumours presented a scarring area after 6 months (Figs 1d and 2d) and after two years (not shown) post-treatment consisted of residual post-inflammatory hypopigmentation and hyperpigmentation.

No systemic features as headache or malaise were reported, and lymph node reaction was neither detected.

Histopathology

Abundant epithelial and superficial dermal necrosis were observed in biopsies taken from Group 1 (3–10 days after treatment initiation). This pattern was accompanied by an important inflammatory cell infiltration of polymorphonuclear (PMN) cells in the entire thickness of the dermis and in the superficial part of the subcutaneous cellular tissue (Fig. 5). In addition, some mononuclear cells were detected in the infiltrates. In biopsies from Group 2 (50 days after treatment initiation), necrosis degree and inflammatory infiltrate were lower than in Group 1, and necrosis was replaced by fibrosis although a mild-moderate mononuclear cell infiltration was still evident in most of the samples (Fig. 5). The immune response in blisters and the erythema areas were similar.

Regarding the adaptive immune response, a similar pattern was observed. A high increase of CD5+ cells was observed at early time-points (P = 0.015 compared to control), mainly composed by cytotoxic CD8+ and CD4+ T cells (P = 0.16 and P = 0.016, respectively, compared to control). T-cell recruitment also decreased over time, but while CD4+ T cells returned to control levels at day 50 after treatment initiation (group 2), residual presence of CD8+ T cells was observed although not statistically significant. These results suggest that some cytotoxic activity was present at the BCC zone at late time-points considering the presence of CD8+ T cells and NK (CD56+) within the infiltrate. Moreover, remaining expression of TIA-1 (cytotoxic marker) was also observed.

Reduction of T-cell recruitment in the late immune response matched with a high presence of regulatory T cells (Tregs) detected as FoxP3-positive cells. This sub-population suppresses effector T-cell activity (CD4+ and CD8+). Moreover, samples showed some B-cell recruitment (CD20-positive cells). We suppose that B-cell recruitment was induced later than T-cell recruitment in the immune response cascade and remained for longer time. However, the tendency of Tregs and B-cells recruitment was not statistically significant (data not shown; Fig. 4).

Regarding apoptotic markers, we observe a high expression of active caspase 3 and granzyme B at early time-points (P = 0.15 and P = 0.06, respectively, compared to control), whereas the levels of the antiapoptotic marker Bcl-2 were lower in group 2 than in group 1, reaching lower levels than in control samples. This apoptosis might be induced to stop the immune response from all recruited inflammatory cells once the tumour is cleared. (Figs 4 and 5). The immune cells undergo apoptosis once finished their role, so after the clearance of the tumour.

DISCUSSION

We demonstrate the efficacy of ingenol mebutate gel 0.05% under occlusion, especially for superficial BCC, where 80% of the cases achieved complete remission which was maintained at 2 years of follow-up.

Published clinical trials of superficial BCC treated with ingenol mebutate gel show similar or lower response rates: in a phase II randomised study, 60 patients with superficial BCC were treated with varying dosing regimens and concentrations of either ingenol mebutate gel or the gel vehicle. Significant histologic clearance at 85 days post-treatment was observed in 65% of patients when ingenol mebutate gel 0.05% was applied for two consecutive days (P < 0.05).5 In a phase I/II clinical study, 82% of patients who failed or refused conventional treatment for superficial BCC achieved a complete clinical response one month after Euphorbia peplus treatment and 57% continued after a mean follow-up of 15 months.5
Safety profile in those trials was favourable with mild to moderate adverse events including erythema, flaking, scaling, erosion and ulceration. Local skin reactions appeared to be dose-dependent and developed rapidly after application (within the first day), peaked in severity shortly after the end of the treatment (1 week) and returned to near baseline levels within 2 weeks.\(^5\)

**Figure 1** Local skin reaction after ingenol mebutate gel treatment. (a) Nodular BCC on the lower limb. (b) Clinical reaction at Day 3. Note the extending erythema with a blister in the center which produced severe pain to the patient. (c) Clinical appearance 50 days from treatment initiation (group 2). (d) Clinical appearance after 6 months of follow-up; no clinical sign of recurrence was detected but a residual hyperpigmentation and a scarring area were observed.

**Figure 2** Follow-up of a patient treated with ingenol mebutate gel. (a) Superficial BCC on the back at baseline. (b) An erosive patch after blister rupture was observed 10 days after treatment. (c) Residual erythema after 2 months of treatment initiation. (d) Residual scar after 6 months of follow-up.
Figure 3  Representative samples showing different degrees of necrosis according to the time-point when biopsies were collected. (a,b) Relevant epidermal and dermal necrosis appearing in certain areas reaching the middle dermis after 3 days from treatment initiation with ingenol mebutate gel were observed. Image (a) emphasises a huge sub-epidermal blister while image (b) emphasises the intense neutrophilic infiltrate accompanied by an *important inflammatory cell infiltration mainly composed by polymorphonuclear cells.* (c) Reduction of necrosis and the intensity of the immune cell infiltration were observed 10 days after treatment initiation with ingenol mebutate gel. Some tumour areas were still present. (d) Day 30 post-treatment biopsy. A residual immune cell infiltration and fibrosis tissue were observed. Original magnification: (a, b, c) HE x4; (d) HE x10

Figure 4  Results of all analysed markers. ANOVA statistical test, *P < 0.05

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There are some case reports and short patient series with BCCs, treated with different ingenol mebutate gel posology (0.015 or 0.05%), time length and number of cycles with complete clearance outcomes. The main difference between our study and those reported in the literature is the use of ingenol mebutate gel under occlusion; it may be the reason why we obtained higher rates of complete response. High efficacy of ingenol

Figure 5 Representative samples showing immunohistochemistry results in the control samples and in the different groups. (a) Control samples. (b) Group 1 (early immune response). (c) Group 2 (late immune response)
mebutate gel 0.05% under occlusion has already been shown in a small series (n = 7) of superficial BCCs on the trunk.10

We would like to highlight that 2/5 patients that were excluded from the study analysis due to incorrect treatment compliance had persisting BCC confirmed by biopsy. However, some weeks later, when those skin lesions were about to be removed by surgery, no tumour was detected. In some cases, the immune system may take longer to completely destroy the tumour since patients that were considered as non-complete responders actually were complete responders. We hypothesise that this finding may correlate with our observation that some cytotoxic cells remained at the tumour site even 50 days after treatment initiation, meaning that some cytotoxic activity may be present at the late immune time-point response, even after local skin reactions resolution.

Ingenol mebutate gel quickly produces a non-specific epidermal and superficial dermal necrosis leading to a release of the cytosolic components within the tumour site, acting as antigens triggering the activation of both the innate and the adaptive immune responses. This papilla dermal necrosis produced by occlusive treatment may be responsible for scarring in some patients and may also be the reason why we observed longer duration of local skin reactions in our patients. It is important to note that six patients with medium or low local skin reaction score did not show tumour regression, suggesting that an incomplete immune response is unable to induce tumour clearance.

Regarding cell recruitment at tumour site, in terms of innate response we observed a high neutrophilic infiltration reinforced by macrophages (P = 0.05) and NK cells (even if not statistically significant).

In terms of adaptive immune response, we observed an important recruitment of T cells (lymphocytes): CD4+ (P = 0.016) and CD8+ (non-statistically significant) at early time-points. Interestingly, CD4+ T cells return to control levels, whereas the recruitment of NK cells, CD8+ T cells, and the presence of TIA-1 show that some cytotoxic activity may remain within the tumour site at late immune response time-points.

There is also a high expression of active caspase 3 and granzyme B at early time-points (10 days after treatment initiation; data not shown), at the same time when we observed an increase of Tregs cells; a T-cell subpopulation able to downregulate and suppress T effector cells by several mechanisms, including apoptosis. The real mechanism of action of Tregs cells has not been fully elucidated,15 but Tregs cells may induce the apoptotic activity to decrease the recruitment and activity of T effector cells (CD4+ and CD8+) as we observe.

Similar results were obtained in a phase 1 randomised trial in which 20 patients with actinic keratosis were treated with ingenol mebutate gel 0.05%. One day after treatment initiation, inflammatory cell infiltration was dominated by CD4+ and CD8+ T cells as well as neutrophils and macrophages within both dermis and epidermis. Fewer changes were observed for CD20+ B-cells which might be produced later, as we observed in our study. Apoptosis (caspase 3) was also found at early time-points after treatment.16

Emmert and colleagues16 demonstrated that ingenol mebutate gel induced a strong inflammatory response in uninvolved skin. These authors also show that short-time immune responses were more pronounced in actinic keratosis lesions compared with uninvolved skin. For instance, the density of CD4+ T cells in the dermis was significantly higher in ingenol mebutate gel-treated keratosis. Finally, these authors propose that a sort of ‘pre-conditioning’ of actinic lesions may contribute to the tumour-preferential activity of ingenol mebutate gel. In agreement with these authors, we have observed infiltration of CD5 T cells in surrounding healthy skin of several samples. Then, we assumed that ingenol mebutate gel triggers an immune response in the treated region either tumour or uninvolved skin. However, we did not collect uninvolved skin systematically, and then, we cannot establish a comparative between BCC and uninvolved skin.

Overall, we found that ingenol mebutate gel was an effective drug to treat BCCs, especially the superficial subtype. Necrosis reaction accompanied by both innate and adaptive immune cells recruitment mainly occurring at early response time-points after treatment initiation may be responsible for the observed efficacy rates. Cytotoxic markers observed at late response time-points may ensure complete response by destroying residual tumour cells.

ACKNOWLEDGEMENTS

This was an investigator-initiated study funded by LEO Pharma. The design of the study and analysis and interpretation of study results are exclusive responsibility of investigators. Work was supported by IRBLleida Biobank (B.0000682) and PLATAFORMA BIOBANCOS (PT13/0010/0014). We would like to thank Lidia Rodriguez, Scientific Affairs LEO Pharma SA for support and medical writing.

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Supporting Information

Additional Supporting Information may be found online in Supporting Information:

Fig S1. Study design (timing and assessment visits).

Table S1. Primary antibodies used in the study to evaluate inflammatory cell infiltration in groups 1 and 2 after treatment with ingenol mebutate gel.

Table S2. Clinical and pathological data from the 16 patients with BCC included in the study.