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INVESTIGATIVE REPORT

Stimulatory Effect of Boron and Manganese Salts on Keratinocyte Migration

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Keratinocyte proliferation and migration are essential for the reconstruction of the cutaneous barrier after skin injury. Interestingly, thermal waters which are rich in trace elements (e.g. boron and manganese), are known to be able to improve wound healing. In order to understand the mechanism of action of this effect, our study investigated the *in vitro* modulation of keratinocyte migration and proliferation by boron and manganese salts, which are present in high concentrations in a thermal water (Saint Gervais). Our *in vitro* study demonstrated that incubating keratinocytes for 24 h with boron salts at concentrations between 0.5 and 10 µg/ml or manganese salts at concentrations between 0.1 and 1.5 µg/ml accelerated wound closure compared with control medium (+20%). As this acceleration was not related to an increase in keratinocyte proliferation we suggest that boron and manganese act on wound healing mainly by increasing the migration of keratinocytes. *Key words: epidermal cell; trace elements; thermal water; wound healing.*

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Wound healing is essential for the reconstruction of the cutaneous barrier after skin injury (1). This phenomenon includes several steps which overlap in time: coagulation, inflammation, new tissue synthesis and maturation (2). The epithelialization involves two keratinocyte phenotypes: migration and proliferation, which occur simultaneously during the formation of the neo-epidermis. Previous clinical and scientific studies have demonstrated the favourable influence of trace elements on wound healing. Benderdour et al. (3, 4) have demonstrated *in vitro* the effect of some boron compounds on wound healing by their action on extracellular matrix (ECM). Indeed, a 3% boric acid solution decreases the intracellular concentrations of proteoglycans and collagen in chick embryo cartilage *in vitro*. This phenomenon is associated with an increase in the release of these ECM macromolecules in culture medium. Boric acid also stimulated the activity of intracellular and extracellular proteases (3, 5). The role of manganese in wound healing has also been investigated. Bang & Dashti (6) observed a significant increase in serum manganese of 40 patients with hypertrophic or keloid scars. Furthermore, Barrat et al. (7) demonstrated a beneficial effect of manganese and copper salts on the healing process of breast chap, and Tenaud et al. (8, 9) demonstrated that treatment of normal human keratinocytes *in vitro* with manganese induced integrin expression during both the proliferative and the differentiated states.

Interestingly, thermal waters that are rich in boron or manganese, such as Saint-Gervais thermal water, are supposed to improve wound healing. These waters are sometimes proposed by dermatologists as a complementary treatment for ulcers or acute and chronic wounds to improve the wound healing. The aim of this study was to investigate the *in vitro* effect of boron and manganese on the migration and proliferation of keratinocytes, to clarify the potential role of these trace elements in the efficacy of thermal waters in wound healing.

MATERIALS AND METHODS

Normal human keratinocyte culture

Human keratinocytes were isolated from children's foreskin after incubation with trypsin 2× overnight at 4°C. Epidermal cells were resuspended and expanded in keratinocyte serum-free medium (KSFM) (Gibco BRL, Cergy-Pontoise, France) containing 5 ng/ml epidermal growth factor (EGF) (Gibco), 25 µg/ml bovine pituitary extract (BPE) (Gibco), 2% penicillin/streptomycin and 1% fungizone at 5% CO_2 and 37°C.

At subconfluence after two passages, cells were resuspended and distributed in Permanox Lab-tek 1-well chambers (Polylabo, France) at a density of 150,000 cells/well or in 24-well plates at a density of 15,000 cells/well in keratinocyte basal medium (KBM) (Promocell, Heidelberg, Germany) containing 0.1 ng/ml EGF, 0.4% BPE, 0.5 µg/ml hydrocortisone, 5 µg/ml insulin, 0.09 mM Ca^{2+}, 50 µg/ml gentamicin and 50 ng/ml amphotericin B. Twenty-four hours before addition of trace elements, culture medium was replaced by KBM without hydrocortisone.

Trace elements

Boron and manganese were diluted in KBM without hydrocortisone and incubated with keratinocytes for 48 h. Culture medium alone was used as a control. The concentrations of sodium borate and manganese gluconate used on

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keratinocytes, were successively: 0.5, 1, 5, 6.5 and 10 μg/ml for boron element and 0.1, 0.2, 0.3, 0.6 and 1.5 μg/ml for manganese element. The concentrations of these two trace elements in Saint-Gervais thermal water were respectively 6.5 μg/ml for boron and 0.3 μg/ml for manganese. Boron and manganese salts were kindly provided by Labcatal, France.

Migration assay

The migration of keratinocytes was studied by the Scratch-Assay method (10) and evaluated in triplicate on keratinocytes from three different donors. Two hours before the formation of the artificial gap, confluent keratinocytes cultured in Lab-tek 1-well chambers were treated with mitomycin 4 μg/ml to stop the proliferation of keratinocytes. A 2-mm wide artificial gap was created and the restoration of the gap was assessed with a microscope at 0, 24 and 48 h after incubation with control medium (KBM) or with the different concentrations of boron and manganese. Results were expressed as the average percentage of gap restoration by migrating keratinocytes in three independent experiments. In each experiment, the different culture conditions were compared by the Student's t-test for paired values.

Proliferation assay

The keratinocyte proliferation was analysed by incorporation of ^{3}H-thymidine, 24 h after incubation of keratinocytes with boron or manganese at the different concentrations or control medium. For each concentration of trace element, proliferation was evaluated in triplicate for the three individual keratinocyte cultures obtained from three foreskins. One μCi of ^{3}H-thymidine was added to each well of multidish 24 wells, 8 h before the end of the incubation. Radioactivity incorporated by cells was counted with a Wallac 1409 Liquid Scintillation Counter and cpm was employed as the unit.

Statistical analysis

The results were expressed as the mean $\pm$ SD of three replicates of keratinocytes isolated from three different donors. Data were compared to controls, consisting of keratinocytes treated in the same manner as the test samples but without trace elements. Student's t-test was used to determine statistically significant differences.

RESULTS

Migration (Scratch-assay method)

In control medium, the initial gap (Fig. 1a) was recovered at 67.5% ($\pm$12.5%) after 24 h and at 72.5% after 48 h by migrating normal human keratinocytes.

Incubation with boron salts stimulated the restoration of the gap (Fig. 1b, Fig. 2a). At a boron concentration of 0.5 μg/ml in the culture medium, the gap recovered 87.5% ($\pm$8.8%) after 24 h (representing an increase of 20% compared with the control) and the recovery was complete at 48 h. Increasing the boron concentration to 1 μg/ml stimulated the recovery to 97.6% ($\pm$1.7 after 24 h), whereas with a boron concentration of 5 μg/ml, the recovery of the gap was complete at 24 h.

For incubation with manganese salts (Fig. 1c and 2b), the results were similar for all the concentrations (0.1, 0.2, 0.3, 0.6 and 1.5 μg/ml) at both 24 h and 48 h, with a nearly complete recovery of the gaps. The recovery rates were between 80 and 95%, i.e. 20% more than in control medium.

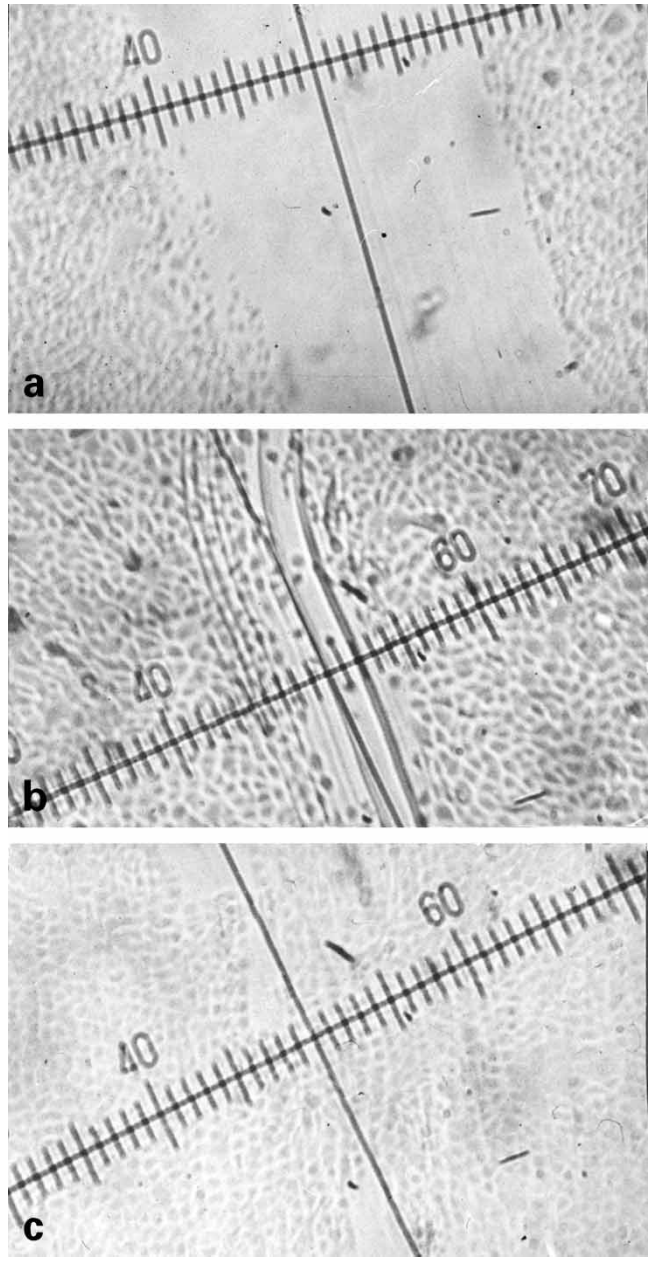

Fig. 1. Recovery of the gap (Scratch-assay) in mitomycin-treated keratinocytes cultures incubated with control keratinocyte basal medium (a), boron 6.46 μg/ml (b) and manganese 0.3 μg/ml (c) for 24 h. Magnification $\times$120.

Proliferation

The proliferation was tested by cellular incorporation of ^{3}H-thymidine, which did not reveal any increase after incubation for either 24 h or 48 h with increasing

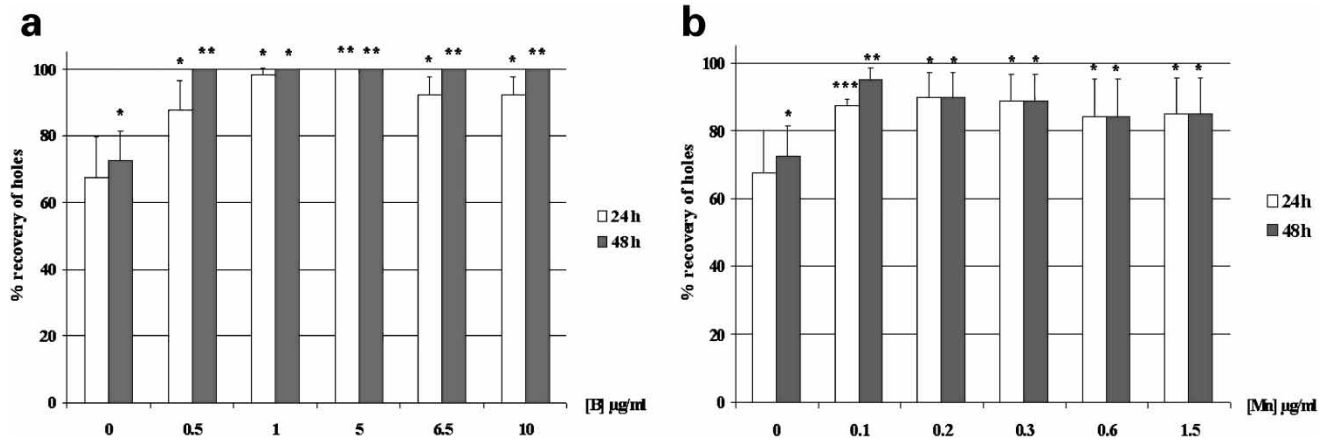

Fig. 2. Effects of increasing concentrations of boron (a) and manganese (b) on *in vitro* keratinocyte migration (Scratch-assay) measured as the percentage of wound recovered after 24 h and 48 h, respectively. Results are the average of three distinct experiments on keratinocytes from three different donors. $*p<0.05$; $**p<0.01$; $***p<0.001$.

concentrations of boron (Fig. 3a) or manganese (Fig. 3b).

DISCUSSION

Our study demonstrates that boron and manganese salts are able to stimulate the epithelial wound healing *in vitro*. Indeed, after incubation for 24 h, both boron at concentrations between 0.5 and 10 µg/ml, and manganese, at concentrations between 0.1 and 1.5 µg/ml, promoted a recovery of the gap by the keratinocytes that was 20% superior to control medium. With manganese, the stimulation of keratinocytes was obtained with the lowest concentrations. Our results are similar to those obtained in previous studies for the migration of keratinocytes (8, 9) suggesting a specific sensitivity of keratinocytes to manganese, but the exact mechanism of action of manganese remain to be determined. Furthermore, our study reveals that this acceleration of the wound healing is not related to an increase in keratinocyte proliferation, but rather a stimulation of keratinocyte migration. These results are in agreement with those of Barrat et al. (7), who reported a beneficial effect of manganese and copper salts on the healing process of breast chaps. Furthermore, Blech et al. reported that a 3% boric acid used as an antiseptic in the clinical treatment of scars improved the healing process (11, 12). Interestingly, in previous studies (8, 9) we demonstrated that the treatment of normal human keratinocytes *in vitro* with manganese salts at a concentration of 0.1 µg/ml induced expression of α_3, α_1 and α_v sub-units of integrins by both pro- liferating and differentiated keratinocytes. As regards our results on wound healing with boron, we also hypothesize that this trace element acts on keratinocyte migration via induction of integrin expression Other studies (5) have demonstrated that a 3% boric acid solution increases the secretion of collagen, allowing the formation of new ECM. Thus boron may act on wound healing at both the epidermal and dermal levels.

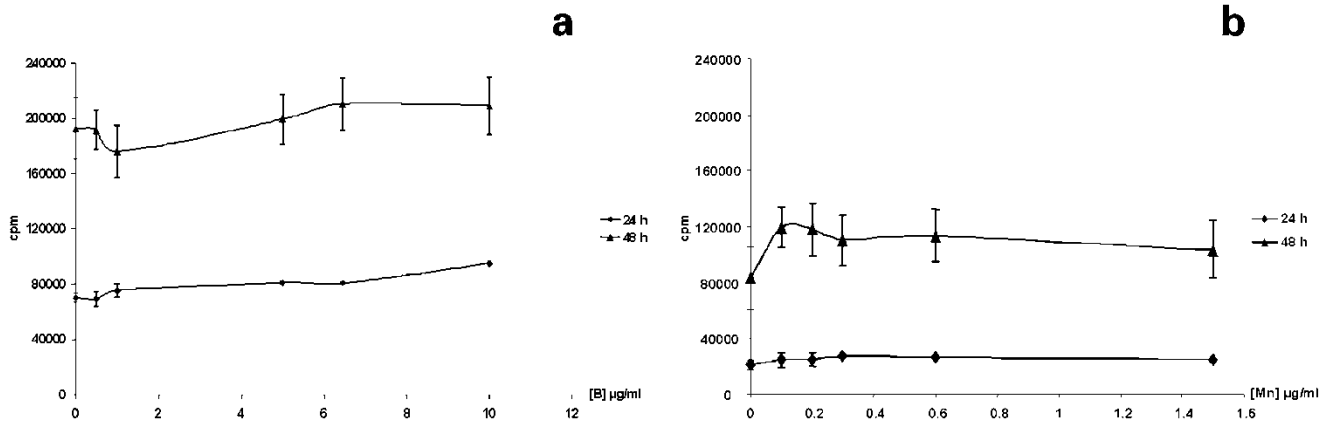

Fig. 3. Effects of increasing concentrations of boron (a) and manganese (b) on keratinocyte proliferation studied by incorporation of ^{3}H-thymidine after 24 h and 48 h. Results are the average of three distinct experiments on keratinocytes from three different donors. No statistically significant differences versus baseline values.

In conclusion, our study provides new arguments to explain why a thermal water rich in boron and manganese can stimulate wound healing in patients with skin ulcers.

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