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Protective Effect of Zinc on Keratinocyte Activation Markers Induced by Interferon or Nickel*

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Zinc therapies exert beneficial effects in several cutaneous pathologies through their antiinflammatory properties, but target cells and mechanisms of action are still uncertain. We wondered whether markers of the keratinocyte activation state, such as the expression of immune surface antigens (ICAM-1 and HLA-DR) and the production of TNF-α, frequently detected in inflammatory reactions, may be reduced by zinc. For this purpose, we used normal human keratinocytes derived from plastic skin surgery and cultured in low-calcium medium (MCDB153). We studied the effects of $ZnSO_4$ (12.5 to 50 μM) alone or in combination with IFN-γ (5 U/ml), a mediator of inflammation produced by activated T-cells, or nickel (5–10 μg/ml), a sensitizing metal hapten.

Using FACS analysis, we showed that the combination of zinc with nickel or the addition of $ZnSO_4$ 24 h before IFN-γ or $NiSO_4$ treatments reduced ICAM-1 expression on the keratinocyte surface ($p < 0.01$). However, zinc did not modify the IFN-γ induced expression of HLA class II antigen on keratinocytes. Zn^{2+} could also reduce the TNF-α secretion of keratinocytes stimulated by IFN-γ or Ni^{2+} during 48 h.

Taken together, these data indicate that zinc can directly reduce some keratinocyte activation markers frequently observed in vivo; this action may be involved in the antiinflammatory effect of Zn^{2+}-associated therapies in cutaneous inflammatory reactions. ***Key words: ICAM-1; TNF-α.***

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Zinc, a necessary nutrient for man, has been proven to play an essential role in skin physiology. Indeed, an adequate zinc level is necessary during migration, proliferation and maturation of the epidermis (1, 2) and for maintaining cutaneous integrity (collagen synthesis and fibroblast proliferation) (3). Moreover, this metal plays a role in the key enzymes involved during the synthesis of nucleic acids and proteins (4), and it also protects against the formation of free radicals (5, 6).

As patients with dermatitis herpetiformis, acne, pustular psoriasis and Darier's disease have a low epidermal zinc concentration, it has been suggested that supplementation of zinc, in regard to its skin action, might be of value in such patients (7). Thus, some cutaneous zinc therapies have been proposed and some beneficial effects have been observed (8). However, the exact mechanisms of action are still uncertain; an effect on neutrophils has been reported (inhibition of chemotaxis function) (9, 10), but data concerning keratinocytes are scarce.

In most inflammatory reactions the keratinocytes are activated (11, 12); they can abnormally express immune-associated surface antigens such as HLA-DR or the intercellular adhesion molecule-1 (ICAM-1), a ligand of the leucocyte function-associated antigen LFA-1 (13–15); they can also produce a large panel of cytokines, and among them tumor necrosis factor-α (TNF-α), which is widely involved in the pathophysiology of the skin (16).

The aim of the present study was to assess the effect of zinc in combination with interferon-γ (IFN-γ), a cytokine synthesized by activated T-cells, or nickel, a sensitizing metal hapten, on the keratinocyte expression of ICAM-1, HLA-DR and the production of TNF-α. For this purpose, keratinocytes cultured in low calcium-defined medium (MCDB153) were appropriately stimulated. Cell surface molecules were analysed by a fluorescence-activated cell sorter (FACS) and TNF-α production was quantified by an enzyme-linked immunosorbent assay (ELISA).

MATERIAL AND METHODS

Keratinocyte cultures

Normal human keratinocytes derived from plastic surgery were used for this study. The cells were seeded into and grown in either 25-cm^3 flask-bottles or Lab-Tek chamber slides (A/S Nunc, Roskilde, Denmark) with low calcium (0.1 mM $CaCl_2$)-defined medium (MCDB 153, Irvine Scientific, Irvine, CA, USA), supplemented with 10 ng/ml epidermal growth factor, 5 μg/ml insulin, 0.1 mM ethanolamine, 0.1 mM phosphoethanolamine and 2% of non-essential amino acid solution (Sigma Chemical Co, St Louis, Mo, USA).

Only subtoxic concentrations of the different compounds were used, and we assessed keratinocyte viability by trypan blue incorporation (viability >95%) and changes in cell morphology and cell detachment by light microscopic observations.

The cells were in an exponentially growing step when stimulations with nickel sulfate ($NiSO_4 \cdot 6H_2O$; Sigma) at 5 to 10 μg/ml, IFN-γ Amersham, Les Ulis, France) at 5 or 80 U/ml, TNF-α (Genzyme, Cambridge, Mass., USA) at 0.15 μg/ml (2500 U/ml) or zinc sulfate ($ZnSO_4.7H_2O$; Sigma) at 12.5 to 50 μM were applied. After different times of stimulation (24 h and 48 h), culture supernatants were collected, centrifuged at 10 000 g for 15 min and stored at −70°C. The flask bottles were then washed in phosphate-buffered saline (PBS) and kept at −70°C until cell extraction.

Three independent experimental series were performed.

Fluorescence-activated cell sorter

All staining procedures were carried out on ice. PBS were used for antibody dilution and washing.

Keratinocytes, either untreated or treated with $NiSO_4$, IFN-γ or $ZnSO_4$, were detached from culture flasks by trypsin treatment. After trypsin inactivation (with addition of fetal calf serum), the cells were pelleted by centrifugation (400 g for 10 min), washed and incubated 45 min with the anti-ICAM-1 monoclonal antibody (1:20) (Immunotech S.A., Marseille, France) or the anti-HLA-DR monoclonal antibody

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Table I. *Level of ICAM-1 expression on keratinocytes after 24 h and 48 h of treatment with $ZnSO_4$ or IFN-γ*

	24 h		48 h	
	% (a)	IR (b)	% (a)	IR (b)
Control	<1	2.9±0.7	<1	3.0±1 0
$ZnSO_4$ 25 μM (c)	<1	2.7		
$ZnSO_4$ 50 μM	<1	6.9±3.7	<1	3.6±1.0
IFN-γ 5 U/ml	94.6±3.4	65.5±7.6	90.8±6.4	138.9±25.3
$ZnSO_4$ 50 μM +IFN-γ 5 U/ml	94.1±4.7	83.2±7.6 (*)	90.2±6.8	219.8±33.3 (*)
$ZnSO_4$ 50 μM (24 h) before IFN-γ 5 U/ml	90.8±5.9	29.6±6.5 (**)	83.6±2.2	67.3±14.0 (*)
$ZnSO_4$ 25 μM (24 h) before IFN-γ 5 U/ml(c)	95.0	47.1		

(a) percentage of positive cells
(b) mean fluorescence intensity expressed on a logarithmic scale
(c) mean of two independent experiments
NB: Student's *t*-test
* $p<0.05$
** $p<0.01$

(1:20) (Becton Dickinson, Mountain View, San Francisco, CA, USA) in suspension at 10^6 cells per incubation. After unbound antibody had been removed by washing, cells were stained for 45 min with fluorescein isothiocyanate-conjugate (FITC) $F(ab')_2$ goat antimouse IgG (1:30) (Zymed Laboratories, San Francisco, CA, USA). As negative control, an unrelated isotype-matched monoclonal antibody was used in place of anti-ICAM-1 or anti HLA-DR antibody in the staining protocol. Cells were subsequently washed and fixed in 1% paraformaldehyde and suspensions were analysed using a FACSTAR plus flow cytometer cell

Table II. *Level of ICAM-1 expression on keratinocytes after 24 h of treatment with $ZnSO_4$ or $NiSO_4$*

	% (a)	IR (b)
Control	<1	2.9±0.7
$ZnSO_4$ 50 μM	<1	6.9±3.7
$NiSO_4$ 5 μg/ml	93.1±2.5	51.3±5.9
$NiSO_4$ 10 μg/ml	87.5±6.3	51.8±1.0
$ZnSO_4$ 50 μM+ $NiSO_4$ 5 μg/ml	86.7±8.2	11.4±3.2 (**)
$ZnSO_4$ 50 μM (24 h) before $NiSO_4$ 5 μg/ml	72.8±3.5	8.7±1.0 (**)
$ZnSO_4$ 50 μM+ $NiSO_4$ 10 μg/ml	92.6±3.6	11.2±0.5 (**)
$ZnSO_4$ 50 μM (24 h) before $NiSO_4$ 10 μg/ml	84.7±3.1	13.5±5.2 (**)

(a) percentage of positive cells
(b) mean fluorescence intensity expressed on a logarithmic scale
NB: Student's *t*-test
** $p<0.01$

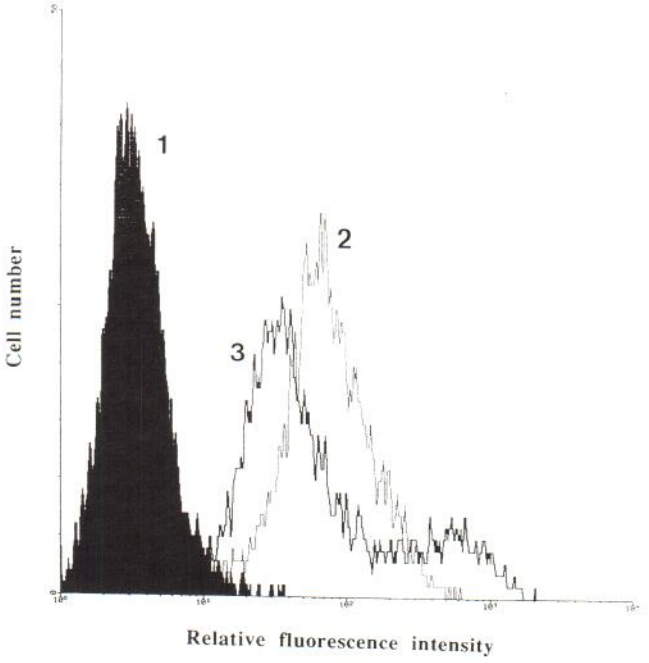

Fig. 1. Keratinocyte ICAM-1 expression analysed by FACS. Control keratinocytes (1). Keratinocytes were incubated for 24 h with 5 U/ml of IFN-γ (2) or $ZnSO_4$ (50 μM) 24 h before the addition of IFN-γ (5 U/ml) (3).

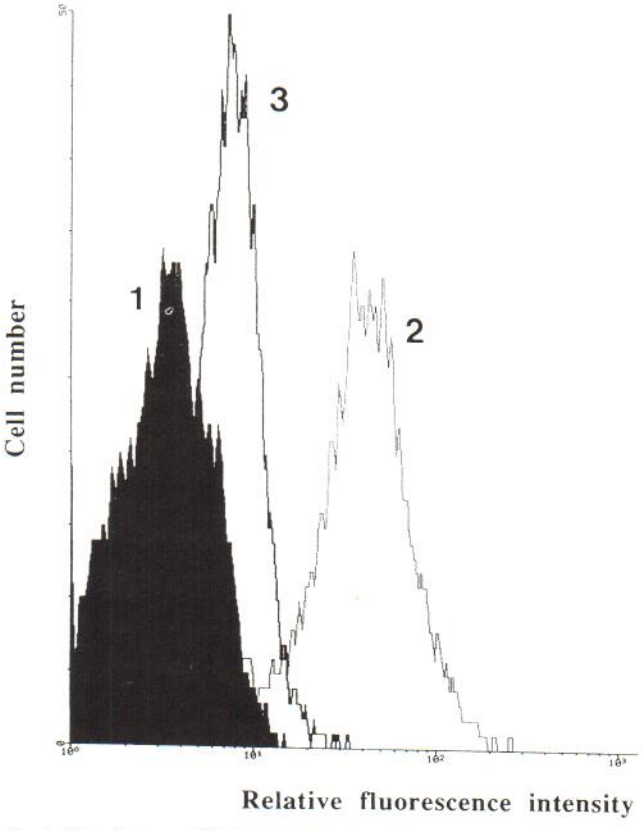

Fig. 2. Keratinocyte ICAM-1 expression analysed by FACS. Control keratinocytes (1). Keratinocytes were incubated for 24 h with 5 μg/ml of $NiSO_4$ (2) or $ZnSO_4$ (50 μM) and $NiSO_4$ (5 μg/ml) (3).

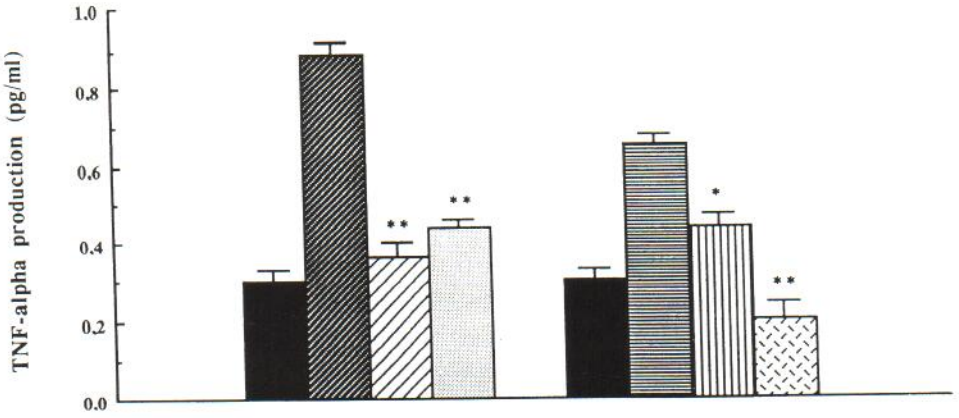

Fig. 3. Effect of $ZnSO_4$ on the production of TNF-α by normal human keratinocytes stimulated with IFN-γ or $NiSO_4$. The cells were treated with 50 μM of $ZnSO_4$ (■), 5 U/ml of IFN-γ (▨), 5 μg/ml $NiSO_4$ (▤), $ZnSO_4$ + IFN-γ (◰), $ZnSO_4$ 24 h before IFN-γ (□), $ZnSO_4$ + $NiSO_4$ (▥) or $ZnSO_4$ 24 h before $NiSO_4$ (▧). Range bars indicate the SEM. $*p<0.05$; $**p<0.01$.

sorter (type IV, Becton Dickinson) equipped with a 5 W argon laser operating at 250 mW at a wavelength of 488 nm. Green fluorescence was collected through a 530/20 nm bandpass filter. Data acquisition was triggered by cell size (forward versus 90° light scatter) in order to eliminate cell debris. The fluorescent signal was read on a logarithmic scale comprising four decades of log. For each assay, the value of the fluorescence intensity corresponded to the mean fluorescence intensity and the percentage of positive cells was determined by comparison with the negative control. For each sample, 10,000 cells were acquired and the data were analysed with the software LYSYS II (Becton Dickinson).

The statistic analysis (unpaired Student's *t*-test) was based on the results obtained from the three independent experimental series performed.

Cell extraction

Cell extracts were prepared by scraping PBS-washed monolayers into extraction buffer containing 10mM Tris-HCl pH 7.4, 2mM $MgCl_2$, 150 mM NaCl, 1% TritonX100, 2mM phenylmethylsulfonyl fluoride. Cell suspensions were briefly sonicated, centrifuged at 10.000 g for 10 min and then the supernatants were stored at −70°C. All procedures were performed on ice.

Total protein content was measured by the Bradford method (17).

ELISAs

TNF-α was measured by ELISA (British Biotechnology, Abingdon, UK) using 96-well microtiter plates according to the manufacturer's instructions. The level of TNF-α was calculated by using a standard curve obtained with hr-TNF-α (from 0.5 to 32 pg/ml). All determinations were performed in duplicate.

The results are expressed as pg/ml (± standard error of the mean (SEM)) and were analysed by an unpaired Student's *t*-test.

RESULTS

ICAM-1 and HLA-DR expression by cultured keratinocytes

With the subtoxic concentrations of $NiSO_4$ (5–10 μg/ml) and $ZnSO_4$ (50 μM) used, cell viability was always superior to 95%.

Flow cytometry analysis allowed us to quantify the ICAM-1 or HLA-DR antigen expression of stimulated cells (Tables I and II; Figs. 1 and 2).

We observed that the control or the zinc-treated normal human keratinocytes did not express ICAM-1 or HLA-DR antigen. IFN-γ (5 U/ml) induced an ICAM-1 expression at 24 h (94.4 ± 3.4% of labelled cells with a mean fluorescence intensity (MFI) of 65.5±7.6) which increased at 48 h (MFI: 138.9 ± 25.3) (Table I). Whereas the addition of 12.5 μM of $ZnSO_4$ 24 h before IFN-γ treatment had no effect, 25 μM and 50 μM reduced ICAM-1 induction by 28% and more than 52% ($p<0.01$), respectively. However, a combination of zinc and interferon increased ICAM-1 (by 27% at 24h and by 58% at 48h; $p<0.05$) (Table I).

Whereas 5 U/ml of IFN-γ was sufficient to obtain a significant expression of ICAM-1, the stimulation of keratinocyte HLA-DR antigen expression required a higher IFN-γ concentration (80 U/ml) for an induction at 24 h (49% positive cells with an MFI of 10.2) which increased at 48 h (81.3% positive cells with an MFI of 10.7).

Although $ZnSO_4$ reduced ICAM-1 expression induced by IFN-γ on cultured keratinocytes, it had no effect on MHC class II expression, whatever the moment of Zn^{2+} introduction (data not shown).

Nickel at 5 or 10 μg/ml also induced ICAM-1 expression in about 90% of keratinocytes at 24 h (MFI > 50). The combination of zinc (25 μM or 50 μM with nickel or the addition of $ZnSO_4$ 24 h before $NiSO_4$ treatments reduced the ICAM-1 expression, by 28% and more than 74% ($p<0.01$), respectively (Table II).

We also explored the ability of zinc sulfate to inhibit TNF-α induction of ICAM-1. We observed that 2,500 UI/ml of TNF-α (0.15 μg/ml) during 48 h is required to induce keratinocyte ICAM-1 expression (90% of labelled cells with a mean fluorescence intensity of 12). However, the combination of TNF-α with $ZnSO_4$ (50 μM) or the addition of $ZnSO_4$ (50 μM) 24 h before TNF-α stimulation, did not significantly reduce ICAM-1 expression (data not shown).

TNF-α production

With normal human keratinocytes no TNF-α production was detectable either in the cell extracts or in the supernatants at 24 h or 48 h.

When the cells were stimulated with either zinc, IFN-γ or Ni^{2+}, a release of TNF-α, in the supernatants was quantified by ELISA after 48 h stimulation (0.3 to 0.9 pg/ml) (Fig 3). $ZnSO_4$ induced a low amount of TNF-α whereas a significant release of the cytotoxin was observed with both $NiSO_4$ and IFN-γ.

The combination of zinc with interferon or nickel or the addition of $ZnSO_4$ 24 h before IFN-γ or $NiSO_4$ treatments reduced TNF-α production compared to that observed with the stimulants alone ($p<0.05$ and $p<0.01$) (Fig 3). Fifty micromolar of zinc sulfate was the minimal concentration required to reduce significantly TNF-α production induced by IFN-γ and nickel sulfate. Indeed, 25 μM of $ZnSO_4$ reduced only slightly

TNF-α production (from 0.9 to 0.8 pg/ml and from 0.65 to 0.6 pg/ml, respectively).

DISCUSSION

This study is the first to demonstrate that the zinc ions exert a direct effect on keratinocytes by reducing the activation state of cells through ICAM-1 expression and TNF-α production.

Among the immune-associated surface antigens of keratinocytes, ICAM-1 plays an important role. This adhesion molecule, frequently expressed by epidermal cells during local inflammatory reactions, facilitates the binding and the recruitment of T-lymphocytes and monocytes through LFA-1 a ligand of this glycoprotein (14, 18, 19). ICAM-1 may be induced on keratinocytes by cytokines (IFN-γ, TNF-α), phorbol ester (i.e. TPA) and allergens (paraphenylenediamine, urushiol, nickel) (13, 20–23). We confirmed that $NiSO_4$ induces an ICAM-1 expression on cultured keratinocytes which is transient (24 h), with a decrease at 48 h probably due to a shedding of the molecule, as previously shown (23).

In contrast, the induction of HLA-DR antigens on keratinocytes is restricted to IFN-γ effect, and the role of this molecule in the antigen presenting function of keratinocytes is limited (24, 25).

The cytotoxin TNF-α, a critical mediator in irritant and contact hypersensitivity reactions (26), is also produced by stimulated keratinocytes (27). The fact that TNF-α protein was undetectable in supernatants of untreated epidermal cells confirms that keratinocytes constitutively secrete little or no TNF-α (16, 27). The induction of this cytokine by nickel and IFN-γ is modest but is a measure of a direct effect of these agents on keratinocytes. As TNF-α has been demonstrated to be a stimulus for Langerhans cells to leave the epidermis in vivo, TNF-α-derived keratinocytes may account for this process (28).

The effects of Zn^{2+} on the modulation of ICAM-1 and HLA-DR antigen expression and on the production of TNF-α under IFN-γ or Ni^{2+} stimulation may be differently interpreted.

While the mechanisms involved in the protective role of zinc observed when it is added together with or before IFN-γ are uncertain, some suppositions can be made. It is well known that keratinocytes possess membrane-specific receptors for IFN-γ (29), and that zinc can readily bind to sulfhydryl groups present on membranes and then stabilize them (6, 30, 31). It is possible that the changes in membrane fluidity upon Zn^{2+} treatment alter the binding of interferon to its receptors and then reduce ICAM-1 induction and TNF-α production. Moreover, it has been reported that zinc ions can compete with certain compounds for receptor binding sites (32).

However, the increase of ICAM-1 expression when zinc is added at the same time as interferon is more difficult to interpret.

In fact, the lack of any effect of Zn^{2+} on HLA-class II antigen expression was unexpected. Indeed, zinc is known to modulate Ca^{2+}-dependent transmembrane function (33) and IFN-γ-induced HLA-DR antigen expression is a calcium/calmodulin event (21). It has been previously shown that calcium channel blockers, such as Verapamil, inhibit contact hypersensitivity reactions, but the precise mode of action remains uncertain (34).

In our study, we have to be aware that the action of Zn^{2+} on this second messager is probably slight and that the high concentration of IFN-γ (80U/ml) may abrogate the competitive effects of zinc to the calcium binding sites. It is also possible that more subtle regulation processes occur.

In the competitive role of zinc in nickel stimulation other mechanisms have to be proposed, since the effects of this sensitizing metal hapten are not mediated by cell surface receptors. Indeed, Ni^{2+} binds to and enters the cells directly and can stimulate the lipoxygenase pathway, with generation of hydroperoxides (35–38). Low levels of hydrogen peroxide are known to activate calcium dependent protein kinase C (PKC) (39, 40), which can in turn explain ICAM-1 induction, as recently reported for phorbol ester (21).

It is noteworthy that zinc protects against the formation of free radicals by several mechanisms : 1) by forming mercaptides with thiol groups of membrane proteins and, thereby, displacing other transition metal ions, such as iron, from binding sites where they promote free radical reactions; 2) by maintaining the activity and the structure of superoxide dismutase; and then 3) by maintaining and elevating the concentration of metallothioneines which, with their high content of sulfhydryl residues (rich cystein protein), scavenge hydroxyl and superoxide radicals (4–6, 41, 42).

Thus, the reduced expression of ICAM-1 and production of TNF-α observed when zinc is added before or during nickel stimulation might be explained by its role against lipid peroxidation.

The unability of Zn^{2+} to reduce ICAM-1 induced by TNF-α could be explained by the fact that a high concentration of TNF-α (2500 UI/ml or 0.15 μg/ml) during 48 h is required to induce a keratinocyte ICAM-1 expression (90% of labelled cells with a mean fluorescence intensity of 12). Moreover, this stimulation is weak compared to those obtained after IFN-γ or nickel sulfate treatments. Thus, as previously shown with IFN-γ-induced HLA-DR, in this case Zn^{2+} was inefficient to abrogate the effect of high doses of cytokines.

Taken together, these data indicate that zinc can reduce keratinocyte activation state; this action may be involved in the antiinflammatory effect of Zn^{2+} associated therapies. Moreover, we should emphasize the fact that zinc could have a preventive role in some cutaneous inflammatory processes, including allergic contact dermatitis.

Such an approach may also represent an interesting alternative to in vivo tests to evaluate putative protective effects of drugs on markers of epidermal inflammation.

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