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Exposure of Human Keratinocytes and Fibroblasts *In Vitro* to Nickel Sulphate Ions Induces Synthesis of Stress Proteins Hsp72 and Hsp90

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Exposure of the epidermis to nickel-containing compounds is one of the most common causes of contact allergy and may indicate that exposure of skin cells to nickel ions causes stress. In this study, cytotoxicity assays upon human keratinocytes and fibroblasts in monolayer culture indicated a 50% decrease in viability of both cell types at nickel sulphate concentrations in excess of 10^{-3} M. To investigate the possible induction of a stress response within these cell types, monolayer cultures were exposed to concentrations of nickel sulphate for 1 h, followed by ^{35}S-methionine labelling. Whole cell lysates were analysed by SDS-PAGE and immunoblotting with specific monoclonal antibodies, or by fluorography. For keratinocytes, increased synthesis of Hsp90 at concentrations of 10^{-5} M and above and induction of the stress-inducible Hsp72 at concentrations of 10^{-4} M and above were observed. For fibroblasts, increased induction of Hsp90 at all concentrations under test and a dose-responsive increase in Hsp72 synthesis were detected. These results indicate that both keratinocytes and fibroblasts react to the toxic effects of nickel ions by mounting a stress response involving the up-regulation of synthesis of key stress proteins. ***Key words: heat shock protein; human skin; nickel allergy; contact dermatitis.***

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Nickel contact allergy is one of the most common causes of contact hypersensitivity (1, 2) and is associated with long-term dermatitis. When pure nickel or nickel-containing alloys are placed in contact with human skin, nickel ions are released on to the surface of the skin due to the hydrating and leaching effects of human sweat. It seems likely that nickel-containing articles inserted into the puncture to prevent wound closure after ear-piercing can bring about the primary sensitization to nickel, leading to dermatitis on further exposure to nickel-containing articles (3).

Many physical and chemical insults are known to initiate the increased expression of certain key cellular proteins, known as stress proteins or heat shock proteins (Hsp). Such challenges include treatment with heat (4), cold (5), ethanol, amino acid analogues, heavy metals, reactive oxygen species and sulphydryl reagents (6). The cytotoxic effects of nickel ions upon several cell types, including human keratinocytes, have been documented by several groups (7, 8). Penetration of nickel ions into the epidermis in such a manner presumably constitutes a toxic challenge or stress to those exposed cells, similar to that observed *in vitro*.

Human keratinocytes and dermal fibroblasts within a monolayer culture obviously have no interaction with the immune cells such as T-lymphocytes or Langerhans' cells, which are likely to play an important part in nickel allergic contact dermatitis. In nickel-allergic individuals, however, a quantitative correlation between lymphocyte mitogenesis and exposure to nickel has been postulated (9), although considerable patient-to-patient variation is observed. Keratinocytes and fibroblasts *in vivo* are likely to play a key role in the activation and modulation of the inflammatory response. Recently, exposure of human keratinocytes to nickel salts *in vitro* has been reported to induce the increased synthesis of several well-characterized markers of inflammation, including intracellular adhesion molecule-1 (10), interleukin-1 and tumour necrosis factor-α (11). In the work reported here, the cytotoxicicity of nickel sulphate upon human keratinocyte and fibroblasts in monolayer culture was studied and the effect of non-cytotoxic concentrations of nickel sulphate on inducing a stress response in the 2 cell types was also investigated.

MATERIAL AND METHODS

Cell culture and cytotoxicity assays

Normal human keratinocytes and fibroblasts were isolated from skin samples obtained from normal healthy individuals, usually via circumcision. To enhance adherence, keratinocytes were grown upon lethally irradiated mouse embryo 3T3 feeder layers (12). Stock solutions of 1M nickel sulphate were prepared by dissolving the nickel salt in sterile water rather than in phosphate-buffered saline (PBS), in order to minimize precipitation of nickel phosphates during the experiments. This stock was serially diluted into culture medium and the pH adjusted to 7.4 before exposing cells, plated overnight in 96-well plates, to final concentrations of nickel sulphate ranging from 10^{-8} M to 10^{-2} M for 24 h. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were then undertaken (13).

Exposure of cells to nickel II sulphate

Before commencing experiments upon keratinocytes, any remaining 3T3 cells were removed by incubation in pre-warmed 0.02% ethylenediamine tetra-acetic acid in PBS for 2 min, followed by vigorous pipetting across the monolayer. Keratinocytes were then allowed to recover in normal culture medium for 24 h. Medium was removed from each flask and the cell monolayers treated with methionine-deficient Modified Eagle Medium (MEM) containing 5% serum with an appropriate concentration of nickel sulphate for 1 h. The medium was removed and the monolayers bathed in methionine-deficient MEM containing 0.1 mCi/ml ^{35}S-methionine (ICN, UK). Cell monolayers were then incubated for 2 h at a 37°C recovery temperature to allow synthesis of new proteins. After removal of excess labelling medium by washing 3 times with PBS, cells were harvested by scraping and centrifugation at 800 rpm, lysed in Laemmli's sample buffer (14) and boiled for 3 min. Total radioactivity for each ^{35}S-labelled sample was then determined (15).

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Analysis of stress protein synthesis

Samples were analysed by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) (14) in 8.5% polyacrylamide gels containing 0.1% SDS. Each well of the gel was loaded with identical numbers of counts per minute as determined by scintillation counting, usually about 100,000 counts per well. The protein bands were stained, destained, dried at 50°C for 1 h on gel-drying apparatus, then exposed to Fuji-X photographic film at −70°C for up to 48 h. Films were developed using an automatic developer. Alternatively, the proteins were transferred by Western blotting to nitrocellulose membranes (16). After saturating binding sites on the membrane overnight at 4°C in 5% non-fat dried milk and 0.2% Tween-20 in PBS, incubation was initiated at room temperature for 2 h with an appropriate (1:500) dilution of 1 of the following monoclonal antibodies (Bioquote, York, UK):

i. the rat 9D2 antibody specific for Hsp90;
ii. the mouse N27F3-4 antibody specific for both Hsp72 and Hsp73 proteins;
iii. the mouse C92F3A-5 antibody specific solely for Hsp72.

After removing excess unbound antibody with 3 washes of fresh PBS for 20 min each, the membranes were further incubated for 1 h at room temperature with a 1:50 dilution in PBS of horseradish-peroxidase conjugated with either anti-mouse IgG for Hsp72 and Hsp73 or anti-rat IgG for Hsp90, and the bands visualized by reaction with 3,3′-diaminobenzidine.

RESULTS

Cytotoxicity of nickel sulphate on keratinocyte and fibroblast monolayers, measured as a percentage of surviving cells relative to untreated controls over a concentration range from 10^{-8} M to 10^{-2} M, is illustrated in Fig. 1. For both cell types, only concentrations of nickel sulphate greater than 10^{-3} M caused cell survival ratios to fall below 50%. Using this result as a guide, it was considered that exposure of keratinocytes and fibroblasts to concentrations of 10^{-5} M, 10^{-4} M and 10^{-3} M nickel sulphate would be suitable for investigating stress protein synthesis.

After the radiolabelling period, extracted cellular proteins were resolved by one-dimensional SDS-PAGE and visualized either by autoradiography or by blotting on to nitrocellulose and stress protein detection by Western immunoblotting with specific monoclonal antibodies. Results displayed were representative of 2 distinct experiments, each performed with cells isolated from 2 distinct pieces of tissue. The autoradiographs and Western immunoblots for keratinocyte total cell lysates are depicted in Fig. 2. For keratinocytes, a slight increase in Hsp90 and a marked increase in total Hsp70 stress proteins was evident at 10^{-3} M nickel sulphate as shown by the autoradiograph. Increased synthesis of a protein of approximate molecular weight of 25 kD, possibly the stress-inducible Hsp28, was also clearly observed. An increase in the inducible Hsp72 was apparent at concentrations of 10^{-4} M and above. The more sensitive immunoblotting

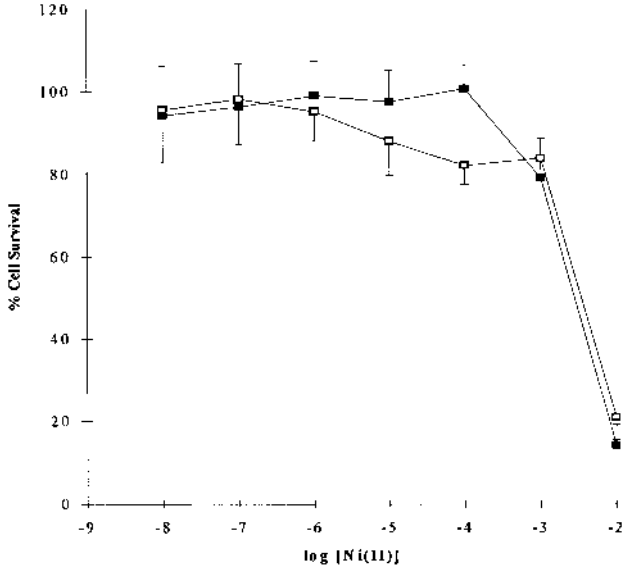

Fig. 1. Relative survival of keratinocytes and fibroblasts after treatment with varying nickel (II) concentrations. Keratinocytes (open symbols) and fibroblasts (closed symbols) were exposed to concentrations of nickel (II) sulphate for 24 h, incubated in 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in PBS for 4 h, then extracted with MTT lysis buffer. Results are expressed as a percentage of control cells containing 1% water only, and represent means (±SEM) from quadruplicate cell wells.

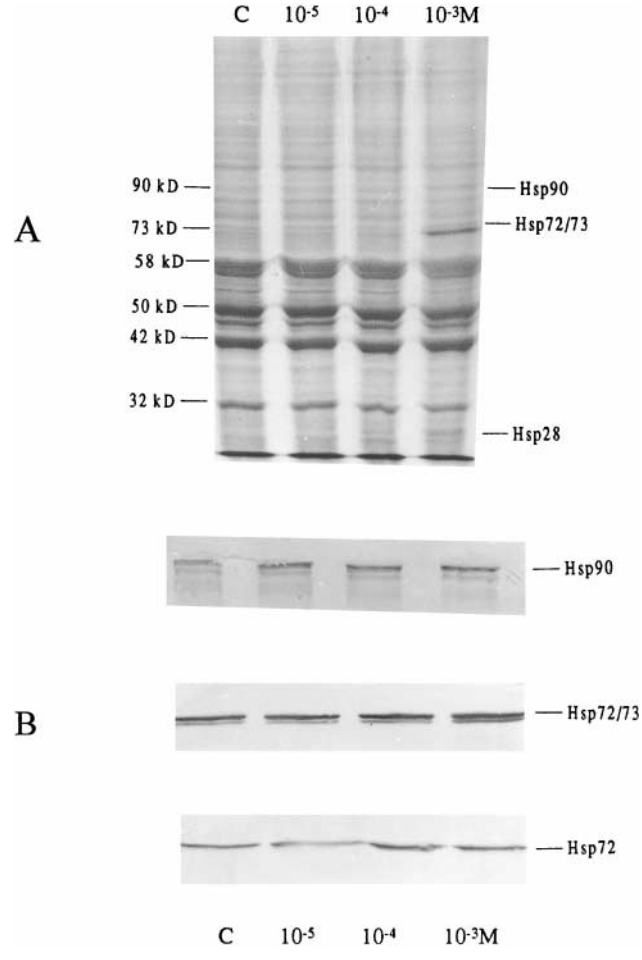

Fig. 2. Expression of stress proteins following nickel sulphate exposure in normal human keratinocytes. After removal of any remaining 3T3 fibroblast feeder layer, keratinocytes were treated for 1 h with the indicated concentration of nickel sulphate, metabolically labelled for 2 h with ^{35}S-methionine, then labelled proteins extracted and analysed by SDS-PAGE and autoradiography (A) or Western immunoblotting (B). Lane C: control, with no nickel sulphate addition.

procedure suggested that induction of Hsp90 was achieved even at the lowest exposure concentration of 10^{-5} M. No significant change in the amount of the constitutive Hsp73 was detected by either method.

For fibroblast monolayers, both the autoradiograph and the Western blot techniques (Fig. 3) showed a marked increase in synthesis of Hsp90 above control quantities at all nickel concentrations under test, with extremely strong and dose-responsive induction of Hsp72 protein evident in both methods. In addition, autoradiography detected increased synthesis of 2 other proteins, one of approximate molecular weight 60 kD, possibly the 58 kD stress protein, and one of 25-30 kD, which probably corresponded to the stress-inducible Hsp28. Increased synthesis of both these proteins was apparently induced at nickel concentrations of 10^{-4} M and above. Again, the results presented here were repesentative of 2 distinct experiments utilizing fibroblast cells isolated from 2 different pieces of tissue.

DISCUSSION

In this study, both human fibroblasts and keratinocytes were exposed to nickel sulphate concentrations between 10^{-9} M and 10^{-2} M, with concentrations only in excess of 10^{-3} M causing significant decreases in cell viability below 70% of control values as quantified by MTT assay. Apart from its obvious action as a cytotoxic agent as demonstrated by the results of several groups (7, 8, 17) and by the MTT assays presented in this study, nickel and certain nickel compounds are also considered to have carcinogenic potential (18). The mechanism for this, as yet, is unclear, but 2 main components are considered to contribute, firstly, the direct effect of nickel ions on DNA and indirect effects as a result of an inflammatory response, and secondly, the promotion of cell proliferation. However, water-soluble nickel compounds, such as the sulphate and the chloride, are not considered to be carcinogenic to experimental animals or in cell culture conditions. For this reason, nickel sulphate in preference to the sulphide or the frequently-used and highly irritant nickel chloride (19) was chosen for experimental purposes here.

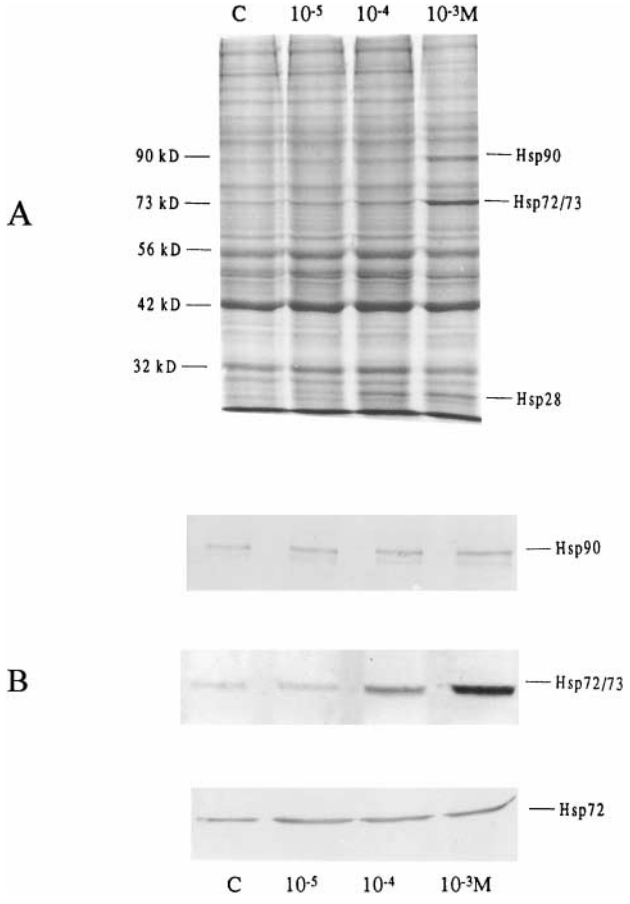

Fig. 3. Expression of stress proteins following nickel sulphate exposure in normal human fibroblasts. Cells were treated for 1 h with the indicated concentration of nickel sulphate, metabolically labelled for 2 h with ^{35}S-methionine, then labelled proteins extracted and analysed by SDS-PAGE and autoradiography (A) or Western immunoblotting (B). Lane C: control, with no nickel sulphate addition.

Exposure of keratinocyte monolayers to concentrations of nickel sulphate of 10^{-5} M and above was sufficient to cause increased synthesis of Hsp90 as demonstrated by the immunoblotting procedure, and a marked increase in Hsp72, the inducible form of the Hsp70 series of proteins, at the lowest concentration under test. This suggests that keratinocytes in culture, during exposure to nickel sulphate concentrations of the same order of magnitude as those utilized in allergic contact dermatitis patch-testing, synthesize stress proteins in the same manner as they would under more conventional stress conditions such as heat, cold or treatment with toxic chemicals, even though keratinocytes demonstrate no significant loss in viability at such concentrations. It is clear, however, that the changes observed in these experiments are a result of toxic, non-allergeneic effects of the nickel solutions, since no immune cells are present in the culture systems.

For human fibroblasts in culture, both the autoradiograph and Western immunoblotting techniques show clear increases in inducible Hsp72 and Hsp90 proteins at all concentrations of nickel sulphate employed in the study. This is perhaps unsurprising considering that human fibroblasts *in vivo* could be unlikely to experience significant environmental or physiological alterations to their dermal surroundings whilst integrity of the surrounding epidermis was intact. Exactly what pathological role, if any, dermal fibroblasts would play during such an insult is unclear.

The epidermis, together with the stratum corneum, is the front line barrier preventing and limiting the permeation and penetration of chemicals into the skin. Nickel penetrates the epidermis very slowly, with a reported lag-time of about 50 h (20). The metal ion does not readily enter animal cells, but may cross the cell membrane by using a magnesium ion transporter system, as has been demonstrated in lower organisms (21). The degree to which the epidermis of a previously sensitized individual will react to a particular nickel concentration varies, with provocation thresholds for the metal ion in petrolatum vehicle varying from 2.5% to as little as 0.01% (3). The highest nickel concentration tested for induction of synthesis of stress proteins, 10^{-3} M, corresponds to approximately 0.025%; hence we should assume that the concentrations under test could possibly exhibit a biological effect *in vivo*, a result of their toxic, rather than their allergeneic properties.

In addition to playing a crucial role in folding and targeting

of steroid receptors as part of a major heteromeric protein complex in the cytosol (22), Hsp90 has been shown to be associated with actin and the cytoskeletal filaments (23) and may use these organelles to target other cell proteins to the nucleus for transcriptional activation and initiation of protein translation. Nickel has been shown to interact and disrupt tubulin assembly and microtubule organization, enhancing the rate of decay of colchicine-binding sites due to its causing conformational changes of the protein (24). It has been suggested that the 28 kD stress protein Hsp28, whose up-regulation was also demonstrated by the experiments outlined here, may be intimately involved in cytoskeletal organization and assembly (25, 26).

To conclude, we have demonstrated that, upon exposure to nickel sulphate at concentrations capable of causing dermatitis *in vivo*, keratinocytes and fibroblasts *in vitro* up-regulate the synthesis of several key stress proteins. It is likely that, along with many other cellular molecular chaperones, Hsp90, Hsp73 and Hsp72 accumulate in the nucleus of keratinocytes and fibroblasts to aid in the recycling, refolding and prevention of hydrophobic aggregation of damaged nuclear proteins during and immediately after exposure to such an insult.

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