CLINICAL STUDY

The effect of nickel sulphate, potassium dichromate, cobalt nitrate and cadmium sulphate on the proteins of cellular contacts and actin skeleton of cultivated human keratinocytes

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Abstract

The authors tested the effects of known allergens, namely nickel sulphate, potassium dichromate, cobalt nitrate and cytotoxic cadmium sulphate on the proteins of cellular contacts (vinculin, talin, E-cadherin, desmoplaktin) and actin cytoskeleton (actin filaments) of cultivated human keratinocytes. The localisation of proteins of cellular contacts was detected by means of direct immunofluorescence. The authors have detected a decrease in, and destruction of cellular contact proteins and actin cytoskeleton after testing the effect of all allergens, while the most significant changes were detected in E-cadherin, vunkulin and actin filaments. Desmoplaktin and talin were less damaged. Potassium dichromate caused damage already in concentration of 1 µg/ml. A similar effect of the other two tested haptanes was brought about in concentration being 100-fold higher. Conclusion: the gained results indicate that the investigation of cellular contact proteins and actin cytoskeleton of cultivated human keratinocytes can possibly become a part of the testing of allergy-triggering potential of chemical substances. ***(Tab. 1, Fig. 4, Ref. 18.)***

Key words: nickel sulphate, potassium dichromate, cobalt nitrate, cadmium sulphate, proteins of cellular contacts, actin skeleton, cultivated human keratinocytes.

A number of chemical substances used currently in modern world grows geometrically. People exposed to many of these chemical substances react by irritation and allergic skin reactions. Such substances must be identified, excluded from manufacture and replaced by less harmful substances. The testing of their toxicity and potential of triggering the allergy can be performed by means of a series of tests, an important part of which include testing on animals. In the past years, an effort has been exerted to develop reliable *in vitro* systems detecting the toxic substances and allergens in order to restrict or substitute the experiments on animals. One of the prospective *in vitro* models of testing is the model of cultivated human keratinocytes.

Review

Four types of intercellular junctions are recognised – gap junctions, tight junctions, desmosomes and adherent junctions, out of which adhesion per se takes place in desmosomes and adherent junctions. In addition to the latter, basal keratinocytes are joined with the basal membrane by means of hemidesmosomes and focal adhesions (1, 2, 3) (Tab. 1).

The function of cellular junctions

The main function of cellular junctions resides in the mutual mechanical junction between individual cells and the junction of the latter with extracellular matrix. It has been found out that the proteins of cellular contact, especially those of cells and extra-

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Tab. 1. Review of cellular junctions of keratinocytes.

Junction	Transmembrane protein	Extracellular ligand	Proteins of intracellular plaque	Contact with cytoskeleton
Adherent junctions	Cadherins (E-cadherin)	Neighbouring cell cadherins	Catenins, vinculin, α-actinin	Actin fillaments
Desmosomes	Cadherins (desmoglein and desmocollins)	Neighbouring cell cadherins	Desmoplakine, plakoglobin	Intermediary fillaments
Focal contacts	Integrins	Extracellular matrix proteins	Talin, vinculin, α-actinin	Actin fillaments
Hemidesmosomes	Integrin	Extracellular matrix proteins	Desmoplakin -like proteins	Intermediary fillaments

cellular matrix, have more functions. The function of focal contacts in coincidence with the regulation of secretion has not been entirely clarified. Kawasugi et al (1995) investigated the regrouping of cellular skeleton and an increase in the expansion of cells during the secretion in a cellular strain of mouse mastocytes. The range of formation of focal contacts was correlated with the range of mediator secretion (4). Intercellular junctions can play a part in the transduction of signals (5). Cellular contacts and their junctions with the cytoskeleton are important in the regulation of cellular motility. Integrines participating in the junctions of keratinocytes with extracellular matrix intermediate or regulate various cellular processes including adhesion, organisation of actin cytoskeleton, cellular proliferation, apoptosis and cellular differentiation.

Cellular junctions of keratinocytes in the pathogenesis of contact eczema

Intact cellular contacts are responsible for the integrity of epidermis. Their gradual destruction contributes to the origin of spongiosis within the epidermis that is characteristic in the histological picture of contact eczema (6). Oxholm et al (1991) compared the differences between allergic and irritation reactions after epicutaneous tests. In addition to the release of cytokines, they compared also the histological pictures. Both types of skin reactions displayed various degrees of epidermal spongiosis, this however appeared in more accentuated allergic contact reactions (7).

E-cadherin represents the main adhesive molecule of adherent junctions of keratinocytes and intermediates the junctions of Langerhans cells (LCs) and keratinocytes (KCs) *in vitro* (8). Jakob et al (1999) demonstrated the focal accumulation of E-cadherin and co-localisation of β-catenin in the sites of contacts between KCs and immature (LC-like) dendritic cells (9).

After the exposure to antigen, LCs migrate from the epidermis into the lymphatic nodes where they initialise the primary immunity T-cell response. Schwarzenberg and Udey (1996) evaluated the expression of E-cadherin on mouse LCs after the exposure to trinitrochlorine benzene and discovered that TNCB induced an increase in class II MHC molecules (the main histocompatible system in mice) to LCs, and at the same time a decrease in the expression of E-cadherin between 12 and 48 hours after the application of allergen. The effect was specific as to the particular allergen. After the application of several contact irritating substances no changes took place in LCs. The authors assume that the increase in the expression of E-cadherin facilitates the migration of LC into the epidermis (10). It is presumed that the main parts in LCs' activation and mobilisation are played by IL-1 and TNF-α. A cutaneous injection of IL-1 and TNF-α decreased the expression of E-cadherins on *in situ* activated LCs. *In vitro,* IL-1 and TNF-α caused a rapid reduction of mRNA for E-cadherin and LC-like dendritic cells preceding the decrease in the expression of E-cadherin on their sufface (11).

In our work we have tried to find out whether the known allergens, namely nickel sulphate, potassium dichromate and cobalt nitrate cause changes in cellular contacts of cultivated keratinocytes in comparison to toxic cadmium sulphate. We have focused our attention on proteins, namely vinculin (part of intracytoplasmatic plaque of adhesive junctions and focal contacts), talin (part of intracytoplasmatic plaque of focal contacts), E-cadherin (transmembrane glycoprotein of adherent junctions), desmoplakin (a part of intracytoplasmic plaque of desmosomes) and actin filaments (actin cytoskeleton). It is known that damage to some part of the cellular contact (either transmembrane glycoprotein, part of intracytoplasmatic plaque, or its junction to the cellular skeleton) disturbs its function, particularly that of mechanical junction.

Material and methods

Nickel sulphate (NiSO4), potassium dichromate ($K_2Cr_2O_7$), cobalt nitrate ($Co(NO_3)_2$) and cadmium sulphate ($CdSO_4$) were acquired from Merck Company, Darmstadt, Germany, primary antibodies as anti-vinculin IgG, anti-talin IgG, anti-E-cadherin IgG from ICN Company, Meckenheim, Germany. Secondary an-

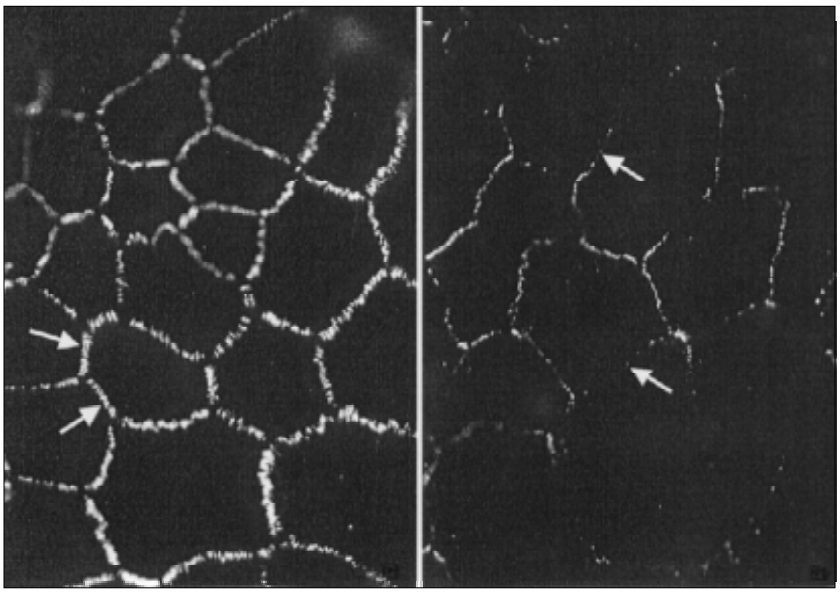

Fig. 1. The localisation of vinculin on cultivated keratinocytes (direct immunofluorescence). a) control keratinocytes, b) keratinocytes with potassium dichromate (concentration 2 μg/ml, 48 h).

tibodies conjugated with rhodamine (Rhodamine-conjugated goat anti-mouse secondary antibody) were supplied by Dianova Company, Hamburg, Germany (Phalloidin-rhodamine).

Cultivation of human keratinocytes

Normal human keratinocytes strains were gained from foreskins of small children, and initiated by means of a modified method of Rheinwald and Green (1975, 1977) (12, 13). Subcultures were cultivated in MCDB 153 medium with 0.1 mM Ca^{2+} serum free (14, 15). All experiments were performed by use of third and fourth passage cells. Prior to the initiation of experiments, the formation of intercellular contacts was induced by the increase in concentration of calcium ions within the cultivation medium (Ca^{2+} 1.1 mM). At the time of the initiation of experiments the cultures had several layers.

The concentrations of the tested substances were chosen after the assessment of toxicity by means of neutral red uptake assay according to Borenfreund and Puemer (1985) (16) – potassium dichromate 2 μg/ml (also tested in concentration of 1 μg/ml), nickel sulphate 10 μg/ml, cobalt nitrate 10 μg/ml, cadmium sulphate 2 μg/ml.

The localisation of cellular contact proteins was performed by the immunofluorescence.

Direct immunofluorescence

The cultivated keratinocytes were allowed to grow down to the surface of slides with 8 fields (ICN, Meckenheim, Germany) and incubated with the tested chemical substances (potassium dichromate 1 and 2 μg/ml, nickel sulphate 10 μg/ml, cobalt nitrate 10 μg/ml, cadmium sulphate 2 μg/ml) for the period of 3, 6, 12, 24, and 48 hours. After incubation, the preparations were fixed in 2 % formalin and permeabilised by 0.2 % Tritone X. Non-specific binding of anti-bodies was blocked by 2 % BSA (bovine serum albumin, ICN, Meckenheim, Germany). Then a two-hour incubation with monoclonal antibodies (anti-vinculin IgG, anti-talin IgG, anti-E-cadherin IgG, ICN, Meckenheim, Germany) and a 45–60-minute incubation with marked secondary antibodies (Rhodamine-conjugated goat anti-mouse secondary antibody, Dianova, Hamburg, Germany) followed at a laboratory temperature. In order to illustrate the actin cytoskeleton, we did not use monoclonal antibodies, but phalloidin conjugated with rhodamine (Phalloidin-rhodamine, Dianova, Hamburg, Germany) that specifically binds directly with actin. After rinsing, the preparations were covered by 40 % glycerine and covered by a slip. The cells were observed under microscope Zeiss Axiophot with epifluorescence illumination and photographs taken on film Kodak Tri-X pan film, Ultrafin SF (Tetenal, Norderstedt, Germany) at 400 ASA.

Results and discussion

The most significant changes in the observed proteins, namely their decrease and changes in their allocation were observed after incubation of keratinocytes with potassium dichromate in concentration of 2 μg/ml, a little weaker, however of the same character were the changes in coincidence with the concentration of 1 μg/ml. Significant was the damage of vinculin, E-cadherin and actin filaments, less changed was desmoplakine and talin. Nickel sulphate and cobalt nitrate in the given concentration did not evoke any changes in observed proteins. The dama-

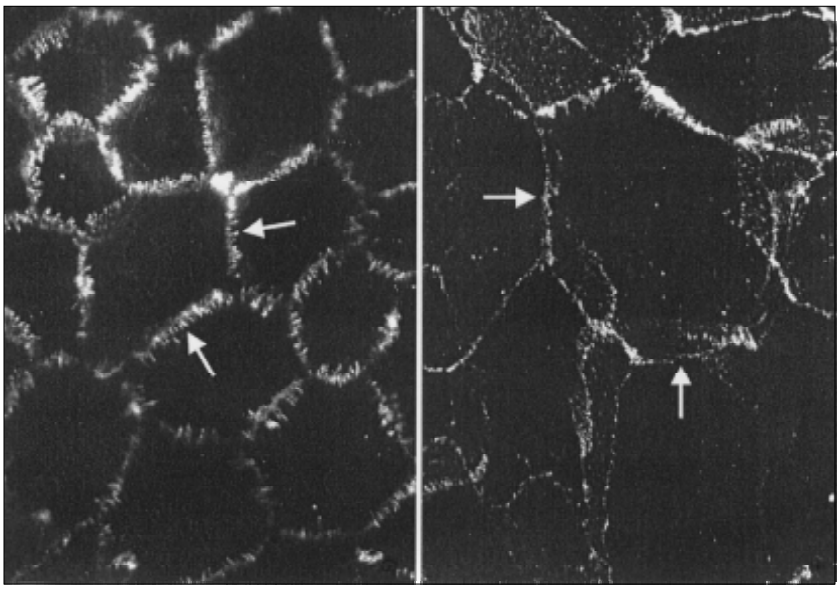

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Fig. 2. The localisation of E-cadherin on cultivated keratinocytes (direct immunofluorescence). a) control keratinocytes, b) keratinocytes with potassium dichromate (concentration 2 µg/ml, 48 h).

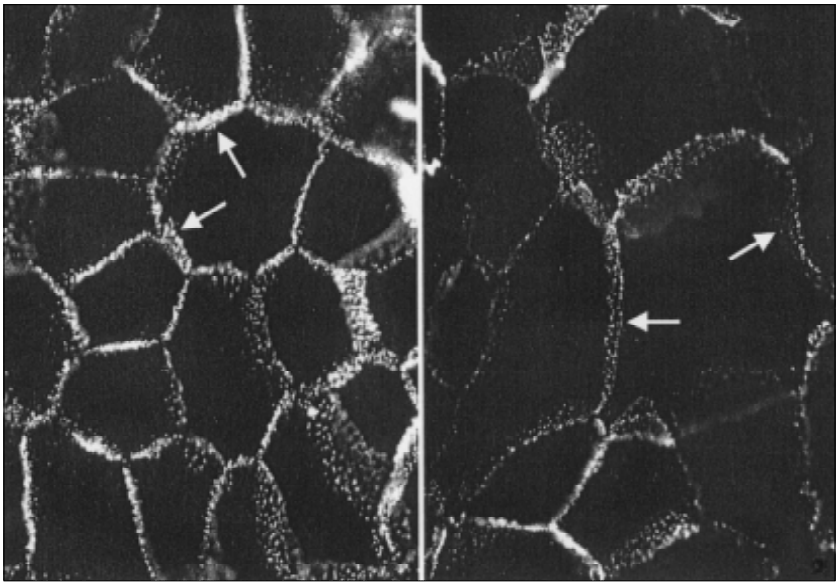

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Fig. 3. The localisation of desmoplakin on cultivated keratinocytes (direct immunofluorescence). a) control keratinocytes, b) keratinocytes with potassium dichromate (concentration 2 µg/ml, 48 h).

ge to cellular contacts could be observed after increasing their concentrations up to 100 and 200 µg/ml (values still below the limit of cytotoxicity). The damage was similar to that caused by the effect of potassium dichromate. Cadmium sulphate had significant effect neither on cellular contacts of keratinocytes, nor on the cytoskeleton, even in concentrations reaching its toxic value (5 µg/ml).

Regarding the fact that the most significant changes in the observed proteins were evoked by the application of potassium dichromate, we concentrated on a detail study or these changes after the incubation of keratinocytes with potassium dichromate in concentration of 2 µg/ml:

Vinculin (Fig. 1) was moderately influenced after 24-hour incubation in intercellular contacts. The contours of cells were

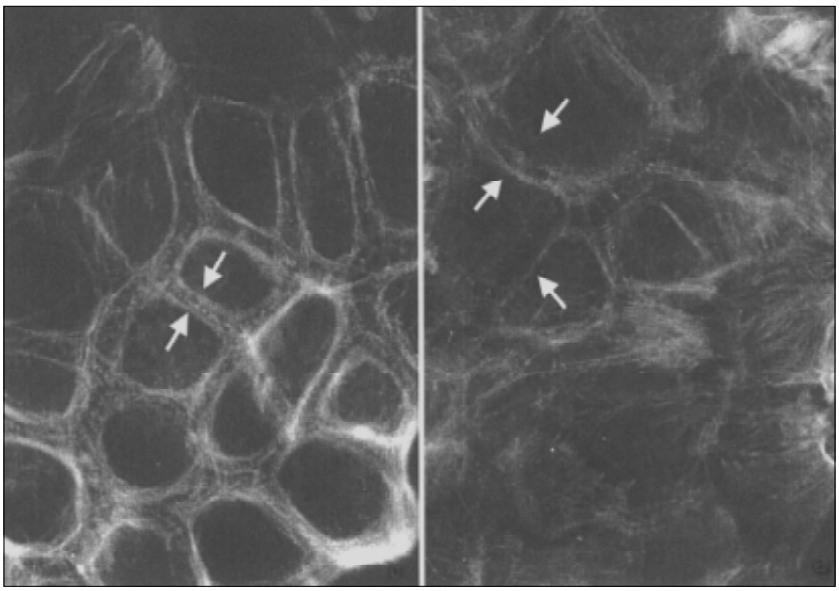

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Fig. 4. The localisation of actin on cultivated keratinocytes (direct immunofluorescence). a) control keratinocytes, b) keratinocytes with potassium dichromate (concentration 2 µg/ml, 48 h).

thinner in comparison to the control. In short sections, the outlines were only dotted. Within the basal layer of keratinocytes as a part of focal adhesions, vinculin was not influenced. After 48 hours, the localisation of protein was significantly changed in comparison with the control group, the outlines were mostly dotted and interrupted in a number of sites. The lines were irregular. Within the basal layer of keratinocytes, vinculin as a part of focal adhesion appeared in spots that were thickened in comparison with the control, multiplied, and shifted in groups to the cellular borders.

Talin. After a 24-hour incubation, the spreading of taline did not differ from the control, after 48-hour incubation, the fluorescence was moderately „blurred".

E-cadherin (Fig. 2). After a 24-hour incubation, the cellular outlines were slightly narrower in comparison to the control. After a 48-hour incubation, the fluorescence was significantly changed. E-cadherin formed very narrow cellular outlines, interrupted in many sites.

Desmoplakin (Fig. 3) was not structurally changed after a 24-hour incubation in comparison to the control. After a 48-hour incubation, the intercellular contacts were reduced, almost disappearing in several sites. In higher layers of keratinocytes, the cellular outlines had a thin thread-like appearance.

Actin (Fig. 4). The system of actin filaments on intact keratinocytes appears as a pronounced ring running parallel with the cellular membrane. After a 24-hour incubation with potassium dichromate, the ring-like structure became less contrast. After 48 hours, a significant, almost entire degradation of rings took place with chaotic spreading of fluorescence in keratinocytes.

On the whole, it is possible to conclude that the most significant decrease was observed in vinculin and E-adherin representing the structural components of adherent junctions.. At the same time also the actin skeleton that joins the adherent junctions was damaged. Less spectacular changes were observed in desmoplakin being a part of desmosomes. The changes in vinculin and talin as parts of focal contacts resided in the character of fluorescence spreading, not in their decrease.

Reversibility of cellular contact damage

In order to judge the reversibility of cellular contact damage induced by 24 and 48-hour periods of incubation of keratinocytes with potassium dichromate, we replaced the medium by pure medium, without the latter substance. The cellular contacts were renewed. Their renewal began already after 3 hours.

So far, the possible task of changes in cellular contacts of keratinocytes in the pathogenesis of contact allergic eczema has not been studied. The only note in literature deals with the effect of trichlorine benzene on the decrease of E-cadherine expression in mouse Langerhans cells (10). The available literature does not provide any studies on the effect of haptenes on the proteins of cellular contact of keratinocytes.

In our study we have proved a decrease in E-cadherine after the application of tested haptanes. This fact could indicate that, parallel with the decrease in E-cadherin expression on Langerhas cells, it also decreases its expression in keratinocytes, the fact of which enhances and speeds up the release of junctions between Langerhans cells and keratinocytes.

It is possible that the weakening of intercellular contacts of keratinocytes takes place in both phases of contact eczema. The first phase could be facilitated by the release of LCs from epidermis in order to migrate into lymphatic nodes together with

the swallowed antigen, and to present it to T-lymphocytes. The second phase of contact eczema, namely the weakening of cellular contact facilitates both migration of LCs and easy infiltration of T-lymphocytes into the epidermis.

In our study, we have proved that the proteins of cellular contact in keratinocytes decrease and are destroyed after the application of known allergens, namely nickel sulphate, potassium dichromate and cobalt nitrate. Potassium dichromate caused damage already in concentration of 1 μg/ml, a similar effect of the other two tested haptenes appeared in concentrations being 100-fold higher. Most significant changes have been proved in coincidence with E-cadherin, vinculin and actin filaments. Desmoplakin and talin were less damaged. Cadmium sulphate had no significant effect on cellular contacts of keratinocytes, not even on cytoskeleton in toxic concentrations (5 μg/ml).

Our study has proved that the investigation of the dynamics of the decrease in cell contact proteins could possibly become a promising *in vitro* model of testing the allergy potential of chemical substances.

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