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SHORT COMMUNICATION

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Contact sensitizers downregulate the expression of the chemokine receptors CCR6 and CXCR4 in a skin dendritic cell line

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Abstract Chemokines are involved in the control of dendritic cell (DC) trafficking, which is critical for the immune response, namely in allergic contact dermatitis (ACD). In this work, we investigated by flow cytometry the effect of the contact sensitizers 2,4-dinitrofluorobenzene (DNFB), 1,4-phenylenediamine (PPD) and nickel sulfate ($NiSO_4$), on the surface expression of the chemokine receptors CCR6 and CXCR4 in DC. As an experimental model of a DC we used a fetal skin-derived dendritic cell line (FSDC), which has morphological, phenotypical and functional characteristics of skin DC. Our results show that all the skin sensitizers studied decreased the membrane expression of the chemokine receptors CCR6 and CXCR4. In contrast, 2,4-dichloronitrobenzene (DCNB), the inactive analogue of DNFB without contact sensitizing properties, was without effect on the surface expression of these receptors. Lipopolysaccharide (LPS), which induces the maturation of DC, also reduced surface CCR6 and CXCR4 expression.

Keywords Skin dendritic cell · Contact sensitizers · CCR6 · CXCR4 · Nickel sulfate · DNFB · PPD

Abbreviations ACD: Allergic contact dermatitis · DC: Dendritic cell · DCNB: 2,4-Dichloronitrobenzene · DMSO: Dimethyl sulphoxide · DNFB: 2,4-Dinitrofluorobenzene · FSDC: Fetal skin dendritic cell line · LC: Langerhans cells · LPS: Lipopolysaccharide · MFI: Mean fluorescence intensity · MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide · PPD: 1,4-Phenylenediamine

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Introduction

Epidermal skin dendritic cells (DC), namely Langerhans cells, play an important role in allergic contact dermatitis (ACD) and other cell-mediated immune reactions. They constantly monitor the epidermal microenvironment by taking up and processing antigens that can be recognized, after migration to the lymph nodes, by T cells [1]. The migration of DC, both to sites of inflammation and to the draining lymph nodes, is dependent on a switch in the expression of chemokine receptors and in the production of chemokines [2]. Chemokines constitute a family of structurally related small (67–127 amino acids, 8–14 kDa) chemotactic cytokines that regulate the migration of leucocytes throughout the body, both under physiological and inflammatory conditions. They are the only cytokines that act on the superfamily of G-protein-coupled serpentine receptors [3], namely class A, which are characterized by high homology with rhodopsin, the prototypical family member [4].

In the skin, the migration and epidermal retention of Langerhans cells is dependent on its expression of CCR6, which is downregulated as DC mature and migrate to the lymph nodes. CXCR4, the membrane chemokine receptor for CXCL12, has been related with the constitutive basal trafficking of leucocytes, namely DC [2]. Scarce information is available concerning direct effects of contact sensitizers on chemokine receptor expression in skin DC. Therefore, the aim of this study was to investigate whether different contact sensitizers, namely 2,4-dinitrofluorobenzene (DNFB), 1,4-pheny-

lenediamine (PPD) and nickel sulfate ($NiSO_4$), modulate the surface expression of the chemokine receptors CCR6 and CXCR4 in DC. As an experimental model of a DC we used a fetal skin-derived dendritic cell line (FSDC), which has morphological, phenotypical and functional characteristics of a skin DC, the Langerhans cells [5]. As a positive control we used lipopolysaccharide (LPS), which was previously shown to induce FSDC activation [6, 7] and DC maturation [8]. As a negative control we used 2,4-dichloronitrobenzene (DCNB), the inactive analogue of DNFB which does not induce ACD.

Materials and methods

Materials

The PE-conjugated monoclonal antibodies against CCR6 and CXCR4 were purchased from R&D Systems (Lille, France). DNFB and DCNB were from Sigma-Aldrich Química (Madrid, Spain) and PPD was from Aldrich Chemical Co. (Milwaukee, WI). LPS from *Escherichia coli* (serotype 026:B6) was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Fetal calf serum was from Biochrom KG (Berlin, Germany) and trypsin from Invitrogen (Paisley, UK). All other reagents were from Sigma Chemical Co. (St. Louis, MO, USA).

Cell culture and chemicals

A fetal mouse skin dendritic cell line (FSDC), kindly supplied by Dr. G. Girolomoni, was used in the experiments. This cell line has a surface phenotype consistent with that of Langerhans cell progenitor (H-$2^{d.b+}$, I-$A^{d.b+}$, $CD54^+$, $MHCII^+$, $MHCI^+$, $CD11c^+$, $CD11b^+$, $B7.2^+$, $CD44^+$ $B220^-$, $CD3^-$). After treatment with cytokines, FSDC stimulate allogeneic or syngeneic T cells in the primary mixed-leukocyte reaction and present haptens to primed T cells in vitro. Moreover, FSDC derivatized with haptens and injected either intravenously or subcutaneously efficiently induce contact sensitivity responses in naïve syngeneic mice [5]. Cells were cultured in endotoxin-free Iscove's medium supplemented with 10% (v/v) fetal calf serum, 1% (w/v) glutamine, 3.02 g/l sodium bicarbonate, 100 µg/ml streptomycin and 100 U/ml penicillin. For flow cytometry analysis and MTT assay, FSDC were plated at 2×10^6 cells/well, in 6-well culture plates, or at 0.2×10^6 cells/well, in 48-well culture plates, respectively, and then stimulated, for 18 h, with culture medium alone, or with LPS (2 µg/ml), or with the following contact sensitizers: PPD (10, 50 µg/ml) and $NiSO_4$ (50 µg/ml), dissolved in saline, DNFB (1, 2.5 µg/ml) and DCNB (2.5 µg/ml) pre-solubilized in dimethyl sulphoxide (DMSO) and diluted in saline. DMSO concentration never exceeded 0.025% in the culture medium.

MTT assay

Assessment of MTT reduction by metabolically active cells was made by a colorimetric assay, using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) as previously reported [6]. In four independent experiments, the average cell viability for FSDC treated, for 18 hours, with the refereed concentrations of nickel and PPD was above 90%, and for DNFB it was respectively 76% and 64%, for 1 µg/ml and 2.5 µg/ml (data not shown). DCNB and the vehicle DMSO had no effect on cell viability evaluated by the MTT assay (data not shown).

Flow cytometric analysis

Briefly, FSDC cells treated with culture medium alone (control), or with the above refereed chemicals (concentrations indicated in the Figs. 1 and 2) were washed with phosphate buffered saline, and cell pellets were incubated with PE-conjugated monoclonal antibodies against CCR6 or CXCR4, for 10 min at room temperature. After washing again with saline, flow cytometry analysis was performed with FACScalibur flow cytometer and CellQuest software (Becton Dickinson). Appropriate isotype controls used at the same concentration as the test antibody showed a mean fluorescence

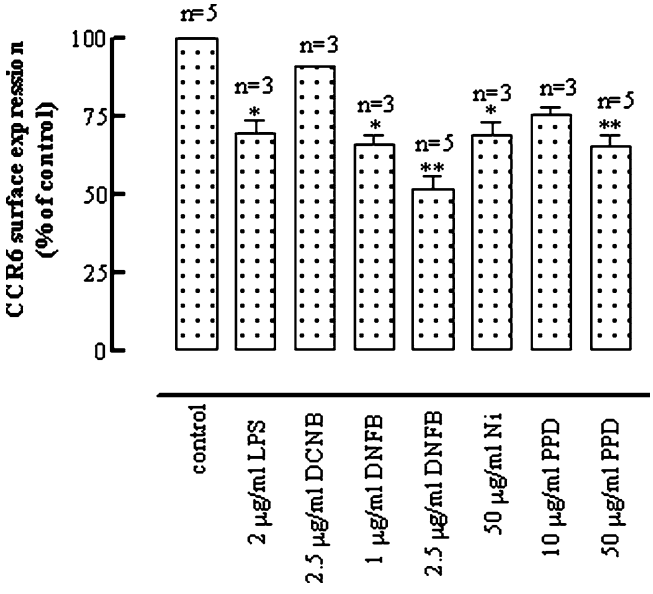

Fig. 1 Effect of contact sensitizers and LPS on CCR6 expression in FSDC cells. FSDC cells (2×10^6 cells) were incubated in culture medium alone (control), or with LPS, or in the presence of different contact sensitizers, at the indicated concentrations, for 18 h. Surface expression of CCR6 was analyzed by flow cytometry as described in material and methods and data are expressed as percentage of non-treated control cells (100% corresponds to a mean fluorescence intensity of 29.6 ± 3.5). Each value represents the mean ± SEM from the indicated number of experiments (*$P < 0.05$; **$P < 0.01$)

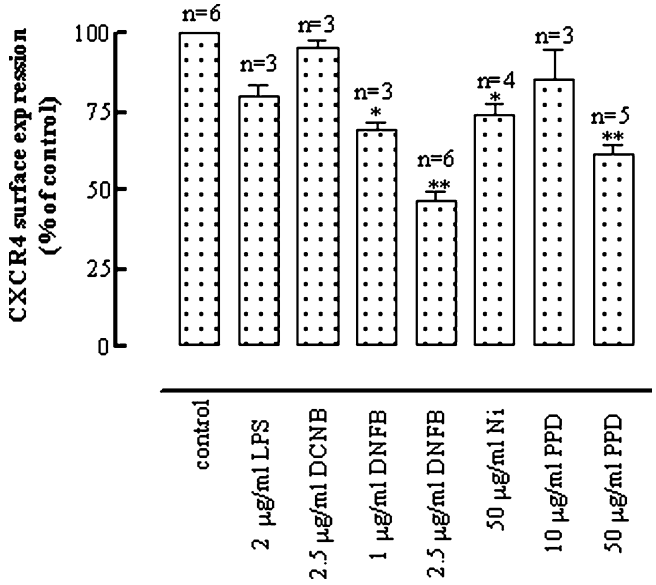

Fig. 2 Effect of contact sensitizers and LPS on CXCR4 expression in FSDC cells. FSDC cells (2×10^6 cells) were incubated in culture medium alone (control), or with LPS, or in the presence of different contact sensitizers, at the indicated concentrations, for 18 h. Surface expression of CXCR4 was analyzed by flow cytometry as described in Materials and Methods and data are expressed as percentage of non-treated control cells (100% corresponds to a mean fluorescence intensity of 52.7 ± 5.3). Each value represents the mean ± SEM from the indicated number of experiments (*$P < 0.05$; **$P < 0.01$)

intensity (MFI) similar to the negative (baseline) control.

Results are presented, in percentage, as the ratio of MFI of the treated and nontreated cells in each lot of experiments, according to the formula: CXCR4 or CCR6 expression (Percentage of control) = (Mean fluorescence intensity of chemical-treated cells/mean fluorescence intensity of nontreated cells)x100. MFI of nontreated control cells (100%) corresponds to 52.7 ± 5.3 and 29.6 ± 3.5, for CXCR4 and CCR6, respectively. FSDC cells cultured in the same conditions and subjected to the same experimental protocol for flow cytometry, but without labeling with monoclonal antibodies (negative baseline control), were always evaluated in parallel experiments (MFI of 3.8 ± 0.6). Cellular debris was eliminated from the analysis using a gate on forward and side scatter.

Data analysis

Results are presented as mean ± SEM of the indicated number of experiments (3–6). Mean values were compared using one-way ANOVA followed by the Dunnett comparison test. The significance level was 0.05.

Results and discussion

Langerhans cells (LC), the principal DC residing in the epidermis, typify the sentinel role of immature DC. LC are known to play a key role in the development of allergic contact dermatitis (ACD). Following an encounter with a chemical allergen, LC become activated and subsequently migrate from the skin to the draining lymph nodes, undergoing a maturation process during this journey [1]. After hapten exposure, LC have been shown to suffer diverse changes, namely internalization of surface MHC class II molecules via endocytosis, induction of tyrosine phosphorylation and modulation of cell surface markers and cytokine expression [9–12]. However, the effect of contact allergens on chemokine receptor expression in LC was seldom addressed before.

The chemokine receptors CCR6 and CXCR4 regulate the recruitment of antigen-presenting and immunocompetent cells during inflammatory and immunological responses, namely ACD. In this work we studied, for the first time, the effect of different skin sensitizers on CCR6 and CXCR4 surface expression. We used an organic contact sensitizer, DNFB and its inactive analogue, DCNB, and two other common allergens, namely nickel sulfate and the arylamine 1,4-phenylenediamine (PPD). FSDC, an immature LC precursor used in these studies [5], constitutively expresses CXCR4 and CCR6 on its surface, with a mean fluorescence intensity of 52.7 ± 5.3 and 29.6 ± 3.5, respectively. All the skin sensitizers decreased membrane expression of the chemokine receptor CCR6 (Fig. 1), with the strongest effect observed for the more potent skin sensitizer used, DNFB. The highest DNFB concentration used (2.5 µg/ml) decreased CCR6 surface expression, to 51.9 ± 9.5% of the control. This contrasts with its inactive analogue, DCNB, which had no effect on CCR6. Accordingly, we have previously shown in the FSDC cell line that, in contrast with DNFB, DCNB had no effect on the expression of other cell activation markers (CD40 and IL-12 receptor) [13].These results suggest that skin sensitizers decrease surface CCR6 expression, and therefore may enhance DC migration out of the skin to the lymph nodes where they can present antigens to T cells. In agreement with our findings, CCR6 is known to be constitutively expressed in immature DC, but it is downregulated as DC mature [3, 14]. LPS, previously shown to induce maturation of dendritic cells [8, 15, 16], also reduced membrane expression of CCR6 in FSDC (Fig. 1), to 69.7 ± 7.8% of the control, although the effect of LPS was not as significant as that of DNFB and PPD. This probably occurs because of their strong skin sensitizing potential, which promote DC activation and maturation, as observed in other DC [11, 17–23]. Moreover, LPS and these contact sensitizers may induce different aspects of DC maturation, since the signaling pathways are also different; LPS acts through TLR4 [15], which is not described as being used by skin sensitizers.

The role of CCR6 in ACD is under current investigation. In DNFB-induced contact hypersensitivity studies, CCR6-deficient mice developed more severe and more persistent inflammation than

wild-type animals [24]. However, in vivo, both CCR6 and its ligand, CCL20, were markedly upregulated in chronic inflammatory skin disorders like contact dermatitis, psoriasis, and atopic dermatitis [25, 26], where there is an accumulation of DC in the epidermis [27].

CXCR4, the membrane chemokine receptor for CXCL12, has been related with the constitutive basal trafficking of leucocytes [2], and was also investigated in this work. Our results show that, as observed with CCR6, all the skin sensitizers tested reduced the amount of plasma membrane-associated CXCR4 in FSDC (Fig. 2). DNFB (2.5 μg/ml) reduced CXCR4 expression to 46.7 ± 7.5% of the control (Fig. 2), being the most potent sensitizer in decreasing CXCR4 surface expression, as observed for the other surface marker. DCNB was without effect on the surface expression of this receptor. LPS also decreased CXCR4 surface expression, to 80.2 ± 5.6%, although this effect was not statistically significant. In contrast to our results, a previous in vitro study, using peripheral blood monocytes-derived DC and methacrylates as skin contact sensitizers, showed an increase in CXCR4 expression [28].

The results obtained in this work demonstrate that, in vitro, skin sensitizers decrease the surface CCR6 and CXCR4 expression on FSDC, which, in vivo, might be related with the enhancement of DC migration out of the skin to the lymph nodes for antigen presentation to T cells. In this work, we did not study other markers of FSDC maturation induced by contact sensitizers and LPS. However, in our previous work we observed that DNFB and $NiSO_4$ increased FSDC expression of the membrane-associated proteins CD40 and interleucin (IL) 12 receptor [13], and that they activated the transcription factor NF-kB [7, 29, 30], which could be regarded as a cellular maturation pathway. Moreover, studies performed in other DC types clearly demonstrate that contact sensitizers induce DC maturation [11, 17–23].

There are two major mechanisms of regulation of the abundance of chemokine receptors on the cell surface: altered gene expression and desensitisation [31]. The skin sensitizers-induced signaling pathway(s) responsible for the decrease of CCR6 and CXCR4 surface expression in dendritic cells have not yet been identified. Our preliminary results suggest that CXCR4 and CCR6 receptors undergo desensitisation, since the decrease in surface expression of the receptors was already observed 2.5 h after incubation with contact sensitizers (data not shown). The understanding of these mechanisms could have profound biological consequences in ACD by allowing to act upstream of the current target of anti-inflammatory agents, through the modulation of surface expression of chemokine receptors. Therefore, targeting chemokines and chemokine receptors may offer new opportunities for therapeutic interventions in ACD and other chronic inflammatory skin diseases.

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