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Research Article

Interleukin 22 early affects keratinocyte differentiation, but not proliferation, in a three-dimensional model of normal human skin

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ABSTRACT

Interleukin (IL)-22 is a pro-inflammatory cytokine driving the progression of the psoriatic lesion with other cytokines, as Tumor Necrosis Factor (TNF)-alpha and IL-17. Our study was aimed at evaluating the early effect of IL-22 alone or in combination with TNF-alpha and IL-17 by immunofluorescence on i) keratinocyte (KC) proliferation, ii) terminal differentiation biomarkers as keratin (K) 10 and 17 expression, iii) intercellular junctions. Transmission electron microscopy (TEM) analysis was performed. A model of human skin culture reproducing a psoriatic microenvironment was used. Plastic surgery explants were obtained from healthy young women (n=7) after informed consent. Fragments were divided before adding IL-22 or a combination of the three cytokines, and harvested 24 (T24), 48 (T48), and 72 (T72) h later. From T24, in IL-22 samples we detected a progressive decrease in K10 immunostaining in the spinous layer paralleled by K17 induction. By TEM, after IL-22 incubation, keratin aggregates were evident in the perinuclear area. Occludin immunostaining was not homogeneously distributed. Conversely, KC proliferation was not inhibited by IL-22 alone, but only by the combination of cytokines. Our results suggest that IL-22 affects keratinocyte terminal differentiation, whereas, in order to induce a proliferation impairment, a more complex psoriatic-like microenvironment is needed.

1. Introduction

The interplay among pro-inflammatory cytokines such as Tumor Necrosis Factor (TNF)-alpha, interleukin (IL)-17 and IL-22 and the two main epidermal cytotypes, i.e. keratinocytes (KCs) and Langerhans cells (LCs), is a central issue in the pathogenesis of psoriasis. Many different stimuli can trigger the onset of psoriasis in genetically predisposed individuals [1]. Psoriasis has been recently defined as "a T-cell mediated inflammatory skin disease with T helper cell type 1 (Th1), type 17 (Th17) and IL-22-producing $CD4^+$ T cells as principal mediators" [2]. On the other hand, cytokine-stimulated KCs may secrete a great variety of pro-inflammatory factors [3]. As a result, this microenvironment is able to promote KC activation and maintain a high rate of KC proliferation. In this milieu, epidermal terminal differentiation is profoundly affected as demonstrated by the expression switch from the typical keratin (K) isoforms K1/K10 towards K17 in the suprabasal layers. K17 is not expressed in normal skin [4], but it is detected in the basal cells of finger nails, hair follicles, sebaceous glands and nail bed epithelium [5]. K17 expression levels are positively associated with psoriasis severity [6] and a K17/T-cell/cytokine autoimmune loop was found, which strengthens the hypothesis of a correlation between K17 and the pathogenesis of psoriasis [7]. Pro-inflammatory cytokines IL-17 and IL-22, both produced by Th17 cells [8], are known to upregulate K17 expression in vitro [9,10] and in psoriatic lesions [11]. Recent in vitro [12] and in vivo [13,14] studies demonstrated the role of IL-22 in the process of KC terminal differentiation, but its effect on proliferation is still debated. IL-22 promoted proliferation and injury repair of hyperglycemic KCs in diabetic mice wounds [14], but it did non induce a proliferative increase in HaCat cells [12]. Considering that much of the data regarding IL-22 has been obtained from animal models and are thus difficult to extrapolate to the human pathology, human systems can greatly help in further exploring the role of IL-22 in psoriasis.

Up to now a specific activity of IL-17 and TNF-alpha was demonstrated on KC proliferation [15] and LC immunophenotype

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[16] in a model of human skin organotypic culture standardized in our laboratory.

As IL-17 and TNF-alpha enhance by powerful synergies the skin effects exerted by IL-22 [17], the present study was aimed at evaluating the epidermal response to IL-22 alone or in combination with IL-17 and TNF-alpha up to 72 h of stimulation in this experimental setting. Immunofluorescence was used to address i) KC proliferation, ii) terminal differentiation biomarkers as K10 and 17 expression and iii) the expression and localization of molecules of intercellular junctions (desmocollin 1 (Dsc 1), E-cadherin, and occludin). Transmission electron microscopy (TEM) was used to analyze ultrastructure.

2. Materials and methods

Normal human skin explants were obtained from abdomen or mammary gland after plastic surgery of healthy 20–40 year-old women (n=7) after informed consent. Procedures were in accordance with the ethical standards of the Institutional committee on human experimentation and with the Helsinki Declaration. The fragments (1 cm^2) were placed in a Transwell system epidermis upwards at air-liquid interface and the dermis immersed in the culture medium (Costar, Corning, NY, USA) thus reproducing as close as possible the physiological condition. Dulbecco's modified Eagle culture medium was used (Euroclone, Milan, Italy) containing 10% fetal bovine serum (FBS; Invitrogen, Life Technologies Ltd., Paisley, UK) supplemented with penicillin/streptomycin, amphotericin B, and glutamine (Sigma-Aldrich, St. Louis, MO, USA) and maintained at 37 °C with 5% CO_2 overnight in order to reduce the acute effects of surgery, as previously described [15,16]. No hydrocortisone was added to avoid an anti-inflammatory activity on epidermal KCs. Specimens were further divided to obtain samples for each culture condition: (A) 100 ng/ml IL-22; (B) 50 ng/ml IL-17; (C) 100 ng/ml TNF-alpha; (D) 100 ng/ml IL-22+50 ng/ml IL-17+100 ng/ml TNF-alpha, (C) no cytokine (control group). All cytokines were from PeproTech (London, UK). Samples were harvested 24 h (T24), 48 h (T48) and 72 h (T72) after cytokine addition and processed for light and transmission electron microscopy as specified below.

2.1. Histology

Three specimens (5 × 5 mm) from each patient were embedded in Killik (Bio Optica, Milan, Italy) frozen at −80 °C and cryosectioned (HM 500 Microm, Zeiss, Oberkochen, Germany).

Other specimens were immersion-fixed in 4% paraformaldehyde buffered with 0.1 mol L^{-1} phosphate-buffered saline (PBS), pH 7.4, for 5 h at room temperature, dehydrated, paraffin embedded and sectioned with a microtome RM2245 (Leica Microsystems GmbH, Wetzlar, Germany). At least five non-consecutive slides for each sample were stained with haematoxylin and eosin to analyze the three-dimensional structure.

2.2. Immunofluorescence of epidermal markers of terminal differentiation and intercellular adhesion

Immunostainings for K10 and occludin were performed after exposure to IL-22 and to the three cytokines together (Triple), while the expressions of K17, Dsc 1, and E-cadherin were evaluated with IL-22, IL-17 or TNF-alpha alone and with the triple. The protocols followed to immunolabel differentiation and adhesion markers are summarized in Table 1. In all experiments, paraffin sections were dewaxed, rehydrated in a decreasing scale of ethanol and treated for antigen unmasking and non-specific binding site were saturated before incubation with specific primary and secondary antibodies. Nuclei were counterstained with 4′, 6-diamidino-2-phenylindole dihydrochloride (DAPI; 1:50,000 in bi-distilled water, 5 min at room temperature). One section on each slide was considered as technical negative control and the primary antibody was thus omitted and replaced with PBS.

Slides were examined in an Eclipse E600 microscope equipped with a digital camera DXM1200 (both from Nikon, Tokyo, Japan).

2.3. Quantitative analysis of epidermal proliferation

In order to evaluate epidermal cell proliferation, 3 h before the end of the experiment the culture medium was enriched with 400 μmol L^{-1} 5-bromo-2′-deoxyuridine (BrdU), a non-radioactive thymidine analogue selectively incorporated in DNA of S-phase cells. The incorporation was revealed using a monoclonal antibody anti-BrdU (Santa Cruz Biotechnology, Dallas, TX; 1:200, 60 min) after DNA denaturation with 2N HCl (30 min), buffering with $Na_2B_4O_7$ (10 min), saturation of non specific binding sites with 3% bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) in PBS (20 min), and digestion with 0.05% pepsin in 20 mmol L^{-1} HCl (20 min). A FITC-conjugated horse anti-mouse IgG (Vector Laboratories, Burlingame; 1:200, 60 min) was used as secondary antibody. All procedures were performed at room temperature except for pepsin incubation, which was done at 37 °C.

For the analysis of cell proliferation at least three experiments were carried out for each sample, with two slides per sample and two sections on each slide. Each section was considered as a sample unit for statistical analysis. Two independent, double-

Table 1.
Summary of the indirect immunofluorescence protocols. Microwave and autoclave antigen unmasking were always performed with slides immersed in citrate buffer (pH 6). Primary, secondary antibodies, and goat serum were diluted in PBS/BSA 1%. RT: room temperature; PBS: phosphate buffered saline; BSA: bovine serum albumin.

Paraffin sections				
Primary antibody	Saturation aspecific sites	Antigen unmasking	Dilution/Incubation time	Secondary antibody
Terminal differentiation				
K10 (DE-K10 - Progen)	Goat Serum (Vector) 1:10/30′ RT	Microwave (750w) boiling 3 × 4″	1:10/overnight 4 °C	Goat anti Mouse FITC-conjugated (Jackson Immuno Research) 1:200/1 h RT
K17 (ab 53,707 - Abcam)	Goat Serum (Vector) 1:10/1 h RT	Autoclave 10′ 120 °C	1:500/overnight 4 °C	Alexa Fluor 488 Goat anti rabbit (Molecular Probes) 1:200/1 h RT
Intercellular adhesion				
Occludin (Invitrogen)	Goat Serum (Vector) 1:10/45′ RT	Microwave (750w) boiling 3 × 1′ and pepsin 0.05% 25′ 37 °C	1:100/overnight 4 °C	Goat anti Mouse FITC-conjugated (Jackson Immuno Research) 1:200/1 h RT
E-cadherin (36/E-Cadherin – BD Bioscience)	Goat Serum (Vector) 2%/45′ RT	Autoclave 10′ 120 °C	1:1000/overnight 4 °C	
Desmocollin 1 (U100 – Progen)	Goat Serum (Vector) 1:10/30′ RT	Microwave (750w) boiling 3 × 4″ and pepsin 0.001% 15′ 37 °C	Prediluted/1 h RT	

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