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Langerhans cell precursors acquire RANK/CD265 in prenatal human skin

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ABSTRACT

The skin is the first barrier against foreign pathogens and the prenatal formation of a strong network of various innate and adaptive cells is required to protect the newborn from perinatal infections. While many studies about the immune system in healthy and diseased adult human skin exist, our knowledge about the cutaneous prenatal/developing immune system and especially about the phenotype and function of antigen-presenting cells such as epidermal Langerhans cells (LCs) in human skin is still scarce. It has been shown previously that LCs in healthy adult human skin express receptor activator of NF-κB (RANK), an important molecule prolonging their survival. In this study, we investigated at which developmental stage LCs acquire this important molecule. Immunofluorescence double-labeling of cryostat sections revealed that LC precursors in prenatal human skin either do not yet [10–11 weeks of estimated gestational age (EGA)] or only faintly (13–15 weeks EGA) express RANK. LCs express RANK at levels comparable to adult LCs by the end of the second trimester. Comparable with adult skin, dermal antigen-presenting cells at no gestational age express this marker. These findings indicate that epidermal leukocytes gradually acquire RANK during gestation – a phenomenon previously observed also for other markers on LCs in prenatal human skin.

Introduction

The receptor activator of nuclear factor-kappaB ligand (RANKL/CD254), its signaling receptor RANK/CD265 and the decoy receptor osteoprotegerin are members of the tumor necrosis factor (TNF) and TNF-receptor superfamily (Sigl and Penninger, 2014). RANKL is found on osteoblasts, basophils, activated keratinocytes and T cells, while RANK is constitutively expressed in osteoclasts and dendritic cells (DCs) (Barbaroux et al., 2008; Huber et al., 2014; Kukita and Kukita, 2013; Leibbrandt and Penninger, 2008; Loser and Beissert, 2007). RANKL/RANK have multiple functions ranging from bone physiology, mammary gland formation, lymph node development, initiation of breast cancer and immune regulation (Arizon et al., 2012; Beristain et al., 2012; Hanada et al., 2011; Kukita and Kukita, 2013; Loser and Beissert, 2007; Sigl and Penninger, 2014).

Langerhans cells (LCs) represent the DC subset in the epidermis and other stratified epithelia. Under steady-state conditions LCs have been implicated to maintain tolerance (Seneschal et al., 2012). Upon infectious challenge they can drive T helper (T_H) T_H1, T_H2 (Furio et al., 2010), T_H17 (Mathers et al., 2009) and T_H22 (Fujita et al., 2009) responses. In healthy adult human epidermis, ~95% of LCs express RANK when analyzed by flow cytometry (Barbaroux et al., 2008) while keratinocytes express low levels of RANKL. Upon ultraviolet irradiation or infection of the skin in mice, keratinocytes upregulate RANKL and trigger RANK expressing LCs (Loser et al., 2006; Yamaguchi and Sakaguchi, 2006). The activated LCs produce cytokines [interleukin (IL)-6, IL-10, TNF-α], upregulate costimulatory molecules (CD86, CD205) and preferentially induce the proliferation of $CD4^+CD25^+$ regulatory T cells (T_{reg}) which are able to suppress local and systemic immune reactions (Loser et al., 2006). Similarly, transgenic mice expressing RANKL under the keratinocyte-specific K14 promotor have increased T_{reg}

Abbreviations: BMP7, bone morphogenetic protein 7; CCL5, chemokine (C–C motif) ligand 5; DC, dendritic cell; EGA, estimated gestational age; GM–CSF, granulocyte–macrophage colony-stimulating factor; IL, interleukin; LC, Langerhans cell; RANK(L), receptor activator of nuclear factor-kappaB (ligand); SCF, stem cell factor; SD, standard deviation; TGF-β, transforming growth factor beta; TNF, tumor necrosis factor; T_{reg}, regulatory T cell.

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numbers in peripheral lymphoid organs which can subsequently reduce and delay the onset of CD40L-induced autoimmune dermatitis in mice (Loser et al., 2006). Depletion of RANKL in mice results in a decrease of LC numbers as well as their proliferation rate without affecting morphology (Barbaroux et al., 2008).

During prenatal human skin development, LC precursors start to populate the epidermis at 7–8 weeks estimated gestational age (EGA). The LC phenotype is acquired in a step-wise manner starting with human leukocyte antigen (HLA) class II followed by CD1c, CD207 and CD1a (Elbe-Bürger and Schuster, 2010; Foster et al., 1986; Fujita et al., 1991; Schuster et al., 2009, 2014). At 18 weeks of gestation, the marker profile is similar to that of adult LCs expressing CD1a, CD1c, CD39, CD207, CCR6, CD324, and HLA class I and II (Elbe-Bürger and Schuster, 2010). The purpose of this study was to examine at which developmental stage LCs acquire RANK expression.

Materials and methods

Embryonic and fetal human skin was obtained from legal abortions, ranging between 10 and 23 weeks EGA ($n = 12$). Healthy adult human breast and belly skin (25–60 years; $n = 7$) was obtained after plastic surgery. The study was approved by the local ethics committee and conducted in accordance with the Declaration of Helsinki principles. The parents and adult volunteers were informed and gave their written permission.

Skin specimens were cut into small pieces, embedded in Tissue-Tek® optimum cutting temperature formulation compound, snap-frozen in liquid nitrogen and stored at −80 °C. Frozen sections (5 μm) of skin were cut, fixed in ice-cold acetone, air-dried and stained.

To prepare epidermal sheets from adult human skin, the subcutaneous tissue was removed, the remaining skin cut into small pieces (~1 cm^2) and floated dermal side down on a 3.8% ammonium thiocyanate solution (Merck, Darmstadt) (1 h, 37 °C). Subsequently, epidermal sheets were carefully peeled from the dermis using forceps, washed with phosphate-buffered saline (pH 7.4; 2× 5 min each), fixed with acetone (10 min, room temperature), washed again and either stained immediately as described below or stored in Eppendorf tubes at −80 °C until use.

Skin sections and epidermal sheets were incubated with an unconjugated anti-RANK mAb (clone 80704; R&D Systems, Minneapolis, MN) in 2% bovine serum albumin/phosphate-buffered saline and subsequently stained with an Alexa Fluor 546-labeled reagent (Invitrogen, San Diego, CA). In the next step mAbs against either HLA-DR (clone L243; FITC-labeled, BD Pharmingen, San Diego, CA) or CD1a (clone HI149; Alexa Fluor 488-labeled, Biolegend, San Diego, CA) were applied. Finally, the samples were washed twice and a nuclear staining was performed with Hoechst dye (Invitrogen). Control samples were stained with appropriate isotype-matched Ab. HLA-DR^+RANK^+ and HLA-DR^+RANK^- cells in human epidermis have been enumerated on cryostat sections and placed in relation to the length of the epidermis (Olympus AX70, Olympus Corp., Tokyo). Single and double positive cells were counted in cryosections and epidermal sheets. Differences between groups were assessed with Student's *t*-test (GraphPad Software, San Diego, CA). The reported *p*-value is a result of a two-sided test. A *p*-value <5% is considered statistically significant.

Results

The majority of LCs in adult human epidermis express RANK

Double immunofluorescence staining of healthy adult human skin using cryostat sections and epidermal sheets revealed that LCs co-express HLA-DR, CD1a and RANK (Fig. 1A–C) confirming previously reported results (Barbaroux et al., 2008). Whereas HLA-DR is expressed on epidermal LCs, dermal antigen-presenting cells and endothelial cells, RANK expression in adult skin is confined to LCs. Based on previous findings which report that not all LCs express RANK when evaluating single cell suspensions by flow cytometry (Barbaroux et al., 2008), it was our goal to investigate whether it is possible to detect $CD1a^+RANK^-$ cells in situ as well. We employed the epidermal sheet staining method as it allows en face view of the whole LC network in a given area compared to the few LCs in a cryostat section. When comparatively analyzing skin from different body regions, we found that the great majority of LCs expresses both CD1a and RANK. In addition, we always detected a small but distinct population of $CD1a^+RANK^-$ cells with a dendritic morphology (Fig. 1C, insert), supplementing previously reported flow cytometry data (Barbaroux et al., 2008). In contrast to CD1a, RANK expression is essentially confined to the perinuclear cytoplasm and is consequently only faintly or not expressed on dendrites. Furthermore, we found that RANK is weakly expressed in some LCs implying either a continuum of $RANK^{low}$ to $RANK^{high}$ or vice versa in adult skin (Fig. 1A,B). When enumerating $CD1a^+RANK^-$ LCs in epidermal sheets, we found that a comparable percentage of $CD1a^+$ LCs in breast, back, and abdominal skin failed to express RANK, indicating that $CD1a^+RANK^-$ cells are similarly distributed in the analyzed body locations (data not shown).

LC precursors in prenatal human skin acquire RANK in a gradual manner

As RANK is (i) an important LC survival factor, (ii) a mediator for cell differentiation and (iii) not expressed in all LCs in adult epidermis (Fig. 1 and Barbaroux et al., 2008), we investigated its expression in human prenatal skin to evaluate when LC precursors start to express this molecule. HLA-DR is the first antigen-presenting cell specific marker to be found in embryonic human skin (Foster et al., 1986 Schuster et al., 2009), and was therefore employed to identify leukocyte precursors in prenatal skin. As shown in Fig. 2, double immunofluorescence staining of cryostat sections confirmed the presence of some rare HLA-DR^+ epidermal (insert) and dermal (arrow) leukocytes none of which express RANK at 10 and 11 weeks EGA. At 13 weeks EGA, we found that some but not all HLA-DR^+ cells in the epidermis express low levels of RANK (Fig. 2, arrow and insert). Surprisingly, we detected strong RANK expression in the periderm – a development-specific cell layer – at this time point of gestation (Fig. 2). With advancing gestational age, we found a spectrum of $RANK^{low-high}$ leukocytes (data not shown) reaching adult-like staining intensity levels at 19–23 weeks EGA (Fig. 2, insert) while the density of $RANK^+$ cells in fetal epidermis is not yet comparable to that in adults (10 vs. 75 cells/5000 μm epidermis) (Fig. 3). HLA-DR^+ dermal cells at no time point of investigation express RANK. Statistical analysis revealed that in first trimester skin 67.7% [standard deviation (SD) 28.0%, $n = 3$] of all HLA-DR^+ epidermal cells lack expression of RANK. The frequency of HLA-DR^+RANK^- epidermal cells decreases with gestational age to 13.0% (SD 12.1%, $n = 3$) in second trimester skin and to 0.2% in adult skin (SD 0.46%; $n = 3$) (Fig. 3).

Discussion

In the current study we provide evidence that LCs undergo a transition regarding the expression of RANK in healthy skin during gestation. We found a major population of HLA-DR^+RANK^- epidermal leukocytes and a minor population of HLA-DR^+RANK^+ cells in embryonic epidermis. Later during development, $RANK^+$ epidermal leukocytes outnumber $RANK^-$ cells. We also identified a small

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