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Epidermal iron metabolism for iron salvage

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ABSTRACT

Background: The epidermis shows a reverse iron gradient from the basal layer to the stratum corneum and consequently, little epidermal intracellular iron is lost by desquamation.
Objective: To clarify the underlying mechanism of iron salvage.
Methods: We first used immunohistochemistry and mRNA quantification to demonstrate the distinctive expression pattern of iron metabolism molecules. The obtained results were confirmed using normal human epidermal keratinocytes (NHEKs) during *in vitro* differentiation. We next examined the effects of reducing ferroportin expression *in vitro* by ferroportin-specific siRNAs or hepcidin on the intracellular iron content of cultured NHEKs. Finally, we compared epidermal and systemic iron metabolism between $Fpn^{Epi\text{-}KO}$ mice and control mice.
Results: The results of both mRNA and protein expression analysis showed that most molecules participating in iron import and storage were expressed in the lower epidermis, while those involved in iron release from heme or iron transport were expressed in the upper epidermis. Consistent with their expression, keratinocyte differentiation reduced intracellular iron content. We next demonstrated that reducing ferroportin expression *in vitro* by ferroportin-specific siRNAs or hepcidin significantly increased the intracellular iron content. Finally, we showed that the iron content of the epidermis and squames was significantly greater in $Fpn^{Epi\text{-}KO}$ mice than in control mice, and that $Fpn^{Epi\text{-}KO}$ exhibited a more rapid decrease in blood hemoglobin concentration than control mice on a low iron diet.
Conclusion: These studies demonstrated that the epidermis is equipped with a machinery by which intracellular iron in differentiated keratinocytes is excreted to the extracellular space before reaching the stratum corneum.

1. Introduction

In the epidermis, proliferating cells localized to the basal cell layer reproduce and migrate toward the outer layer of skin with progressive differentiation, and finally desquamate from the cornified layer. Humans lose approximately 1000 cells per square centimeter per hour from the cornified layer [1]. Therefore, it is assumed that cellular contents in the epidermis, and in particular cellular iron, are constantly lost from the body. However, Cavil et al. demonstrated in a study involving the injection of ^{59}Fe-ferric citrate into human subepidermal tissue that only approximately 10% of the iron incorporated in epidermal cells was lost by desquamation, suggesting that most of the iron in epidermal keratinocytes is not lost from the skin by desquamation [2]. In addition, previous studies using a variety of procedures, such as proton induced X-ray emission [3], a histochemical technique [4], and synchrotron micro X-ray fluorescence [5], have shown that the iron content in the human epidermis is highest in the basal layer and decreases toward the stratum corneum. These observations imply the existence of an epidermal iron salvage system whereby iron is retained in the body by excretion from differentiated keratinocytes before the keratinocytes are shed. This implication was supported by the observation that, when ^{55}Fe was injected into the skin as transferrin-bound Fe, the amount of ^{55}Fe incorporated in the basal and suprabasal layer 35 days after injection was comparable to that 4 days after injection [6]. However, despite this accumulating evidence, the mechanism by which iron is excreted from differentiated keratinocytes and, moreover, the significance of this system in systemic iron metabolism, remains unknown.

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To demonstrate the presence of an iron metabolism system for recovering the intracellular iron of differentiated keratinocytes prior to shedding, we here first examined the expression of iron metabolism molecules in human epidermis and cultured normal human epidermal keratinocytes (NHEKs) during calcium-induced differentiation. These studies revealed a distinctive expression pattern of iron metabolism molecules in which molecules participating in iron import and storage are expressed in the lower epidermis or undifferentiated keratinocytes, while those used for iron release from heme or iron transport are expressed in the upper epidermis or differentiated keratinocytes. Next, to clarify whether or not these iron metabolism molecules function in the epidermis, we demonstrated an increase in iron content in cultured keratinocytes after reducing their ferroportin expression *in vitro* by using ferroportin-specific siRNAs and hepcidin. Finally, we demonstrated that the iron content of the epidermis and squames in the dorsal skin was significantly higher in Fpn^{Epi-KO} mice than in $Fpn^{fl/fl}$ mice, and that the blood hemoglobin (Hb) concentration of Fpn^{Epi-KO} mice on a low iron diet decreased more rapidly compared to $Fpn^{fl/fl}$ mice fed the same diet.

2. Materials and methods

2.1. Tissue samples

After obtaining ethics approval and informed consent in compliance with the Declaration of Helsinki Principles, we collected 7 non-lesional skin samples of resected benign skin tumors for particle induced X-ray emission (PIXE) and laser microdissection, and 6 archival formalin-fixed paraffin-embedded specimens of non-lesional skin of benign skin tumors for immunohistochemistry. None of the donor patients suffered from iron-deficiency anemia. This study was approved by the Ethics Committee of Tohoku University Graduate School of Medicine.

2.2. Immunohistochemistry

Formalin-fixed paraffin-embedded tissue samples cut at 3 μm thicknesses were deparaffinized, followed by incubation with the following antibodies overnight at 4 °C: ferroportin (ab85370), IRP2 (ab110070), and FtH1 (ab65080) (from Abcam, Cambridge, England), HOX1 (SPA-896) (from Stressgen, Ann Arbor, MI, USA), and DMT1 (HPA032139), TfR1 (HPA028598), IRP1 (HPA024157), ceruloplasmin (HPA001834), hephaestin (HPA005824), and transferrin (HPA005692) (from Atlas Antibodies, Stockholm, Sweden). Visualization was performed using a peroxidase-conjugated anti-rabbit immunoglobulin (Histofine MAX-PO (R) kit; Nichirei, Tokyo, Japan) and 3,3-diaminobenzidine tetrahydrochloride (Wako), followed by counterstaining with hematoxylin. Controls for the specificity of the immunoperoxidase reactions omitted the primary antibodies.

2.3. Laser capture microdissection

Snap–frozen skin biopsies (8 μm thick sections) were placed on RNase-free PEN membrane slides (Leica), then immediately fixed in cold ethanol/acetic acid (19:1, v/v) for 1 min and stained with 0.1% Toluidine blue. Laser-capture microdissection was performed using a Leica LMD7000 system (Leica).

2.4. Cell culture and calcium treatment

NHEKs purchased from Kurabo (Osaka, Japan) were cultured in HuMedia-KG2 containing 10 μg/ml insulin, 0.1 ng/ml human recombinant epidermal growth factor, 0.5 mg/ml hydrocortisone, 0.4% bovine pituitary extract, 50 μg/ml gentamicin sulfate, 50 ng/ml amphotericin B (Kurabo), and $FeSO_4$ (Sigma–Aldrich, St. Louis, MO, USA) at 0.05, 0.5, 1.25, 2.5 and 5 μM. In the study examining the effects of Ca concentration on intracellular iron content and the expression of iron metabolism proteins, we used HuMedia-KG2 with 1.25 μM Fe (iron low culture medium) containing various concentrations of Ca. The cultures at passage 2 were used for experiments. Keratinocyte differentiation was induced by culturing confluent cells in culture medium containing 1.5 mM calcium, as previously described [7].

2.5. Iron measurement

Keratinocytes were harvested using a cell scraper (BD, Franklin Lakes, NJ, USA), followed by sonication (Branson 350 Sonifier, Hielscher, Teltow, Germany) on ice. Quantitative measurement of the iron content in cells was performed using a colorimetric ferrozine-based assay kit (Metallo Assay Iron LS, Metallogenics, Chiba, Japan) following the manufacturer's instructions.

2.6. RNA extraction

To obtain RNA from the different layers of epidermis, RNA was extracted with an RNeasy Micro Kit with DNase I digestion (Qiagen, Venlo, the Netherlands) from cultured NHEKs, or the lower or upper epidermis separately obtained by laser capture microdissection according to the manufacturer's instructions. RNA (2 μg) was used for complementary DNA synthesis using a SuperScript VILO cDNA Synthesis Kit (Thermo Fisher Scientific) in a total volume of 20 μL.

2.7. qRT-PCR

qRT-PCR products were amplified in 20 μl from 1 μl complementary DNA with Brilliant III FAST QPCR Master Mix (Agilent Technologies, Santa Clara, CA, USA) using Mx3000P (Agilent) and 1 μl TaqMan probe (Thermo Fisher Scientific). Relative gene expression was calculated from a standard curve generated for each run using MxPro ver 4.1 software (Agilent). Raw data were normalized to the internal control RPLP0, previously suggested as an appropriate housekeeping gene to study gene expression during epidermal differentiation [8]. The data were presented as the relative expression level calculated using the $2^{\Delta\Delta}$Ct method [9]. The TaqMan probes used in these studies are listed in Supplementary Table S1. All experiments were performed in triplicate.

2.8. Protein extraction and measurement

NHEKs cultured for various time periods were harvested using a cell scraper, followed by sonication on ice. Sonicated cell aliquots were lysed using Lysis Buffer (CST, Danvers, MA, USA) supplemented with 1 mM phenylmethanesulfonyl fluoride (Roche, Basel, Switzerland). Protein concentrations were determined using bicinchoninic acid protein assay reagent (Thermo Fisher Scientific) according to the manufacturer's instructions.

2.9. Western blot analysis

Equal amounts of total cellular protein (10 μg) were dissolved in SDS sample buffer (CST) and 40 mM dithiothreitol (CST). Cell lysates were incubated at 100 °C for 5 min, cooled on ice, separated by 10% SDS-PAGE, and transferred to polyvinylidene difluoride membranes (Immobilon-P Membranes, Millipore, Bedford, MA, USA) for 1.5 h at 40 mA. Electroblotted membranes were blocked with TBST (50 mM Tris (pH 7.5) and 0.5% Tween) containing 5% nonfat dried milk and probed with antibodies against ferroportin

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