Namba K, Horie Y, Takeno M, Sugita S, Mochizuki M, Bahram S, Ishigatsubo Y, Inoko H.					
Kitaichi N, Kitamura M, Namba K, Ishida S, Ohno S	Elevation of surfactant protein D, a pulmonary disease biomarker, in the sera of uveitis patients with sarcoidosis.	**Jpn J Ophthalmol**	54	81-4	2010
Abe R, Yoshioka N, Murata J, Fujita Y, Shimizu H	Granulysin as a marker for early diagnosis of the Stevens-Johnson syndrome	**Ann Intern Med**	151	514-515	2009
Hamasaka A, **Abe R**, Koyama Y, Yoshioka N, Fujita Y, Hoshina D, Sasaki M, Hirasawa T, Onodera S, Ohshima S, Leng L, Bucala R, Nishihira J, Shimizu T, Shimizu H	DNA vaccination against macrophage migration inhibitory factor improves atopic dermatitis in murine models	**J Allergy Clin Immunol**	124	90-99	2009
Ando S, **Abe R**, Sasaki M, Murata J, Inokuma D, Shimizu H	Bone marrow-derived cells are not the origin of the cancer stem cells in ultraviolet-induced skin cancer	**Am J Pathol**	174	595-601	2009
Shinkuma S, **Abe R**, Nishimura M, Natsuga K, Fujita Y, Nomura T, Nishie W, Shimizu H	Secondary syphilis mimicking warts in an HIV-positive patient	**Sex Transm Infect**	85	484	2009
Inokuma D, Sawamura D,	Scleroedema adultorum associated with	**Clin Exp Dermatol**	34	e428-429	2009

Shibaki A, **Abe R**, Shimizu H.	sarcoidosis				
Honda A, **Abe R**, Yoshihisa Y, Makino T, Matsunaga K, Nishihira J, Shimizu H, Shimizu T	Deficient deletion of apoptotic cells by macrophage migration inhibitory factor (MIF) overexpression accelerates photocarcinogenesis	**Carcinogenesis**	30	1597-1605	2009
Arita K, **Abe R**, Baba K, McGrath JA, Akiyama M, Shimizu H	A novel OSMR mutation in familial primary localized cutaneous amyloidosis in a Japanese family	**J Dermatol Sci**	55	64-65	2009
Bohgaki T, Atsumi T, Bohgaki M, Furusaki A, Kondo M, Sato-Matsumura KC, **Abe R**, Kataoka H, Horita T, Yasuda S, Amasaki Y, Nishio M, Sawada K, Shimizu H, Koike T	Immunological reconstitution after autologous hematopoietic stem cell transplantation in patients with systemic sclerosis: relationship between clinical benefits and intensity of immunosuppression	**J Rheumatol**	36	1240-1248	2009
Qiao H, Shibaki A, Long HA, Wang G, Li Q, Nishie W, **Abe R**, Akiyama M, Shimizu H, McMillan JR.	Collagen XVII participates in keratinocyte adhesion to collagen IV, and in p38MAPK-dependent migration and cell signaling	**J Invest Dermatol**	129	2288-2295	2009
Wu C, Mino K , Akimoto H, Kawabata M,	In vivo far-red luminescence imaging of a biomarker based on	**Proc Nat Acad Sci USA**	106 (37)	15599-15603	2009

Nakamura K, **Ozaki M**, Ohmiya Y.	BRET from Cypridina bioluminescence to an organic dye				
Ogino T, **Ozaki M**, Hosako M, Omori M, Okada S, Matsukawa A.	Activation of c-Jun N-terminal kinase is essential for oxidative stress-induced Jurkat cell apoptosis by monochloramine	**Leuk Res**	33	151-158	2009
Akimoto H, Kwon HJ, **Ozaki M**, Yasuda K, Honma K, Ohmiya Y	In vivo bioluminescence imaging of bone marrow-derived cells in brain inflammation	**Biochemical and Biophysical Research Communications**	380	844-849	2009
Ikeda O, **Ozaki M**, Murata S, Matsuo R, Nakano Y, Watanabe M, Hisakura K, Myronovych A, Ohkohchi N	Autonomic regulation of liver regeneration after partial hepatectomy in mice	**J Surg Res**	52	218-223	2009
Haga S, **Ozaki M**, Inoue H, Okamoto Y, Ogawa W, Takeda K, Akira S, Todo S	The survival pathways phosphatidylinositol-3 kinase (PI3-K)/phosphoinositide -dependent protein kinase 1 (PDK1)/Akt modulate liver regeneration through hepatocyte size rather than proliferation	**Hepatology**	49	204-214	2009
Kitaichi N, Miyazaki A, Stanford MR, Iwata D, Chams H, Ohno S	Low prevalence of juvenile-onset Behcet's disease with uveitis in East/South Asian people	**Br J Ophthalmol**	93	1428-1430	2009

Horie Y, Meguro A, Ota M, **Kitaichi N**, Katsuyama Y, Takemoto Y, Namba K, Yoshida K, Song YW, Park KS, Lee, EB, Inoko H, Mizuki N, Ohno S.	Association of TLR4 polymorphism with Behcet disease in Korean population	**Rheumatology**	48	638-642	2009
Horie Y, **Kitaichi N**, Katsuyama Y, Yoshida K, Miura T, Ota M, Asukata Y, Inoko H, Mizuki N, Ishida S, Ohno S.	Evaluation of PTPN22 polymorphisms and Vogt-Koyanagi-Harada disease in Japanese patients	**Mol Vis**	15	1115-1119	2009
Kitamei H, **Kitaichi N**, Namba K, Kotake S, Goda C, Kitamura M, Miyazaki A, Ohno S	Clinical features of intraocular inflammation in Hokkaido, Japan	**Acta Ophthalmol**	87	424-428	2009

Ⅳ. 研究成果の刊行物・別刷

Accepted Date : 21-Feb-2012

Article type : Item of Correspondence

Prolonged elevation of serum granulysin in drug-induced hypersensitivity syndrome

N. Saito, R. Abe, N. Yoshioka, J. Murata, Y. Fujita, H. Shimizu

Department of Dermatology, Hokkaido University Graduate School of Medicine, Sapporo, Japan

Key words

Drug-induced hypersensitivity syndrome (DIHS), Eosinophilia and systemic symptoms (DRESS), Granulysin

Correspondence to:

Riichiro Abe MD/PhD

Department of Dermatology

Hokkaido University Graduate School of Medicine

N15 W7, Kita-ku, Sapporo 060-8638, Japan.

Tel: +81-11-706-7387

Fax: +81-11-706-7820

e-mail: aberi@med.hokudai.ac.jp

MADAM,

Drug-induced hypersensitivity syndrome (DIHS)[1], also known as drug rash with eosinophilia and systemic symptoms (DRESS) [2], has been established as a clinical entity in severe cutaneous adverse drug reactions. DIHS is characterized by the limited number of causative drugs, late onset, clinical similarity to infectious mononucleosis-like syndrome and prolonged clinical course due to relapse[1].

Granulysin is a cytotoxic molecule produced against virus-infected cells, tumor cells, transplant cells, bacteria, fungi and parasites[3]. It plays an important role in the host defense against pathogens. A recent paper reported that granulysin is highly expressed in blisters of two other severe cutaneous adverse drug reactions: Stevens-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN)[4]. In addition, we found that serum granulysin is more elevated in patients with early-stage SJS/TEN than in those with ordinary drug-induced skin reactions[5].

This paper investigates the serum granulysin level of DIHS patients. We assembled serum samples of patients with DIHS and analyzed the correlation between granulysin concentrations with clinical manifestations and disease courses.

Sera of 15 patients with DIHS (10 men and 5 women; average age: 55.4 ±19.9 years) were obtained from multiple institutions. All the patients had actively progressing reactions meeting the criteria for DIHS, as previously defined[1]. The disease onset (day 1) was defined as when the skin eruption appeared. Sera of patients with ordinary drug skin reactions (ODSRs) (n=24) and healthy controls

(n=31) were also obtained. ODSRs included maculopapulartype and erythema multiforme-type reactions. The granulysin concentrations of the serum samples were measured with an ELISA as previously described[6]. In brief, serum samples were incubated on plates coated with RB1 antibody (MBL) and then reacted with biotinylated RC8 antibody (MBL). We performed assays in blind of the clinical features.

In serum samples taken from day 1 to day 10 (n=9), 8 samples showed elevated serum granulysin levels over 10 ng/ml (88.9%, 21.9 ±12 ng/ml). In serum samples taken from day 11 to day 20 (n=11), we detected prolonged high serum granulysin levels (63.6%, 16.1±14.8ng/ml). Serum granulysin levels decreased gradually after day 21 (n=20) (30%, 7.6 ± 3.4) (Figure 1). By day 20, the skin eruptions of all the DIHS patients had disappeared. As we reported previously, in 31 healthy control subjects, no increase of granulysin level was detected (0%, 1.6±0.6 ng/ml) and in 24 patients with ODSRs, elevated granulysin was detected in only one patient (4.16%,3.5±3.4 ng/ml) [5].

To distinguish DIHS from ODSRs, the following clinical information is helpful: limited causative drugs, late onset after medication, manifestations similar to infectious mononucleosis such as fever, lymphoadenopathy, hepatitis and hematological abnormalities. However, because of the diversity of ODSRs and similarity to viral exanthema, DIHS sometimes poses a diagnostic challenge. In addition, some cases suffer from multiple organ failure. Therefore, early diagnosis and appropriate treatment is essential.

Unique mechanisms have been implicated in DIHS development, including detoxification defects leading to reactive metabolite formation and subsequent

immunological reactions[7], and reactivation of HHV[8]. In addition, it is increasingly apparent that there is a genetic predisposition to adverse drug reactions. Human leucocyte antigen-related genes have been identified as predictors of DIHS[9].

In particular, the observation that HHV reactivation occurs during the acute phase of DIHS has led to suggestions of a pathogenic link. Shiohara *et al.* identified early reactivation of HHV6 and EB virus, with later involvement of HHV7 and CMV[8]. The resulting expansion of virus-specific T cells might mediate the clinical disease. Recent paper showed that cutaneous and visceral symptoms of DIHS/DRESS are mediated by activated $CD8^+$ T lymphocytes, which are directed against herpes viruses such as EBV[10].

Granulysin exhibits potent cytotoxicity against a broad panel of microbial targets, including tumor cells, transplant cells, bacteria, fungi and parasites, damaging negatively charged cell membranes because of its positive charge[3]. Granulysin plays important roles in the host defense against pathogens and induces apoptosis of the target cells in a mechanism involving caspases and other pathways [3]. In the present study, we showed that granulysin levels of sera were significantly elevated in DIHS patients compared to those of ODSRs. It is suggested that, in DIHS, activation of virus-specific cytotoxic T cells resulted in granulysin release in circulated blood. In contrast, granulysin was identified as the most highly expressed cytotoxic molecule in blisters of SJS/TEN resulting massive keratinocyte apoptosis[4], and we revealed that serum granulysin increased in early stage of SJS/TEN[5]. We speculated that granulysin is involved in SJS/TEN pathogenesis, inducing keratinocyte death in the early stage of

these diseases, whereas serum granulysin in DIHS might be released against virus-infected cells. This speculation is consistent with the present data that show the duration of DIHS manifestation to coincide with the timing of elevated serum granulysin levels. Recently we developed a rapid immunochromatographic test to detect high serum granulysin level in 15 minutes[6]. We expect that monitoring of serum granulysin by the rapid test might contribute to the early diagnosis of DIHS as well as of SJS/TEN. In conclusion, serum granulysin might help early diagnosis and predict disease prognosis.

Figure Legend

Fig 1. Granulysin levels of healthy controls, and of ODSRs patients and DIHS patients at different stages of the diseases.

In DIHS patients, we examined the concentration of granulysin for three terms: day 1 to 10, day 11 to 20, and after day 21. The granulysin level was elevated from day 1 to 20, compared to those for ODSRs and normal controls (*:$p<0.01$).

References

1 Tohyama M, Hashimoto K. New aspects of drug-induced hypersensitivity syndrome. *J Dermatol* 2011; **38**: 222-8.

2 Kardaun SH, Sidoroff A, Valeyrie-Allanore L *et al.* Variability in the clinical pattern of cutaneous side-effects of drugs with systemic symptoms: does a DRESS syndrome really exist? *Br J Dermatol* 2007; **156**: 609-11.

3 Kaspar AA, Okada S, Kumar J *et al.* A distinct pathway of cell-mediated apoptosis initiated by granulysin. *J Immunol* 2001; **167**: 350-6.

4 Chung WH, Hung SI, Yang JY *et al.* Granulysin is a key mediator for disseminated keratinocyte death in Stevens-Johnson syndrome and toxic epidermal necrolysis. *Nat Med* 2008; **14**: 1343-50.

5 Abe R, Yoshioka N, Murata J *et al.* Granulysin as a marker for early diagnosis of the Stevens-Johnson syndrome. *Ann Intern Med* 2009; **151**: 514-5.

6 Fujita Y, Yoshioka N, Abe R *et al.* Rapid immunochromatographic test for serum granulysin is useful for the prediction of Stevens-Johnson syndrome and toxic epidermal necrolysis. *Journal of the American Academy of Dermatology* 2011.

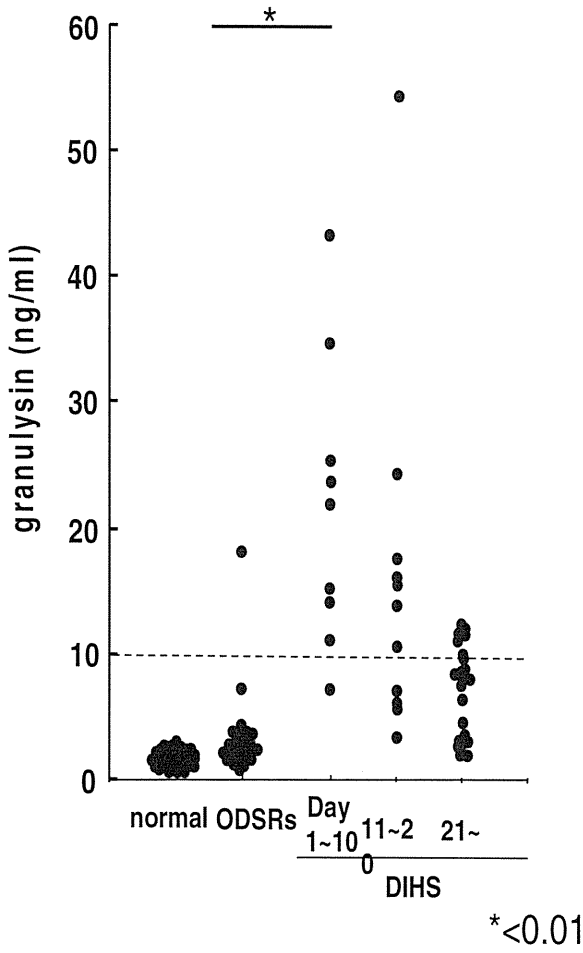
7 Shear NH, Spielberg SP. Anticonvulsant hypersensitivity syndrome. In vitro assessment of risk. *The Journal of clinical investigation* 1988; **82**: 1826-32.

8 Shiohara T, Inaoka M, Kano Y. Drug-induced hypersensitivity syndrome (DIHS): a reaction induced by a complex interplay among herpesviruses and antiviral and antidrug immune responses. *Allergology international : official journal of the Japanese Society of Allergology* 2006; **55**: 1-8.

9 Ozeki T, Mushiroda T, Yowang A *et al.* Genome-wide association study identifies HLA-A*3101 allele as a genetic risk factor for carbamazepine-induced cutaneous adverse drug reactions in Japanese population. *Human molecular genetics* 2011; **20**: 1034-41.

10 Picard D, Janela B, Descamps V *et al.* Drug reaction with eosinophilia and systemic symptoms (DRESS): a multiorgan antiviral T cell response. *Science translational medicine* 2010; **2**: 46ra62.

Fig 1


*
60
50
40
30
20
10
0
granulysin (ng/ml)
normal
ODSRs
Day
1~10
11~2
21~
0
DIHS
*<0.01

CORRESPONDENCE

Intraepidermal neutrophilic IgA pemphigus successfully treated with dapsone

A 25-year-old woman presented with a 2-month history of erythematous, intensely itchy macules and vesicles on the extremities and trunk. Before onset, she was in good health and took no medication. Physical examination revealed pinkish or reddish, edematous, well-demarcated erythema *(figure 1A)*. The lesions tended to coalesce, forming annular patterns, some of which had vesicles around the margins, forming a sunflower-like configuration. The oral cavity and genital area were unaffected. Histopathological findings of a pustule revealed intraepidermal blisters with neutrophil infiltrates without prominent acantholysis *(figure 1B)*. Laboratory examinations, including serum immunoglobulins, and ELISA for anti-desmoglein 1 and 3 were within normal ranges. Chest X-ray, electrocardiogram, and blood tests revealed no other related diseases and monoclonal gammopathy. DIF of the erythematous lesion revealed IgA deposition in the intercellular space throughout the epidermis *(figure 1C)*. IIF revealed circulating IgA autoantibodies binding to the cell surfaces of the entire epidermis of normal human skin (titer: 64×). Immunoblot analysis using epidermal extracts from normal human skin and recombinant desmocollin 3 showed no specific bands for either IgA or IgG antibodies. These findings led to the diagnosis of IEN-type IgA pemphigus. Treatment was initiated with topical corticosteroids, achieving only a slight effect; dapsone (50 mg per day) was therefore started. The pruritus and lesions improved but the symptoms recurred after four weeks. For that reason the dose was raised to 75 mg dapsone and the itchness subsided within a few days. Two weeks later, only pigmented macules with no active lesions were observed. The titer of IIF also decreased from 64× to 16×.

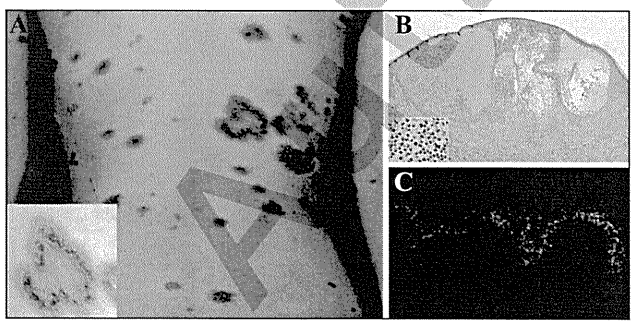


Figure 1. A) Pinkish and reddish edematous erythema with vesicles around the margins are scattered on the trunk. **B)** Histopathological findings of a pustule reveal intraepidermal blisters with neutrophil infiltrates. **C)** Direct immunofluorescence of the perilesional skin biopsy specimen reveals IgA deposits on the keratinocyte cell surfaces.

IgA pemphigus is a distinct group of auto-immune intraepidermal blistering diseases that present with vesiculopustular eruption, neutrophil infiltration with or without acantholysis. IgA autoantibodies that target keratinocyte cell surfaces and desmosomal components in the epidermis have been detected in DIF and IIF [1]. IgA pemphigus is divided into two major subtypes: the IEN type, and the SPD type. While SPD-type IgA pemphigus shows subcorneal pustules, the IEN type is characterized by pustule formation, mainly in the middle or lower epidermis.

In DIF, SPD-type IgA pemphigus involves cell surface IgA binding only in the upper epidermis, whereas IEN-type IgA pemphigus shows binding throughout the epidermis [2]. Desmocollin 1 has been identified as an autoantigen in SPD-type IgA pemphigus, suggesting that it plays an important role in the pathogenesis of this disease subtype [3]. Although autoantibodies against desmogleins [4] and desmocollins [5] have been reported in some cases of IEN-type IgA pemphigus, the specific autoantigen remains unidentified. In our case, we were also unable to detect specific autoantibodies using immunoblot analysis. Interestingly, a case with clinical and histological features compatible with SPD-type IgA pemphigus, but for which anti-desmocollins antibodies were not detected, was diagnosed as IEN-type IgA pemphigus [6]. That report suggested that the subtypes of IgA pemphigus might be considered to be divided by autoantigens.

In contrast to the common types of pemphigus, like pemphigus vulgaris, treatment for some cases of IgA pemphigus does not require corticosteroid or other immunosuppressive therapy. These cases of IgA pemphigus are well controlled using only anti-inflammatory treatments, such as dapsone, colchicine or isotretinoin [1]. Dapsone may be useful in treating IgA pemphigus due to its effect in suppressing neutrophilic infiltration. However refractory cases require plasmapheresis or cyclophosphamide. In the present case, oral administration of dapsone quickly caused the symptoms to subside. In IgA pemphigus, it is important to make the correct diagnosis and to choose a suitable therapy to avoid the side effects by the prolonged use of systemic corticosteroids. ■

Disclosure. *Financial support: none. Conflict of interest: none.*

doi:10.1684/ejd.2012.1662

To cite this article: Hirata Y, Abe R, Kikuchi K, Hamasaka A, Shinkuma S, Nomura T, Nishie W, Arita K, Shimizu H. Intraepidermal neutrophilic IgA pemphigus successfully treated with dapsone. *Eur J Dermatol* 2012; 22(2): 1-2 doi:10.1684/ejd.2012.1662

Department of Dermatology,
Hokkaido University Graduate
School of Medicine
N15 W7,
Sapporo 060-8638, Japan
<aberi@med.hokudai.ac.jp>

Yu HIRATA
Riichiro ABE
Kazuhiro KIKUCHI
Asuka HAMASAKA
Satoru SHINKUMA
Toshifumi NOMURA
Wataru NISHIE
Ken ARITA
Hiroshi SHIMIZU

1. Hashimoto T. Immunopathology of IgA pemphigus. *Clinics in dermatology* 2001; 19: 683-689.
2. Hashimoto T, Ebihara T, Nishikawa T. Studies of autoantigens recognized by IgA anti-keratinocyte cell surface antibodies. *Journal of dermatological science* 1996; 12: 10-17.
3. Hashimoto T, Kiyokawa C, Mori O, *et al.* Human desmocollin 1 (Dsc1) is an autoantigen for the subcorneal pustular dermatosis type of IgA pemphigus. *The Journal of Investigative Dermatology* 1997; 109: 127-131.
4. Karpati S, Amagai M, Liu WL, *et al.* Identification of desmoglein 1 as autoantigen in a patient with intraepidermal neutrophilic IgA dermatosis type of IgA pemphigus. *Experimental Dermatology* 2000; 9: 224-228.
5. Yasuda H, Kobayashi H, Hashimoto T, et al. Subcorneal pustular dermatosis type of IgA pemphigus: demonstration of autoantibodies to desmocollin-1 and clinical review. *The British journal of Dermatology* 2000; 143: 144-148.
6. Iida K, Sueki H, Ohyama B, *et al.* A unique case of intraepidermal neutrophilic dermatosis-type IgA pemphigus presenting with subcorneal pustules. *Dermatology* 2011; 222: 15-19.

doi:10.1684/ejd.2012.1662

Experimental dermatology • **Original article**

CED
Clinical and Experimental Dermatology

Conversion from human haematopoietic stem cells to keratinocytes requires keratinocyte secretory factors

Y. Fujita, D. Inokuma, R. Abe, M. Sasaki, H. Nakamura, T. Shimizu* and H. Shimizu

*Department of Dermatology, Hokkaido University Graduate School of Medicine, Sapporo, Japan; and *Department of Dermatology, Faculty of Medicine, University of Toyama, Toyama, Japan*

doi:10.1111/j.1365-2230.2011.04312.x

Summary

Background. Recent studies have reported that bone-marrow-derived stem cells (BMSCs), including haematopoietic stem cells (HSCs) and mesenchymal stromal cells, differentiate in order to regenerate various cellular lineages. Based on these findings, it is known that BMSCs can be used clinically to treat various disorders, such as myocardial infarction and neurotraumatic injuries. However, the mechanism of HSC conversion into organ cells is incompletely understood. The mechanism is suspected to involve direct cell–cell interaction between BMSCs, damaged organ cells, and paracrine-regulated soluble factors from the organ, but to date, there have been no investigations into which of these are essential for keratinocyte differentiation from HSCs.

Aim. To elucidate the mechanism and necessary conditions for HSC differentiation into keratinocytes *in vitro*.

Methods. We cultured human (h)HSCs under various conditions to try to elucidate the mechanism and necessary conditions for hHSCs to differentiate into keratinocytes.

Result. hHSCs cocultured with mouse keratinocytes induced expression of human keratin 14 and transglutaminase I. Only 0.1% of the differentiated keratinocytes possessed multiple nuclei indicating cell fusion. Coculture of hHSCs with fixed murine keratinocytes (predicted to stabilize cellular components) failed to induce conversion into keratinocytes. Conversely, keratinocyte-conditioned medium from both human and mouse keratinocytes was found to mediate hHSC conversion into keratinocytes.

Conclusions. Human HSCs are capable of differentiation into keratinocytes, and cell fusion is extremely rare. This differentiating is mediated by the plasma environment rather than by direct cell–cell interactions.

Introduction

Recent studies have reported that bone marrow-derived stem cells (BMSCs), including haematopoietic stem cells (HSCs) and multipotent mesenchymal stromal cells (MSCs), differentiate into various cellular lineages.[1–3] Based on these findings, BMSCs have been used to treat several disorders in animal models, including myocardial infarction, Parkinson disease and neurotraumatic injuries.[4–6] We previously used a murine bone-marrow transplantation model to show that HSCs can differentiate into functional keratinocytes *in vivo*.[7,8] Cutaneous T-cell-attracting chemokine (CTACK)/CCL27 was found to be the major regulator involved in the migration from bone marrow of keratinocyte precursor cells, which expressed CCR10, the receptor for the CTACK ligand.[7] In addition, we reported that bone marrow-derived MSCs also convert into keratin14-positive keratinocytes *in vivo* and *in vitro*.[9]

Correspondence: Dr Yasuyuki Fujita, Department of Dermatology, Hokkaido University Graduate School of Medicine, N15 W7, Kita-ku, Sapporo 060-8638, Japan
E-mail: yfujita@med.hokudai.ac.jp

The first two authors contributed equally to this work, and should be considered joint first authors.

Conflict of interest: none declared.

Accepted for publication 8 October 2011

The factor or mechanism governing the differentiation of HSCs into injured organ cells is not fully understood. Possible mechanisms include direct cell–cell interaction between peripheral haematopoietic progenitor cells and damaged organ cells, and involvement of paracrine-regulated soluble factors from the organ. Requirement of feeder cells such as 3T3 cells when culturing keratinocytes implies that direct cellular interactions play a major role in keratinocyte differentiation, proliferation and homeostasis. Previous papers have reported that use of secretory factors from damaged liver tissue enables HSCs to take on many of the characteristics of liver cells.[10]

We cultured human (h)HSCs under various conditions to elucidate the mechanism and necessary conditions of hHSC differentiation into keratinocytes.

Methods

The study was approved by the ethics committee of Hokkaido University Graduate School of Medicine, and volunteers signed consent forms approved by the Hokkaido University Graduate School of Medicine and the Hokkaido Red Cross Blood Centre Committee for the Protection of Human Subjects.[11] All animal procedures were conducted according to guidelines provided by the Hokkaido University Institutional Animal Care and Use Committee under an approved protocol.

Cells

Human peripheral blood CD34-positive cells, which are considered to be hHSCs, were collected. Recombinant human granulocyte colony-stimulating factor (G-CSF; Chugai Pharmaceutical Co. and Kyowa Hakko Pharmaceutical Co., Tokyo, Japan) was administered to the healthy subjects. Mobilized hHSCs were then isolated from peripheral blood using immunomagnetic beads with an antibody against CD34 as described previously.[11,12]

Pam 212, a murine keratinocyte cell line, was derived from spontaneously transformed BALB/c keratinocytes. Murine dermal fibroblasts were obtained from the dorsal skin surface of C57BL/6 mice. Normal human epidermal keratinocytes (NHEKs; Cambrex, East Rutherford, NJ, USA) were used as controls.

Coculture of hHSCs

To distinguish the differentiated HSC-derived keratinocytes and cocultured keratinocytes, we chose hHSCs and murine keratinocytes Pam 212 to coculture. PAM 212 and murine dermal fibroblasts were grown separately on eight-well culture slides to 80% confluence in DMEM (Invitrogen, Grand Island, NY, USA), and NHEKs were grown in Konjac glucomannan medium (KGM, Lonza Walkersville, Walkersville, MD, USA). Each cell was washed twice with phosphate-buffered saline (PBS) and then 1×10^5 hHSCs were added to each well and cocultured for 5 days in RPMI medium (Invitrogen). Each experiment was repeated three times.

Paraformaldehyde fixation of keratinocytes

In coculture with fixed cells, Pam 212 cells or NHEKs were grown on eight-well culture slides to 80% confluence, and then fixed with 0.5% paraformaldehyde for 15 min at 25 °C. Each well was washed twice with PBS, and 1×10^5 hHSCs per well were cultured with fixed keratinocytes for 5 days with RPMI.

Preparation of conditioned medium

Pam 212 cells or NHEKs at 80% confluence were washed twice with PBS, and cultured for 48 h with RPMI. The conditioned media were centrifuged at 2500 ***g*** for 10 min, filtered through 0.22-μm filters (Millipore, Billerica, MA, USA), and stored at − 80 °C until use. These conditioned media were then used to culture 1×10^5 hHSCs on eight-well culture slides with the conditioned media collected from the Pam 212 cells or NHEKs.

Culture with secreted factors

The hHSCs (1×10^5) were plated onto eight-well slides as before, and cultured in keratinocyte basal medium (Invitrogen) containing 0.5 nmol/L bone morphogenetic protein-4 (R&D Systems, Minneapolis, MN, USA), keratinocyte growth factor (KGF; Invitrogen) or interleukin-1-induced growth factor (IGF; Invitrogen). After 48 h of culture, hHSCs were stained to investigate their differentiation into keratinocytes.

Immunocytochemistry

Skin samples were embedded in optimal cutting temperature compound (Sakura Finetek Japan, Tokyo, Japan), then cut on a cryostat into 5 μm sections, which were placed onto microscope slides. The slides were used for indirect immunofluorescence using the following primary antibodies: human cytokeratin (CK)5 (catalogue no. RCK103) and human CK14 (LL002) (both Santa Cruz Biotechnology, Santa Cruz, CA, USA), human transglutaminase 1 (B.C1) and human involucrin (rabbit polyclonal (both Biomedical Technologies, Stoughton,

MA, USA), human N-cadherin (GC4; Sigma-Aldrich, St. Louis, MO, USA), anti-pankeratin goat polyclonal, human α6-integrin (GoH3) and human HLA-ABC (G46-2.6) (all BD Biosciences Pharmingen, San Jose, CA, USA), and human nuclei (235-1; Millipore).

Fluorescence staining was investigated using a confocal laser scanning fluorescence microscope (Laser Scanning Confocal Imaging System MRC 1024; Bio-Rad, Richmond, CA, USA). Cells that stained positive to human (human nuclei or HLA-ABC) and keratinocyte markers were counted as positive for bone marrow-derived keratinocytes.

Statistical evaluation of results

Statistical analysis of differences in the means for each experimental group was carried out using the Student *t*-test, with significance set at $P < 0.05$.

Results

Human nuclei-positive and cytokeratin-positive cells are derived from human haematopoietic stem cells

No cells stained positively with antibodies against pankeratin, human CK5 or CK14, human transglutaminase I or human involucrin. Furthermore, culture of hHSCs in RPMI medium for 5 days did not result in any cells positive for any of these five antibodies. Spontaneous conversion of hHSCs to keratinocytes seldom happened.

Next, we investigated whether coculture with keratinocytes mediates hHSC differentiation into keratinocytes. Using a specific antibody against human nuclei,[13] we detected human nuclei+/cytokeratin+ cells (hNCs) after coculture of hHSCs and Pam 212 cells for 5 days (Fig. 1a). We also found human nuclei+/cytokeratin 14+ cells and human cytokeratin 5+ cells (Fig. 1b,c). These hNCs expressed HLA-ABC as a human origin marker (Fig. 1d). Furthermore, hNCs expressed human transglutaminase 1 and involucrin as other specific markers of keratinocytes (Fig. 1e). The number of hNCs increased relative to the coculture time, from $0.03 \pm 0.002\%$ at 6 h to $0.46 \pm 0.11\%$ at 48 h (Fig. 1f). However, coculture for 5 days did not introduce any colonies of hHSCs or hNCs.

Human haematopoietic stem cells cultured with fixed keratinocytes do not express keratinocyte markers

To determine whether the hNCs were generated through cell fusion or true differentiation, we examined whether multinucleate cells could result from cell fusionh between HSCs and keratinocytes.[14] Most hNCs were microscopically uninucleate, and only 0.1% of hNCs were binucleate, with human and nonhuman (presumed mouse) nuclei (Fig. 2a). To exclude the possibility of cell fusion at the initial transition of hHSCs to keratinocytes, hHSCs were cultured with 0.5% paraformaldehyde-fixed Pam 212 cells or NHEKs. It was predicted that the fixed cells would stabilize the cellular components, rendering the live hHSCs resistant to fusion. This method has been shown to prevent fusion of live cells with fixed cells, while not disrupting receptor-mediated recognition and association of these cell types.[15] This procedure failed to convert hHSCs to hNCs (Fig. 2b), suggesting that hHSCs seldom fuse with keratinocytes, and that the cell-surface molecules of keratinocytes do not induce hHSC differentiation into keratinocytes.

Human haematopoietic stem cells cultured with keratinocyte-conditioned media mediate their differentiation into keratinocytes

To clarify the potential role of the secretory factors released by keratinocytes, we treated hHSCs with the keratinocyte-conditioned medium from Pam 212 cells or NHEKs; interestingly, both types of medium induced hHSC conversion into keratinocytes (Fig. 2c). The number of hNCs in 10^4 hHSCs after 48 h in culture was 35 (0.35%) in NHEK-conditioned medium and 9 (0.09%) in Pam-212-conditioned medium (Table 1). The greater number in NHEK-conditioned medium compared with Pam-212-conditioned medium implies an association with species-specific factors. However, there were no significant differences between cultures with NHEK-conditioned medium and those with Pam-212-conditioned medium. Furthermore, 40 (0.40%) hNCs were detected in culture with fixed NHEKs in NHEK-conditioned medium, and 29 (0.29%) in culture with fixed Pam 212 cells in Pam-212-conditioned medium. Fixed keratinocytes seemed to accelerate the conversion of hHSCs with keratinocyte-conditioned medium, but the difference was not significant. In addition, as a control of cell type for the conditioned media, fibroblast-conditioned media never induced hHSCs to convert into keratinocytes.

To explore any additional effects of keratinocyte surface molecules on differentiation, we added blocking antibodies against keratinocyte surface molecules during culture. We chose human α6-integrin and human N-cadherin as the surface molecules, as these molecules are expressed on various stem cells, including epidermal

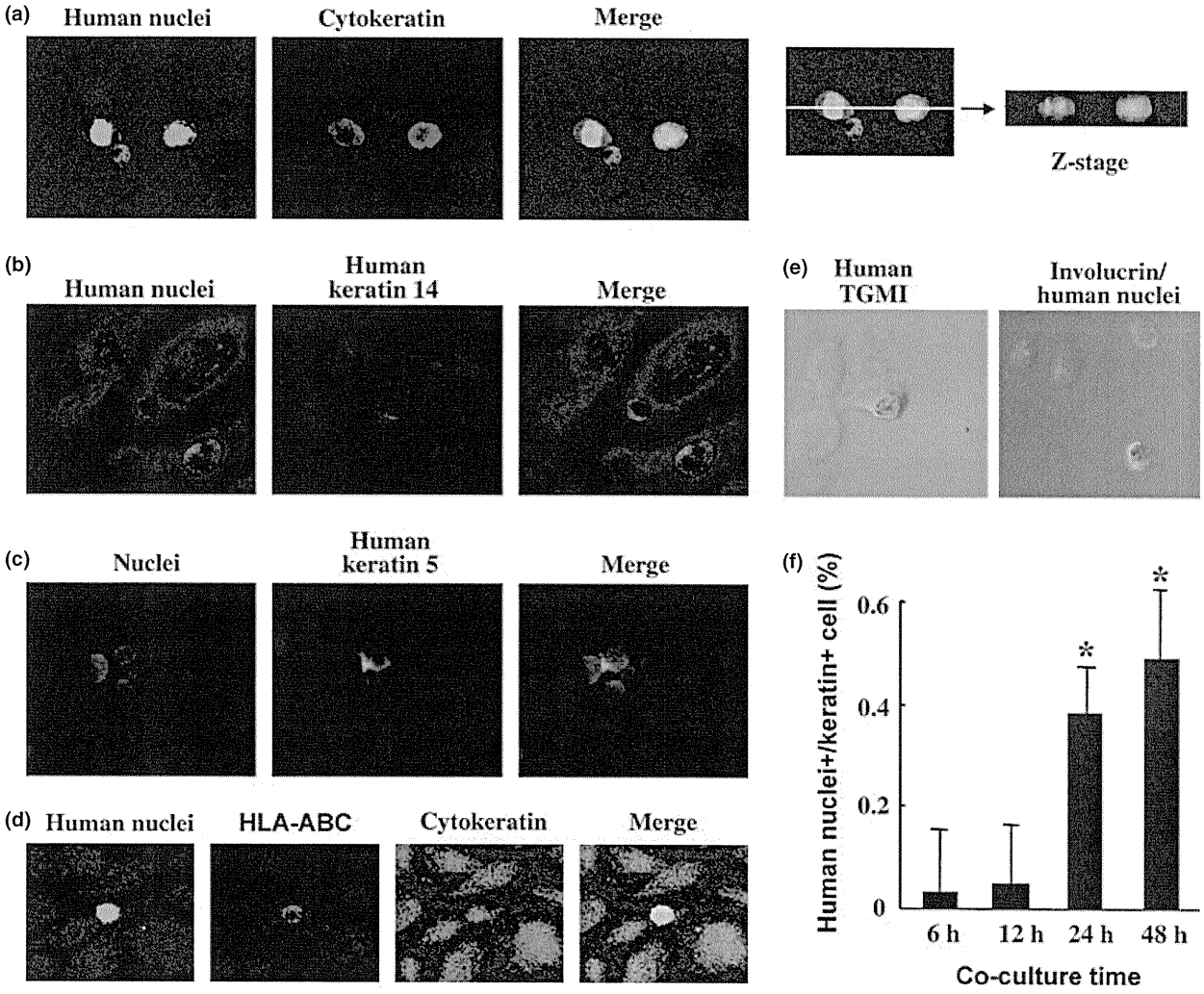


Figure 1 Coculture of human haematopoietic stem cells (hHSCs) and a mouse keratinocyte cell line, Pam212. (a) Expression of human nuclei (green) and cytokeratin (red) after 2 days in coculture, and cross-sectional analysis of the same cells (Z-axis). (b) Expression of human nuclei (green) and human cytokeratin 14 (red) after 2 days in coculture. (c) Expression of human cytokeratin 5 (green) and nuclei (propidium iodide staining, red) after 2 days in coculture. (d) Expression of human nuclei (green), human leucocyte antibody-ABC (red) and cytokeratin (blue). (e) Left: expression of human transglutaminase I (green) with transmission after 2 days in coculture; right: expression of involucrin (green) and human nuclei (red) with transmission after 2 days in coculture. (f) Percentages of hHSCs expressing keratin after 6, 12, 24 and 48 h in culture. $^*P < 0.05$ vs. 6 h.

stem cells, playing an important role in differentiation.[16,17] Blocking antibodies during coculture of hHSCs and fixed NHEK with NHEK-conditioned medium did not influence the keratinocyte conversion (data not shown).

It is possible that the humoral induction of keratinocyte differentiation is mediated by a specific growth factor such as KGF and IGF.[18] However, we did not observe hNCs with exposure of hHSCs to KGF or IGF, which are secreted exclusively from keratinocytes (data not shown). These findings suggest that soluble factors other than KGF and IGF in keratinocyte supernatant may mediate HSC differentiation.

Discussion

We have shown that hHSCs differentiate into keratinocytes in the presence of factors secreted from keratinocytes, without cell fusion. In this study, hHSCs converted into keratinocytes when cocultured with keratinocytes. By contrast, hHSCs cocultured with fixed keratinocytes were found never to convert into

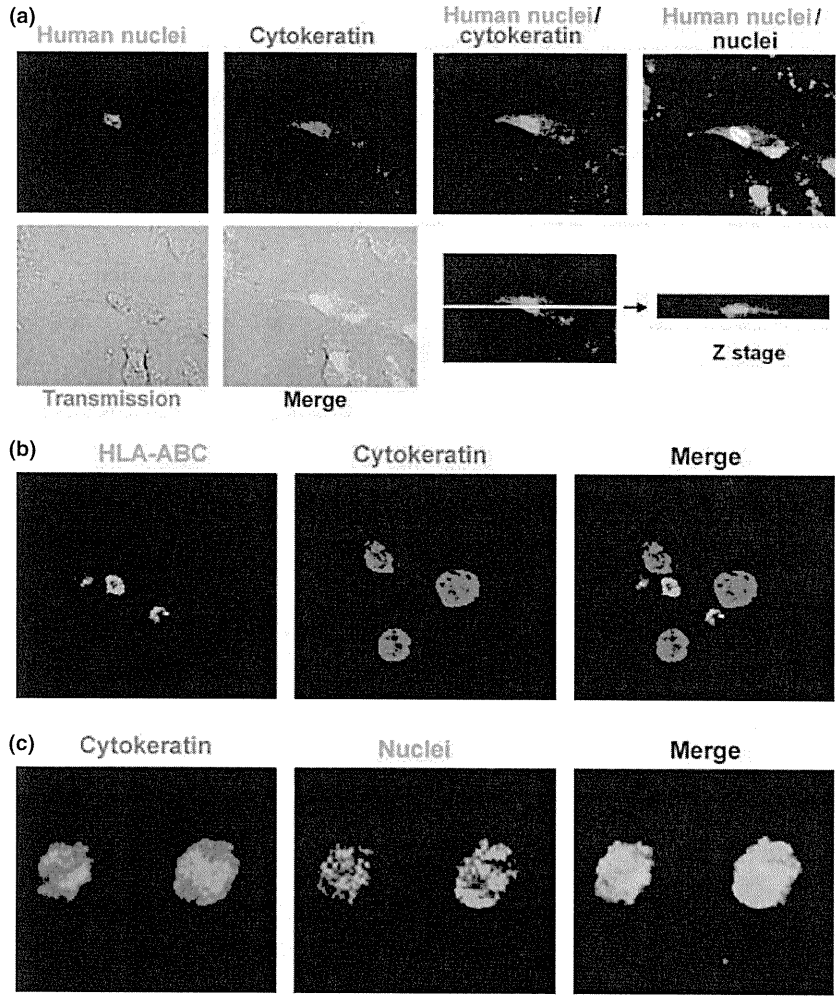


Figure 2 Coculture of human haematopoietic stem cells (hHSCs) and Pam212 cells fixed with 0.5% paraformaldehyde, and culture of hHSCs in conditioned medium of Pam 212 cells. (a) Expression of human nuclei (green), mouse nuclei (blue) and cytokeratin (red) after 2 days in coculture of hHSCs and fixed Pam212 cells, and analysis of the same cells by Z-axis or transmission. (b) Expression of human leucocyte antibody-ABC (green) and cytokeratin (red). (c) Expression of cytokeratin (blue) and human nuclei (green) in culture of hHSCs in the conditioned medium of Pam 212 cells.

Table 1 Frequency of cytokeratin-positive cells derived from human haematopoietic stem cells (hHSCs).

Treatment	CK-positive cells, *n**
Coculture with Pam 212 cells	49
Coculture with fixed Pam 212 cells	0
Coculture with mouse fibroblasts	0
Culture in Pam 212 CM	9
Culture with fixed Pam 212 in Pam-212 CM	29
Culture in NHEK CM	35
Culture with fixed NHEKs in NHEK CM	40

CK, cytokeratin; CM, conditioned medium; NHEK, normal human epidermal keratinocyte. *In 10^4 hHSCs.

keratinocytes, and hHSCs cultured with keratinocyte-conditioned medium expressed keratinocyte-specific markers. These data support the existence of factors secreted from keratinocytes or the existence of relatively paraformaldehyde-sensitive cell-surface molecules that induce hHSCs to differentiate into keratinocytes.

We did not observe differentiation after exposure of hHSCs to the growth factors KGF or IGF, which suggests that other soluble factors might mediate HSC differentiation. Indeed, a previous report on hepatocyte differentiation showed that the specific growth factors hepatocyte growth factor and fibroblast growth factor 4 failed to

mediate such conversion.[10] Further investigation is required to identify specific soluble factors that affect differentiation of HSCs to keratinocytes.

Recently Mortier *et al.*[19] succeeded in generating a skin equivalent model from human cord-blood cells, which contains heterogeneous cells including hHSCs and MSCs. Although the origin of the induced keratinocytes was not investigated, we propose that most of these cells are mesenchyme-derived, as our observation showed that purified hHSCs seldom convert to keratinocytes.

Murine BMSCs can contribute to the regeneration of injured adult tissues of various organs, including brain, liver and heart tissue, after bone-marrow transplantation.[1,3,20] These unexpected events were initially attributed to BMSC transdifferentiation, supporting the emerging idea of extended plasticity of adult stem cells. The alternative hypothesis of spontaneous cell fusion has also been proposed as the primary cause of unexpected cell-fate switches of BMSCs into various cell lineages.[21,22]

We found that the number of fused multinucleate cells (which are unlikely to undergo further cell division) in the skin was very low. Conversely, Fujino *et al.*[23] reported the observation of fused functional hepatocytes after hHSC injection into immunodeficient mice. Taking these results into consideration, it is likely that both cell fusion and conversion from HSCs play some role in the repair of damaged tissue.

Previously, we reported that CTACK/CCL27 accelerates skin regeneration via accumulation of BMDCs.[7] Furthermore, bone-marrow transplantation improves type XVII collagen-knockout epidermolysis bullosa (EB) mice, in which the deficient type XVII collagen, a cutaneous structure protein produced by keratinocytes, was restored by BMSCs.[8] Because there have been ethical and safety concerns in using embryonic stem cells and induced pluripotent stem cells, therapies using HSCs are thought to be safer.[24] In the near future, stem-cell therapies might be a candidate for the treatment of severe EB, for which there is no effective treatment other than palliative care.[25]

Conclusion

When exposed to skin tissue, hHSCs are capable of taking on many characteristics of the skin cell types, and this is mediated by the plasma environment rather than by direct cell–cell interactions, including the specific gene and/or protein expression and function of the cells.

Learning points

- It is known that HSCs have the potential for conversion into keratinocytes.
- Several mechanisms, including direct cell–cell interaction between HSCs and damaged skin, and involvement of paracrine-regulated soluble factors from the organ, have been suggested; however, there have been no reports identifying the precise mechanism involved.
- In this study, we found that the conversion of HSCs into keratinocytes is mediated by the plasma environment rather than by direct cell–cell interactions.

Acknowledgements

We are grateful to Ms Yuika Osaki for her excellent technical assistance. This work was supported in part by grants-in-aid for Scientific Research (No. 13357008 to HS and no. 15790563 to RA) and by the Project for Realization of Regenerative Medicine (to HS) from the Ministry of Education, Culture, Sports, Science and Technology, Japan and by grant-in-aid for Young Scientist (A, No. 23689053 to YF) from Japan Society for the Promotion of Science and by the Health and Labor Sciences Research Grants (No. H13-Measures for Intractable Disease-02 HS) from the Ministry of Health, Labor and Welfare of Japan.

References

1 Jackson KA, Majka SM, Wang H *et al.* Regeneration of ischemic cardiac muscle and vascular endothelium by adult stem cells. *J Clin Invest* 2001; **107**: 1395–402.
2 Orlic D, Kajstura J, Chimenti S *et al.* Bone marrow cells regenerate infarcted myocardium. *Nature* 2001; **410**: 701–5.
3 LaBarge MA, Blau HM. Biological progression from adult bone marrow to mononucleate muscle stem cell to multinucleate muscle fiber in response to injury. *Cell* 2002; **111**: 589–601.
4 Chang SA, Lee EJ, Kang HJ *et al.* Impact of myocardial infarct proteins and oscillating pressure on the differentiation of mesenchymal stem cells: effect of acute myocardial infarction on stem cell differentiation. *Stem Cells* 2008; **26**: 1901–12.
5 Dezawa M, Kanno H, Hoshino M *et al.* Specific induction of neuronal cells from bone marrow stromal cells and application for autologous transplantation. *J Clin Invest* 2004; **113**: 1701–10.