Material and methods

Immunofluorescence analysis

Direct immunofluorescence (IF) for detecting deposits of IgG, IgA, IgM, C3 and C1q was performed on perilesional skin biopsy specimens from the patient. Indirect IF was performed on normal human skin and 1M NaCl-split normal human skin as described previously [10].

Immunoblot analysis

Epidermal and dermal extracts of normal human skin, supernatants of cultured HaCaT cells and recombinant proteins (NC16A and the C-terminal (BP915) domains of COL17) were prepared as described previously [11-14]. Purified laminin-332 was supplied as a gift from Dr. S. Amano of Shiseido Life Science Research Center, Yokohama, Japan [15,16]. Immunoblotting was performed as described previously [5]. For IgG detection, nitrocellulose membranes were incubated with 1 : 20 diluted serum overnight at 4℃. Bound antibodies were visualized enzymatically using 1 : 100 diluted HRP-conjugated rabbit antihuman IgG for 3 h at room temperature. For IgA detection, membranes were incubated with 1 : 20 diluted serum overnight at 37℃, then incubated in 1 : 50 diluted HRP-conjugated rabbit antihuman IgA for 3 h at room temperature. Colour was

developed with 4-chloro-1-naphthol in the presence of H_2O_2 [5].

Results

Immunofluorescence analysis

Direct IF microscopy showed linear deposition of IgG (Fig. 2a), IgA (Fig. 2b) and C3 (data not shown) at the basement membrane zone (BMZ). Indirect IF of normal human skin demonstrated circulating IgG (titre 1 : 320) and IgA (titre 1 : 32) reacting with the BMZ (data not shown). Indirect IF using 1M NaCl-split normal human skin revealed linear deposition of IgG (Fig. 2c) (titres of 1 : 320 and 1 : 80, epidermal and dermal sides, respectively) and IgA (Fig. 2d) (titres of 1 : 32 and 1 : 16, epidermal and dermal sides, respectively).

Immunoblot analysis

Immunoblot analysis using epidermal and dermal extracts from normal human skin, the recombinant NC16A and C-terminal domains of COL17, purified laminin-332 and a cell culture supernatant of HaCaT cells, from which the 120-kDa soluble ectodomain (LAD-1) of COL17 was isolated[17], were performed. We found circulating IgG autoantibodies against the NC16A domain (Fig. 3a), the C-terminal domain (Fig. 3b) and the 120-kDa soluble ectodomain (Fig. 3c) of COL17, and the γ2 subunit of laminin-332 (Fig. 3d).

Further immunoblotting revealed that IgA autoantibodies reacted with the γ2 subunit of laminin-332 (Fig. 3d) and faint reactivity with the NC16A domain and the 120-kDa soluble ectodomain of COL17 (data not shown). IgA autoantibody against the C-terminal domain of COL17 was negative.

Neither IgG nor IgA against BP230, type VII collagen or p200 protein were detected in epidermal and dermal extracts (data not shown).

Discussion

We describe an uncommon case of MMP with multiple mucosal involvement as well as generalized blisters, which predominantly healed with scar formation. Initially, the widespread bullae and circulating IgG against the NC16A domain of COL17 led us to diagnose bullous pemphigoid (BP). Detailed immunohistochemical examination, however, showed that both IgA and IgG reacted with laminin-332 as well as with COL17. From these results, the patient's disease could be diagnosed as MMP with generalized blisters, BP with extensive mucosal involvement or subepidermal autoimmune blistering disease with overlapping features of MMP and BP. In this case, because of multiple mucosal lesions and the unusual scar formation, we finally made the diagnosis of MMP with generalized blisters.

A variety of different autoantigens are recognized by circulating autoantibodies from patients with MMP and it is possible that the unusual autoimmune profile developed as a result of epitope spreading. In this case, in addition to IgG directed against laminin-332 and the noncollagenous 16A (NC16A) and C-terminal domains of COL17, circulating IgA reacted with laminin-332 and with the NC16A domain of COL17 were also detected. Previously, antibodies against laminin-332 are found in about 10% to 20%

of MMP patients [18]. Passive transfer studies in newborn and adult mice have shown that polyclonal antibodies to human laminin-332, generated from rabbits, bind epidermal basement membrane and produce subepidermal blisters of skin and mucous membranes like those seen in patients with MMP [6,19]. In contrast, approximately 40 to 70% of patients with MMP show autoantibodies to multiple sites on COL17 [19]. Rabbit antibodies, generated against the murine homologue of the NC16A domain of human COL17, induce subepidermal blisters when passively transferred into neonatal mice, and that resemble those seen in patients with BP and some forms of MMP [19,20]. The *in vivo* pathogenicity of autoantibodies to the C-terminal domain of COL17 has not yet been demonstrated [21].

In contrast to BP, which is the most common autoimmune blistering disorder induced by autoantibodies against the NC16A domain of COL17, cutaneous involvement of MMP is usually limited to small areas [22-24]. To date, there have been only a few cases of MMP with widespread blisters [23,24]. Interestingly, these cases had autoantibodies against the NC16A domain of COL17 [23,24]. Our patient also presented with widespread cutaneous blisters, and ELISA and immunoblot analysis revealed antibodies against the NC16A domain of COL17. These findings suggest that the presence of autoantibodies against

the NC16A domain of COL17 may be associated with the clinical involvement of wide spread blisters in MMP patients.

IgG is the main immunoglobulin subtype that has been confirmed as an autoantibody against laminin-332 and/or COL17 in sera from patients with MMP [4,5]. In addition, IgA autoantibodies against COL17 are another major immunoglobulin subtype found in sera from MMP patients, and the presence of both IgG and IgA anti-COL17 antibodies has been associated with more severe and persistent clinical features [21,25,26]. However, to the best of our knowledge, there are no reports of IgA autoantibodies against both laminin-332 and COL17 being detected in MMP sera [5]. In the previous study, a patient with IgA autoantibodies against laminin-332 had severe conjunctival involvement with multiple bullae on the extremities and was refractory to systemic steroid therapy [5]. Our patient was also intractable to steroid treatment and needed relatively high doses of oral prednisolone, azathioprine and diaphenylsulfone. The presence of IgA anti-laminin-332 might be associated with poor response to steroid treatment, although the correlation between clinical manifestations and the profile of autoantibody subtypes is difficult to determine due to limited number of patients.

Acknowledgements

We thank Dr. Satoshi Amano for providing purified laminin-332, Mr. Mike O'Connell for his proofreading, and Ms. Mika Tanabe for her technical assistance.

References

1 Chan LS, Ahmed AR, Anhalt GJ *et al.* The first international consensus on mucous membrane pemphigoid: definition, diagnostic criteria, pathogenic factors, medical treatment, and prognostic indicators. *Arch Dermatol* 2002; **138**: 370-9.

2 Nakatani C, Muramatsu T, Shirai T. Immunoreactivity of bullous pemphigoid (BP) autoantibodies against the NC16A and C-terminal domains of the 180 kDa BP antigen (BP180): immunoblot analysis and enzyme-linked immunosorbent assay using BP180 recombinant proteins. *Br J Dermatol* 1998; **139**: 365-70.

3 Lazarova Z, Salato VK, Lanschuetzer CM *et al.* IgG anti-laminin-332 autoantibodies are present in a subset of patients with mucous membrane, but not bullous, pemphigoid. *J Am Acad Dermatol* 2008; **58**: 951-8.

4 Schmidt E, Skrobek C, Kromminga A *et al.* Cicatricial pemphigoid: IgA and IgG autoantibodies target epitopes on both intra- and extracellular domains of bullous pemphigoid antigen 180. *Br J Dermatol* 2001; **145**: 778-83.

5 Natsuga K, Nishie W, Shinkuma S *et al.* Circulating IgA and IgE autoantibodies in antilaminin-332 mucous membrane pemphigoid. *Br J Dermatol* 2010; **162**: 513-7.

6 Lazarova Z, Yee C, Darling T *et al.* Passive transfer of anti-laminin 5 antibodies induces subepidermal blisters in neonatal mice. *J Clin Invest* 1996; **98**: 1509-18.

7 Lazarova Z, Hsu R, Yee C *et al.* Human anti-laminin 5 autoantibodies induce subepidermal blisters in an experimental human skin graft model. *J Invest Dermatol* 2000; **114**: 178-84.

8 Zone JJ, Egan CA, Taylor TB *et al.* IgA autoimmune disorders: development of a passive transfer mouse model. *J Investig Dermatol Symp Proc* 2004; **9**: 47-51.

9 Nishie W, Sawamura D, Goto M *et al.* Humanization of autoantigen. *Nat Med* 2007; **13**: 378-83.

10 Gammon WR, Briggaman RA, Inman AO *et al.* Differentiating anti-lamina lucida and anti-sublamina densa anti-BMZ antibodies by indirect immunofluorescence on 1.0 M sodium chloride-separated skin. *J Invest Dermatol* 1984; **82**: 139-44.

11 Dmochowski M, Hashimoto T, Bhogal BS *et al.* Immunoblotting studies of linear IgA disease. *J Dermatol Sci* 1993; **6**: 194-200.

12 Schäcke H, Schumann H, Hammami-Hauasli N *et al.* Two forms of collagen XVII in keratinocytes. A full-length transmembrane protein and a soluble ectodomain. *J Biol Chem* 1998; **273**: 25937-43.

13 Zillikens D, Rose PA, Balding SD *et al.* Tight clustering of extracellular BP180 epitopes recognized by bullous pemphigoid autoantibodies. *J Invest Dermatol* 1997; **109**: 573-9.

14 Nie Z, Garrod DR, Chan LS *et al.* Complementary peptides against the major epitope in the NC16A domain of BP180 show no specificity as vaccines to bullous pemphigoid. *J Dermatol Sci* 1999; **21**: 157-64.

15 Tsunenaga M, Adachi E, Amano S *et al.* Laminin 5 can promote assembly of the lamina densa in the skin equivalent model. *Matrix Biol* 1998; **17**: 603-13.

16 Amano S, Nishiyama T, Burgeson RE. A specific and sensitive ELISA for laminin 5. *J Immunol Methods* 1999; **224**: 161-9.

17 Pas HH, Kloosterhuis GJ, Heeres K, *et al.* Bullous pemphigoid and linear IgA dermatosis sera recognize a similar 120-kDa keratinocyte collagenous glycoprotein with antigenic cross-reactivity to BP180. *J Invest Dermatol* 1997; **108**: 423-9.

18 Kikuchi K, Natsuga K, Shinkuma S, *et al.* Subepidermal blistering disease with 3 distinct autoantibodies: anti-BP230, anti-laminin gamma-1, and anti-laminin-332. *J Am Acad Dermatol* 2011; **65**: 878-80.

19 Sitaru C, Zillkens D. Mechanisms of blister induction by autoantibodies. *Exp Dermatol* 2005; **14**: 861-75.

20 Liu Z, Diaz LA, Troy JL, *et al.* A passive transfer model of the organ-specific autoimmune disease, bullous pemphigoid, using antibodies generated against the hemidesmosomal antigen, BP180. *J Clin Invest* 1993; **92**: 2480-8.

21 Kurosh AS, Yancey KB. Pathogenesis of mucous membrane pemphigoid. *Dermatol Clin* 2011; **29**: 479-84.

22 Ujiie H, Nishie W, Shimizu H. Pathogenesis of bullous pemphigoid. *Dermatol Clin* 2011; **29**: 439-46.

23 Inaoki M, Ishii N, Hashimoto T *et al.* Cicatricial pemphigoid with widespread bullous eruption. *J Dermatol* 2006; **33**: 727-9.

24 Mulyowa GK, Jaeger G, Sitaru C *et al.* Scarring autoimmune bullous disease in a Ugandan patient with autoantibodies to BP180, BP230, and laminin 5. *J Am Acad Dermatol* 2006; **54**: S43-6.

25 Setterfield J, Shirlaw PJ, Kerr-Muir M *et al.* Mucous membrane pemphigoid: a dual circulating antibody response with IgG and IgA signifies a more severe and persistent disease. *Br J Dermatol* 1998; **138**: 602-10.

26 Setterfield J, Shirlaw PJ, Bhogal BS *et al.* Cicatricial pemphigoid: serial titres of circulating IgG and IgA antibasement membrane antibodies correlate with disease activity. *Br J Dermatol* 1999; **140**: 645-50.

Figure legends

Fig. 1. Clinical manifestations and histopathological findings

Multiple erythema, bullae and erosions on the back (a) and forearm (b), and esophageal erosions (c) were observed at the initial visit. Refractory ulcers on the perianal area were also observed (d). After 3 months of treatment with systemic prednisolone, azathioprine and diaphenylsulfone, the skin lesions gradually healed with hyperpigmentation and scar formation (e, f). The esophageal erosions also slowly resolved (g).

A skin biopsy specimen obtained from the edge of blister on the back showed a subepidermal blister with inflammatory cell infiltrates, including eosinophils, lymphocytes and neutrophils (h). (Haematoxylin and eosin stain, original magnification x200)

Fig. 2. Immunofluorescence studies

Direct immunofluorescence of the cutaneous lesions revealed *in vivo* linear IgG (a) and IgA (b) deposition at the BMZ (arrowheads). Indirect immunofluorescence microscopy of 1M NaCl-split normal human skin showed that circulating IgG (c) and IgA (d) anti-BMZ antibodies reacted with both epidermal and dermal sides (arrowheads). (original magnification x200; indirect immunofluorescence, serum dilutions were 1 : 40 (c) and 1 :

8 (d).)

Fig 3. Immunoblot analysis using the recombinant NC16A and C-terminal domains of COL17, the 120-kDa soluble ectodomain of COL17 and purified laminin-332

(a) The recombinant NC16A-COL17 and GST fusion protein (lane 1: control bullous pemphigoid (BP) IgG; lane 2: the patient's IgG). Circulating IgG autoantibodies reacted with NC16A-COL17 (arrow).

(b) BP915-COL17 (the recombinant protein of the C-terminal domain of COL17 amino acids spanning 1227 to 1532) and the GST fusion protein (lane 1: control BP IgG; lane 2: the patient's IgG). IgG against the C-terminal domain of COL17 was detected (arrow).

(c) The 120-kDa soluble ectodomain of COL17 (lane 1: control BP IgG; lane 2: the patient's IgG).

(d) The purified laminin-332 (lane 1: ponceau S stain sample; lane 2: the patient's IgG; lane 3: the patient's IgA). Both IgG and IgA from the patient reacted with the 105-kDa, γ2 subunit of laminin-332 (arrow).

Lichen Planus in Childhood Showing Various Cutaneous Features

Yohei Hamade[1], Ken Arita[1], Ellen Toyonaga[1], Nao Saito[1], Daisuke Inokuma[1], Kokichi Hamasaka[2] and Hiroshi Shimizu[1]
[1]Department of Dermatology, Hokkaido University Graduate School of Medicine, North 15 West 7, Kita-ku, Sapporo 060-8638 and [2]Sapporo City, Japan.
E-mail: hinhira@yahoo.co.jp
Accepted August 17, 2011.

Lichen planus (LP) in childhood is less common than in adulthood, with cases in individuals under 14 years of age estimated to account for no more than 10% of cases of LP (1). We describe here a child with LP who showed various cutaneous presentations.

CASE REPORT

A 5-year-old boy presented with papules on his right leg lasting for 6 months, which had gradually progressed to his trunk and extremities. He had a history of cytomegalovirus (CMV) hepatitis. His general condition was good and he was not taking any medications. On physical examination, keratotic papules and plaques with a peachblow-colour were observed on his legs. On the right leg, the papules were arranged linearly (Fig. 1a). On the dorsum of the right foot, hyperkeratosis and hypertrophy of the eruptions was conspicuous, showing a verrucous appearance (Fig. 1b). Miliary, lustrous eruptions were scattered on his abdomen, most of which were folliculo-centred (Fig. 1c). The oral mucosae and nails were normal.

Blood tests showed a small number of atypical lymphocytes (1% of 5,200/mm^3 white blood cells (WBC)) and liver dysfunction (aspartate transaminase (AST) 99 IU/l, alanine transaminase (ALT) 80 IU/l). Cytomegalovirus-immunoglobulin G (CMV-IgG) and antibodies for Epstein-Barr (EB) virus (EB nuclear antigen (EBNA)-IgG and viral capsid antigen (VCA)-IgG) were positive, although CMV-IgM and VCA-IgM were negative. Antibodies for the hepatitis B virus (HBV) and the hepatitis C virus (HCV) were negative.

Histopathological examination of a skin biopsy specimen taken from a verrucous papule on the dorsum of the right foot showed epidermal ortho-hyperkeratosis, wedge-shaped hypergranulosis, serrated change in the epidermal rete ridge, and band-like infiltration of lymphocytes in the superficial dermis. In addition to above-mentioned features, a skin biopsy specimen taken from a papule on the abdomen showed marked vacuolar degeneration of the epidermis and many Civatte bodies.

DISCUSSION

This case presented various clinical phenotypes of LP, including linear LP, hypertrophic LP and follicular LP. Linear LP and hypertrophic LP each has been reported as accounting for 10% of cases of childhood LP, and follicular LP as accounting for approximately 4% of cases of childhood LP (2). Linear LP is less common in adult (< 1% of all LP) (2) and is considered a characteristic feature of childhood LP. The simultaneous existence of different phenotypes of LP in a single patient has been reported in several papers (3); however, to our knowledge, no report has shown simultaneous presentation of linear, hypertrophic and follicular LP, which were shown in our case.

LP is often induced by drugs, dental metals, chronic liver disease, viral infection, etc. The association of HBV or HCV infection with LP has been given particular focus (4). In this case, laboratory data of liver dysfunction together with the finding of atypical lymphocytes suggested the reactivation of EBV or CMV, which might be related to the aetiology of the LP.

Topical tacrolimus was applied as a treatment, and the eruptions gradually subsided within 6 months. Several reports have shown the usefulness of topical tacrolimus against LP, especially against LP in the oral cavity (5). This case showed a favourable clinical course during the topical treatment with tacrolimus.

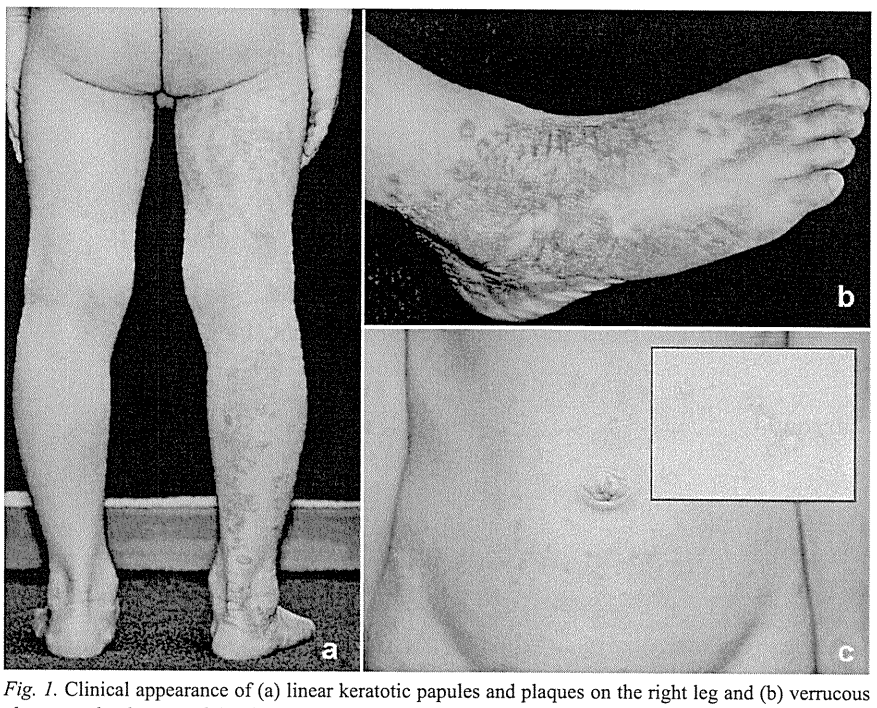

Fig. 1. Clinical appearance of (a) linear keratotic papules and plaques on the right leg and (b) verrucous plaque on the dorsum of the right foot. (c) Miliary, lustrous papules distributed almost consistently with hair follicles on the abdomen.

The authors declare no conflicts of interest.

 doi: 10.2340/00015555-1260
 ISSN 0001-5555

REFERENCES

1. Kumar V, Garg BR, Baruah MC, Vasireddi SS. Childhood lichen planus (LP). J Dermatol 1993; 20: 175–177.
2. Handa S, Sahoo B. Childhood lichen planus: a study of 87 cases. Int J Dermatol 2002; 41: 423–427.
3. Kanwar AJ, De D. Lichen planus in childhood: report of 100 cases. Clin Exp Dermatol 2010; 35: 257–262.
4. Rebora A, Robert E, Rongioletti F. Clinical and laboratory presentation of lichen planus patients with chronic liver disease. J Dermatol Sci 1992; 4: 38–41.
5. Vente C, Reich K, Rupprecht R, Neumann C. Erosive mucosal lichen planus: response to topical treatment with tacrolimus. Br J Dermatol 1999; 140: 338–342.

C E D | 4 3 1 2 | B
Journal Name | Manuscript No.
Dispatch: 16.12.11 | Journal: CED | CE: Gayathri J.
Author Received: | No. of pages: 7 | PE: Radhika

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 mechanisms of HSC conversion into organ cells are 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. We tried 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 differentiation 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's 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 been 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 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, Bedford, 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 were used: 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), and human nuclei (235-1; Millipore, Billerica, MA, USA).

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 cells 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 ± 0.002% at 6 h to 0.46 ± 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 hHSC to keratinocyte transition, 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 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 difference between cultures with NHEK-conditioned medium and those with Pam-212-conditioned medium. Furthermore, 40 (0.40%) hNCs were detected in culture with fixed NHEK 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 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 alpha 6-integrin and human N-cadherin as the surface molecules, as these molecules express on various stem cells, including

