



Contents lists available at ScienceDirect

Journal of Dermatological Science

journal homepage: www.jdsjournal.com



Research Paper

Altered balance of epidermis-related chemokines in epidermolysis bullosa

Inkin Ujiie^a, Yasuyuki Fujita^{a,*}, Chihiro Nakayama^a, Wakana Matsumura^a,
Shotaro Suzuki^a, Satoru Shinkuma^a, Toshifumi Nomura^a, Riichiro Abe^b,
Hiroshi Shimizu^{a,*}

^a Department of Dermatology, Hokkaido University Graduate School of Medicine, N15 W7, Kita-ku, Sapporo 060-8638, Japan

^b Department of Dermatology, Niigata University Graduate School of Medicine, 1-757, Asahimachi-dori, Chuo-ku, Niigata 951-8510, Japan

ARTICLE INFO

Article history:

Received 4 October 2016

Received in revised form 7 December 2016

Accepted 28 December 2016

Keywords:

Epidermolysis bullosa

Chemokine

CCL27

HMGB1

SDF-1

ABSTRACT

Background: Epidermolysis bullosa (EB) is a congenital, refractory skin disease and there are no fundamental treatments. Recently, allogenic cell therapies are beginning to be applied as potential treatments, that are based on the concept that the allogenic cells can migrate into the skin and reconstitute the skin components. Although the mechanisms of cell migration into skin are not fully understood, chemokines are regarded as key factors in recruiting bone marrow-derived cells.

Objectives: Our study aims to elucidate the expression of chemokines in the EB patients.

Methods: We determined the expression of wound-healing related chemokines in the sera, keratinocytes, and skin tissues of EB patients and compared them to those of healthy volunteers by enzyme-linked immunosorbent assays, quantitative reverse transcription PCR, and immunofluorescence staining.

Results: The serum levels of CXCL12 and HMGB1 were found to be significantly elevated in the EB patients. Conversely, the serum levels of CCL21 were found to be lower in the EB patients than in healthy controls. In addition, the serum levels of CXCL12 tended to increase and the serum levels of CCL27 tended to decrease with an increase in the affected body surface areas. To detect the origin of the circulating chemokines, we performed immunofluorescence staining. CCL21, CCL27, HMGB1 and CXCL12 were stained more broadly in the EB patient tissues than those in the control tissues.

Conclusions: These results suggest that fluctuations in chemokine levels may contribute in a coordinated way to the wound-healing process and lend clues toward efficient cell therapies for EB.

© 2017 Japanese Society for Investigative Dermatology. Published by Elsevier Ireland Ltd. All rights reserved.

Abbreviations: BM, bone marrow; BMSC, bone marrow stem cells; BSA, body surface area; CTACK, cutaneous T cell-attracting chemokine; DDEB, dominant dystrophic epidermolysis bullosa; DEJ, dermo-epidermal junction; EB, epidermolysis bullosa; EBK, epidermolysis bullosa keratinocyte; EBS, epidermolysis bullosa simplex; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; HMGB1, high-mobility group box 1; HSC, hematopoietic stem cell; JEB, junctional epidermolysis bullosa; MIP, macrophage inflammatory protein; MSC, mesenchymal stromal cell; NHEK, normal human epidermal keratinocyte; qRT-PCR, quantitative reverse transcription polymerase chain reaction; RAGE, receptor for advanced glycation end-products; RDEB, recessive dystrophic epidermolysis bullosa; SDF-1, stromal cell-derived factor-1; SEM, standard error of the mean; SLC, secondary lymphoid-tissue chemokine.

* Corresponding authors.

E-mail addresses: yfujita@med.hokudai.ac.jp (Y. Fujita), shimizu@med.hokudai.ac.jp (H. Shimizu).

<http://dx.doi.org/10.1016/j.jdermsci.2016.12.021>

0923-1811/© 2017 Japanese Society for Investigative Dermatology. Published by Elsevier Ireland Ltd. All rights reserved.

1. Introduction

Epidermolysis bullosa (EB) is a group of genetic dermatoses that are caused by the absence or structural abnormality of proteins in the dermo-epidermal junction (DEJ). Insufficiency of the proteins leads to skin fragility and blisters or erosions by mechanical stimulation. At present, there are no fundamental treatments other than symptomatic management.

Recently, allogenic cell therapies, such as hematopoietic stem cell (HSC) transplantation and mesenchymal stromal cell (MSC) infusion have gained attention as possible treatments [1,2]. These are based on the concept that these donor-derived cells could migrate into the skin, reconstitute the skin components, and lead to the restoration of normal DEJ proteins [3–5]. The frequency of donor-derived cells in the epidermis varies depending on the underlying skin condition. In one study, bone marrow (BM)-

derived keratinocytes were present as $0.025\% \pm 0.009\%$ of all keratinocytes and comprised approximately 0.1% of the basal cells of re-epithelized skin in healthy mice [3]. In contrast, $1.08\% \pm 0.39\%$ of the basal cells were found to be derived from the BM in non-Herlitz junctional EB (JEB) model mice [5]. In humans, skin chimerism was reported in up to 93% of HSC-transplanted EB patients, however, many of these donor-derived cells are considered to be inflammatory leukocytes [6]. These facts suggest that injured skin, especially EB skin tissue, expresses some factors that partly circulate in the peripheral blood, and they play a role in recruiting BM-derived cells such as MSCs or HSCs to the affected skin.

To date, several chemokines are considered to play a role in the migration of BM-derived cells to the skin. CCL21/secondary lymphoid-tissue chemokine (SLC) and CCL27/cutaneous T-cell-attracting chemokine (CTACK) are reported to have a strong relation with the migration of BM-derived cells to wounded skin and refurbish the damaged skin in mice experiments [3,4]. CXCL12/stromal cell-derived factor-1 (SDF-1) was reported to be elevated at the damaged skin, and to contribute to wound-healing through the recruitment of BM-derived MSCs in mouse models [7–9]. In addition, high-mobility group box 1 (HMGB1) was suggested as playing a role in the migration of BM-derived cells into the bloodstream in mouse models [10]. It was also reported that serum HMGB1 levels are elevated in recessive dystrophic epidermolysis bullosa (RDEB) patients and correlate with the disease severity [10,11]. However, there have been no investigations into injury-associated chemokines in EB patients, other than investigations into HMGB1.

In this study, we examined these chemokine expressions of EB patients that could be related to the wound-healing process. We also analyzed the expressions of CCL28 and CCL19, which bind to the receptors of CCL27 and CCL21, respectively. Elucidating the chemokine profiles in EB patients could help to clarify the mechanism of BM cell migration into the skin, which could contribute to efficient cell therapies.

2. Materials and methods

2.1. Patients and controls

This study was performed following approval from the human research ethics committee of Hokkaido University Hospital (approval number: 011-0089), and all informed consent was obtained from all participants.

Serum samples of 45 EB patients were collected from multiple Japanese hospitals and institutions (Supplementary Table A1). The diagnosis of EB was made by certified dermatologists according to the international criteria, which were recommended by Fine JD, et al. [12]. We also assembled the following clinical information on the patients: sex, age and affected body surface area (BSA) (%). The affected area was defined as including erythema, erosions, ulcers and scars. Serum samples from 39 healthy volunteers without any dermatological disorders were also collected as controls.

Frozen skin specimens of 19 EB patients that had been collected in our department from 2000 to 2009 were investigated. The skin specimens were taken from the trunk or the extremities. Skin samples from five unaffected volunteers were also collected as controls.

For the *in vitro* scratch model, primary keratinocytes were isolated from two RDEB patients and two control subjects. These cells were cultured in CnT-PR from CELLnTEC and were used for the assay with no more than six passages. The cultures were performed in a humidified atmosphere with 5% CO₂ at 37 °C.

2.2. Measurement of the chemokine levels in the serum samples

The chemokine concentration in each sample was quantitatively analyzed by using the following enzyme-linked immunosorbent assay (ELISA) kits: human CCL19/macrophage inflammatory protein (MIP)-3 β DuoSet ELISA (R&D Systems, Minneapolis, MN), human CCL21/6CKine DuoSet ELISA (R&D Systems), human CCL27/CTACK DuoSet ELISA (R&D Systems), human CCL28 DuoSet ELISA (R&D Systems), HMGB1 ELISA Kit (Shino-Test Corp., Tokyo, Japan) and human CXCL12/SDF-1 DuoSet ELISA (R&D Systems), according to the manufacturers' instructions. Optical densities were measured at 450 nm with a Bio-Rad Model 550 microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA). The protein levels were calculated from a standard curve generated by a curve-fitting program.

2.3. Scratch assay of keratinocytes from EB patients and controls

Supernatant from cultured normal human epidermal keratinocytes (NHEKs) and EB keratinocytes (EBKs) was collected after confluence. Meanwhile, cultured NHEKs and EBKs after confluence on other dishes were scratched with a 200 μ l pipette tip and the supernatant was collected 24 h later. The chemokine levels of supernatants from cultured NHEKs and EBKs with or without scratching were measured by ELISAs as described above.

2.4. Quantitative reverse transcription PCR (qRT-PCR) analysis

Total RNA was isolated from NHEKs and EBKs with or without scratching using the RNeasy Plus Mini Kit (Qiagen, Hombrechtikon, Switzerland). RT-PCR analysis of mRNA from the chemokines and 18S rRNA was performed in a thermocycler (GeneAmp PCR system 9600; PerkinElmer Life and Analytical Sciences, Boston, MA). TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA) were used to analyze the expressions of CCL21 (assay ID: Hs99999110_m1), HMGB1 (assay ID: Hs01590761_g1), CXCL12 (assay ID: Hs03676656_mH), and 18S rRNA (assay ID: 4319413E) per the manufacturer's instructions. 18S rRNA was used as an internal control to validate the RNA for each sample. Aliquots from each amplification reaction were analyzed by the StepOnePlus Real-Time PCR Systems (v. 2.0, Applied Biosystems). Values are calculated as the relative expression using $\Delta\Delta$ Ct methods.

2.5. Immunofluorescence staining

Frozen skin sections of 5 μ m in thickness were fixed in ice-cold acetone for 30 min. The sections were then incubated for 15 min at 37 °C with phosphate buffered saline containing 10% bovine serum albumin for blocking before the addition of primary antibodies for 30 min at 37 °C. The following primary antibodies were used: mouse monoclonal anti-human CCL19/MIP-3 β (54909, 20 μ g/ml, R&D Systems), goat polyclonal anti-human CCL21/SLC (10 μ g/ml, R&D Systems), mouse monoclonal anti-human CCL27/CTACK antibody (124302, 20 μ g/ml, R&D Systems), mouse monoclonal anti-human CCL28 antibody (62705, 20 μ g/ml, R&D Systems), mouse monoclonal anti-HMGB1 antibody (115603, 20 μ g/ml, R&D Systems), mouse monoclonal anti-Human/Mouse CXCL12/SDF-1 antibody (79018, 12 μ g/ml, R&D Systems), rabbit monoclonal anti-human CCR7 antibody (ab32527, 2 μ g/ml, Abcam, Cambridge, UK), goat polyclonal anti-human CCR10 antibody (ab1661, 20 μ g/ml, Abcam), and rabbit polyclonal anti-human RAGE (sc-5563, 4 μ g/ml, Santa Cruz Biotechnology, Santa Cruz, CA). Subsequently, sections were incubated for 30 min with secondary antibodies as follows: fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (15 μ g/ml, Jackson ImmunoResearch, West Grove, PA), FITC-conjugated goat anti-rabbit IgG (15 μ g/ml, Jackson

Table 1
The profiles of the serum samples.

	EB patients (n=45)					Controls (n=39)
	RDEB	DDEB	DEB	EBS	unknown	
Subtype Number	23	6	2	9	5	39
Age (y) ± SD (range)	24 ± 22.0 (2-83)	20 ± 15.5 (6-53)	6 ± 5.5 (0-11)	19 ± 15.7 (1-48)	21 ± 20.9 (3-61)	30 ± 4.2 (25-42)
Sex M:F	13:10	2:4	2:0	7:2	2:3	22:17
Affected BSA%	48.5 ± 37.0	29.8 ± 24.5	67.5 ± 27.5	14.2 ± 28.9	17.6 ± 11.6	-

Abbreviation: BSA, body surface area; SD, standard deviation.

ImmunoResearch), and FITC-conjugated donkey anti-goat IgG (15 µg/ml, Jackson ImmunoResearch).

Then, sections were mounted with mounting medium with propidium iodide (PI) (ImmunoBioScience Corp., Mukilteo, WA) to visualize the nuclei. Fluorescence staining was detected using a confocal laser scanning fluorescence microscope (Fluoview FV1000, Olympus, Tokyo, Japan).

2.6. Statistical analyses

Comparisons of chemokine levels of EB patients and control subjects were performed using the student's *t*-test. A comparison of chemokine levels and subtypes of EB was performed using the Kruskal-Wallis test with Dunnett's post test. The chemokine levels in the keratinocytes with scratching and in the controls were compared using paired *t*-test. Differences with *P* < 0.05 were considered statistically significant. Microsoft Excel (Microsoft, Redmond, WA) with the add-in software Statcel2 [13] and

GraphPad Prism ver. 7.00 for Windows (GraphPad Software, San Diego CA) were utilized for statistical analyses.

3. Results

3.1. The profiles of the EB patients

Sera from the following patients were obtained: 31 dystrophic EB patients (23 RDEB, 6 dominant dystrophic EB (DDEB), and 2 dystrophic EB with unknown inheritance), 9 EB simplex (EBS) patients, and 5 unclassified EB patients. For analysis, 2 dystrophic EB with unknown inheritance and 5 unclassified EB patients were excluded. The mean affected BSA (%) ± standard error of the mean (SEM) of the RDEB, DDEB and EBS patients was 48.5% ± 8.1, 29.8% ± 12.2 and 14.2% ± 10.2, respectively (Table 1). Skin tissue specimens from the following patients were obtained: 6 dystrophic EB patients (4 RDEB, 1 DDEB, and 1 dystrophic EB with unknown inheritance), 2 JEB patients, 7 EBS patients, 1 Kindler

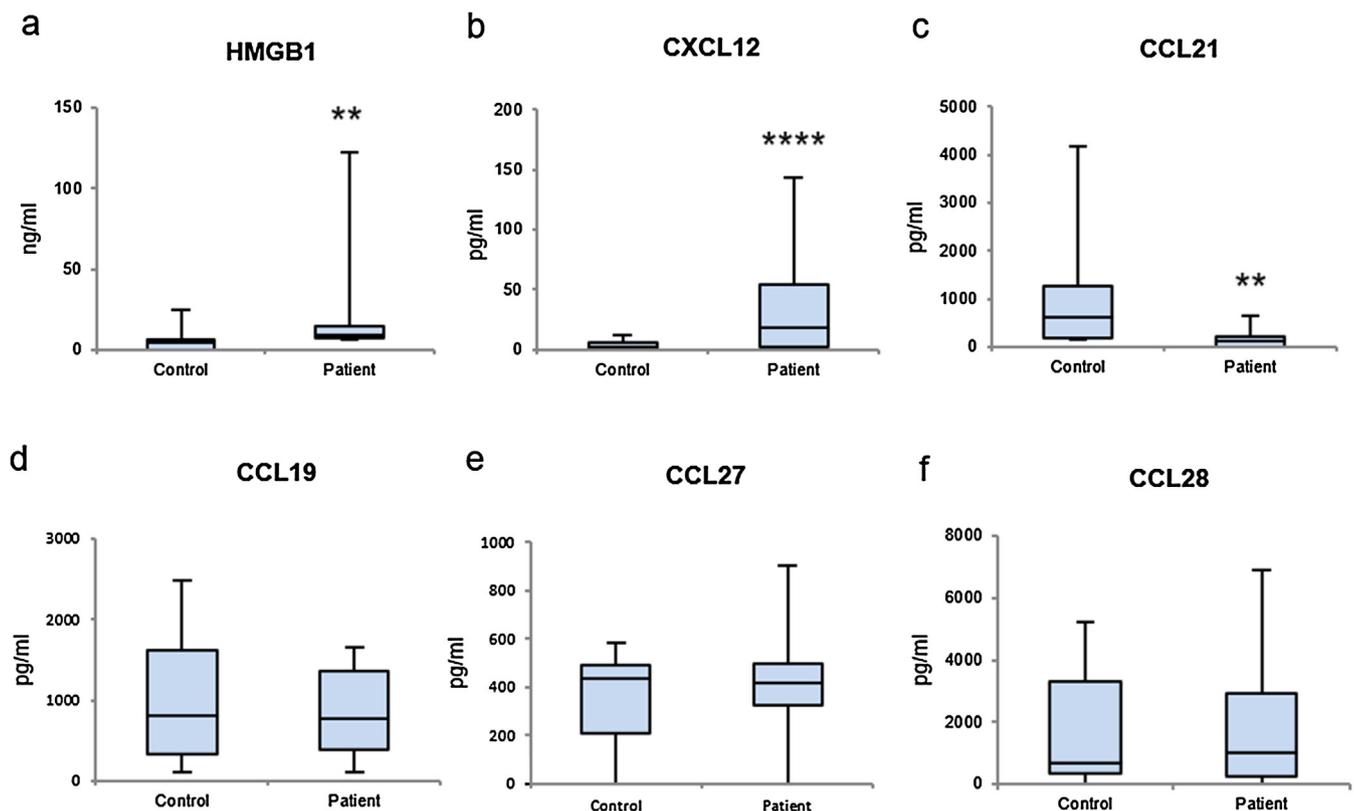


Fig. 1. The serum chemokine levels. The serum chemokine levels were measured by ELISAs: (a) HMGB1 (EB patients, n=37; controls, n=18); (b) CXCL12 (EB patients, n=34; controls, n=15); (c) CCL21 (EB patients, n=37; controls, n=19); (d) CCL19 (EB patients, n=33; controls, n=13); (e) CCL27 (EB patients, n=37; controls, n=19); (f) CCL28 (EB patients, n=33; controls, n=15). Box plots show the distribution of chemokine levels in each group, and error bars are SEM. The statistical differences were determined by student's *t*-test. ***P* < 0.01, *****P* < 0.0001.

syndrome and 3 unclassified EB patients. Epidermal keratinocytes were obtained from 2 RDEB patients.

3.2. Serum HMGB1 and CXCL12 levels are elevated whereas CCL21 levels are decreased in EB patients

We used ELISA to measure 6 chemokines: CCL19/MIP-3 β , CCL21, CCL27, CCL28, HMGB1 and CXCL12. HMGB1 levels in the sera of EB patients were found to be significantly higher than in the control subjects, as previously reported [10,11]. The mean \pm SEM serum HMGB1 level in the EB patients and the control subjects was 13.8 ± 2.7 and 4.9 ± 1.4 ng/ml, respectively ($P < 0.01$; Fig. 1a). In addition, CXCL12 levels were elevated in the EB patients. The mean \pm SEM serum CXCL12 level in the EB patients and the control subjects was 34.2 ± 7.3 and 3.8 ± 1.1 pg/ml, respectively ($P < 0.0001$; Fig. 1b).

In contrast, CCL21 levels in the sera of EB patients were significantly lower than in normal control subjects. The mean \pm SEM serum CCL21 level in the EB patients and the control subjects was 123.5 ± 18.8 and 996.2 ± 270.3 pg/ml, respectively ($P < 0.01$; Fig. 1c).

There were no significant differences between serum levels in EB patients and control subjects with respect to CCL19, CCL27 and CCL28. The mean \pm SEM serum CCL19, CCL27 and CCL28 levels in the EB patients and the control subjects were 887.2 ± 90.4 and 1067.2 ± 235.5 pg/ml, 410.8 ± 28.5 and 363.6 ± 45.4 pg/ml, and 1807.4 ± 332.7 and 1766.5 ± 502.0 pg/ml, respectively (Fig. 1d–f). Since blister depth differs by EB subtype and that difference can

affect the chemokine levels, we further analyzed the controls and the DEB group separately. The results of comparison between DEB patients and controls showed similarity with the results of comparison between all subtypes of EB patients and controls (Supplementary Figure A1).

3.3. CXCL12 levels in the sera of EB patients are tend to increase, whereas CCL27 levels tend to decrease in accordance with the area of affected skin

Next, we investigated the association between the chemokine levels and the profiles of the EB patients. There were no significant correlations between subtypes of EB and the chemokine levels, except for the HMGB1 levels (control versus all types of EB), the CXCL12 levels (control versus RDEB) and the CCL21 levels (control versus RDEB and EBS) (Fig. 2a–f). We also analyzed the relationship between serum chemokine levels and age and sex, and we found no correlations (data not shown).

Furthermore, we analyzed the relationship between the area of affected skin and the serum chemokine levels. The CXCL12 levels in the EB patients tended to increase with the increase in the affected BSA (Fig. 3b). In contrast, serum CCL27 levels tended to decrease with the increase in the affected BSA (Fig. 3e). There were no significant correlations between the affected BSA and the serum levels of HMGB1, CCL21, CCL19 or CCL28 (Fig. 3a, c, d, f). The serum chemokine levels of RDEB patients exhibited the similar trend (Supplementary Figure B1).

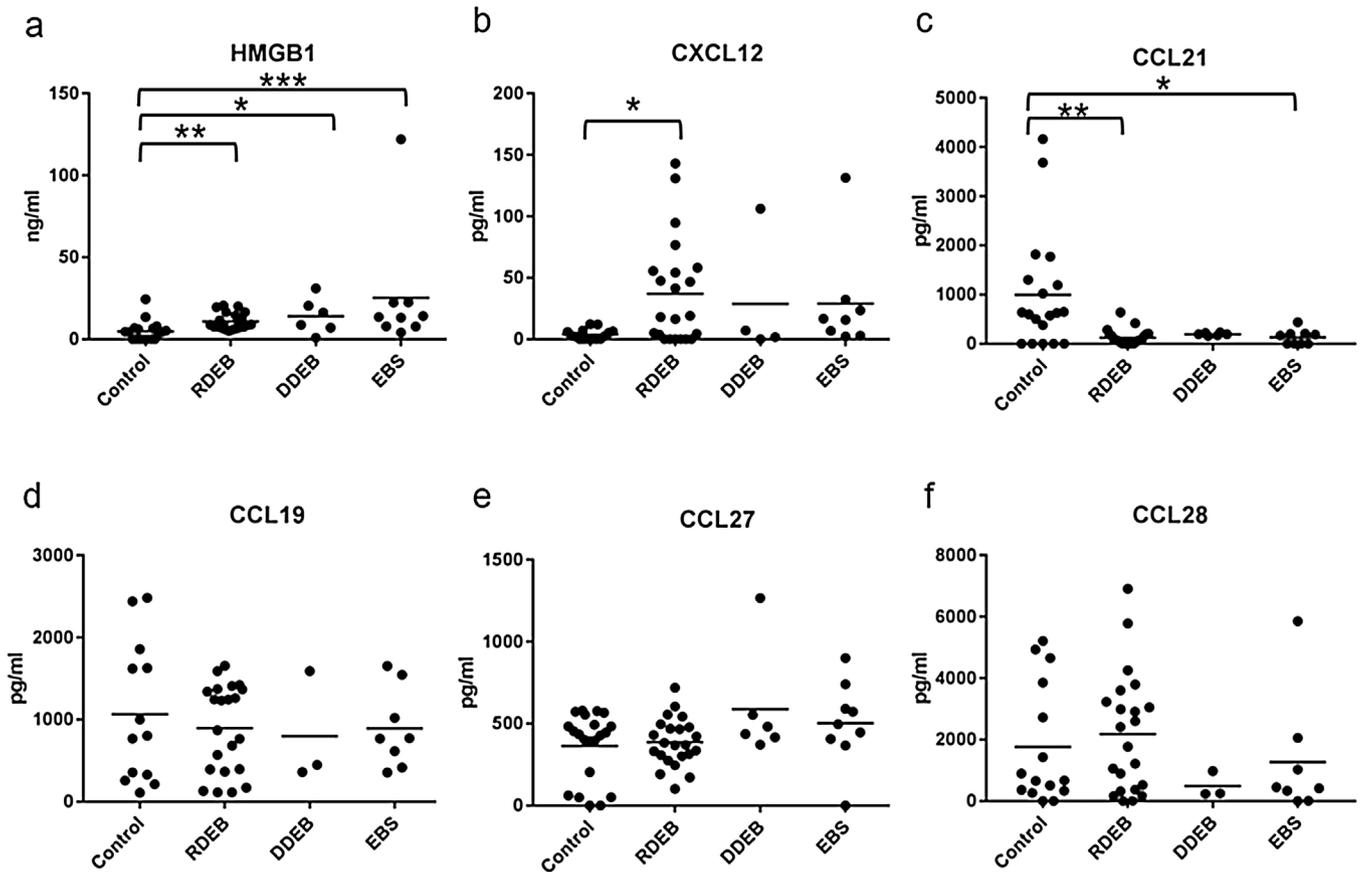


Fig. 2. The serum chemokine levels and EB subtypes. The serum chemokine levels are shown as scatterplots for each group: (a) HMGB1 (RDEB, n = 22; DDEB, n = 6; EBS, n = 9; control, n = 18); (b) CXCL12 (RDEB, n = 22; DDEB, n = 4; EBS, n = 8; control, n = 15); (c) CCL21 (RDEB, n = 23; DDEB, n = 6; EBS, n = 9; control, n = 19); (d) CCL19 (RDEB, n = 22; DDEB, n = 3; EBS, n = 8; control, n = 13); (e) CCL27 (RDEB, n = 23; DDEB, n = 6; EBS, n = 9; control, n = 21); (f) CCL28 (RDEB, n = 22; DDEB, n = 3; EBS, n = 8; control, n = 15). The mean is represented by a bold dash for each group. The statistical differences were determined by Kruskal–Wallis test with Dunnett’s post test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

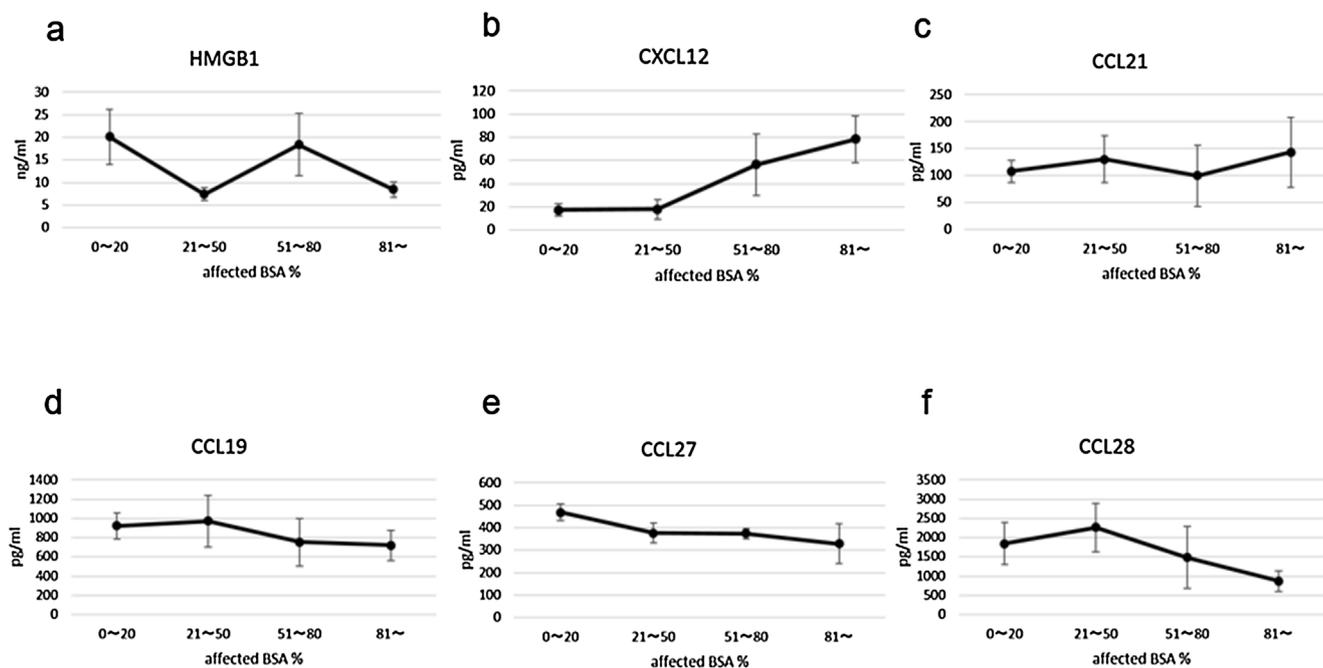


Fig. 3. The serum chemokine levels and affected BSA.

The affected BSA was divided into four groups: 0–20%, 21–50%, 51–80%, 80%<. Data are expressed as the mean \pm SEM. (a) HMGB1 (EB patients, n = 35); (b) CXCL12 (EB patients, n = 32); (c) CCL21 (EB patients, n = 35); (d) CCL19 (EB patients, n = 32); (e) CCL27 (EB patients, n = 33); (f) CCL28 (EB patients, n = 32).

3.4. CCL21, CCL27, HMGB1 and CXCL12 are stained more broadly in the EB patient tissues than those in the control tissues

We then tried to detect expression of chemokine and its receptors in the EB skin, which is a potential origin of the altered circulating chemokines. Approximately half of the EB skin expressed CCL21 from the basal cells to the middle of the epidermis (EB: 10/19 specimens; control: 1/5 specimens). Both EB and control samples showed CCL27 expression in the basal cells (EB: 10/19 specimens; control: 3/5 specimens), and 1 EB sample expressed CCL27 broadly in the epidermis. The expression of HMGB1 was limited to the upper layer above the basal cells in the control skins. Conversely, significant numbers of EB skin expressed HMGB1 throughout the epidermis (EB: 6/19 specimens; control: 0/5 specimens). Similarly, the expression of CXCL12 was localized to the upper layer above the basal cells in the control subjects, whereas CXCL12 was detected throughout the epidermis in 2/10 of the EB skin specimens (Fig. 4, Table 2). Staining patterns were unrelated to EB subtypes. The expressions of CCL19, CCL28, CCR7, CCR10 and receptor for advanced glycation end-products (RAGE) were also investigated, and there were no differences between the EB skin and the control skin (data not shown). All the skin specimens showed very mild inflammatory cell infiltration. We presumed that elevated expressions of CCL21, CCL27, HMGB1 and CXCL12 in the epidermis were potential factors affecting serum concentrations of chemokines.

3.5. RDEB keratinocytes produce less HMGB1 in response to scratching than normal keratinocytes

To investigate whether injured keratinocytes express these chemokines, we performed scratch assays as an *in vitro* model of wound healing in confluent keratinocytes, and then we investigated the chemokine expressions by ELISA and qRT-PCR.

HMGB1 protein levels in the supernatant from cultured NHEKs with scratching were elevated relative to the controls ($P < 0.05$; Fig. 5a). However, HMGB1 levels in the supernatant of the EBKs of

the RDEB patients showed only slight elevation after scratching. In addition, EBKs tended to express less HMGB1 than NHEKs and responded more weakly to scratch than NHEKs. CXCL12 levels showed no significant differences between supernatant with scratching and the control (Fig. 5b). The concentration of CCL19, CCL21, CCL27 and CCL28 were too low to be detected by ELISA.

qRT-PCR analysis showed that *HMGB1* mRNA was expressed in the cultured NHEKs and EBKs. However, it did not show any significant differences between NHEKs and EBKs nor between scratching and control (Fig. 5c). The mRNA expressions of *CCL21* and *CXCL12* were too low to be detected.

4. Discussion

Wound healing and tissue regeneration generally occurs in three phases: inflammation, proliferation and remodeling [14]. It is well known that numerous growth factors and cytokines, including chemokines, released from wounds play pivotal roles in coordinating the process. However, chemokine levels are influenced by many factors. As previously reported, a skin injury's etiology, e.g., burn or excision, can affect the immune response, and this response can lead to fluctuations in chemokine levels [15]. Altered chemokine levels are also seen in various skin diseases, including autoimmune blistering diseases such as bullous pemphigoid [16–18]. In this study, HMGB1 and CXCL12 levels in the sera of EB patients were found to be significantly higher than in control subjects. In contrast, the levels of serum CCL21 were decreased in the EB patients. Furthermore, CCL27 and CXCL12 levels in EB patients showed a tendency to decrease or increase with an increase in the affected BSA.

We previously reported that the intradermal injection of CCL21 increased the migration of MSCs and promoted the wound-healing process in a mice model [4]. We also proved that MSCs expressed several chemokine receptors, especially CCR7, whose ligands are CCL21 and CCL19 [4]. CCL19 and CCL21 are constitutively expressed mostly in lymphocytes and control the movement of lymphocytes and dendritic cells into lymph nodes [19]. In cases of inflammatory

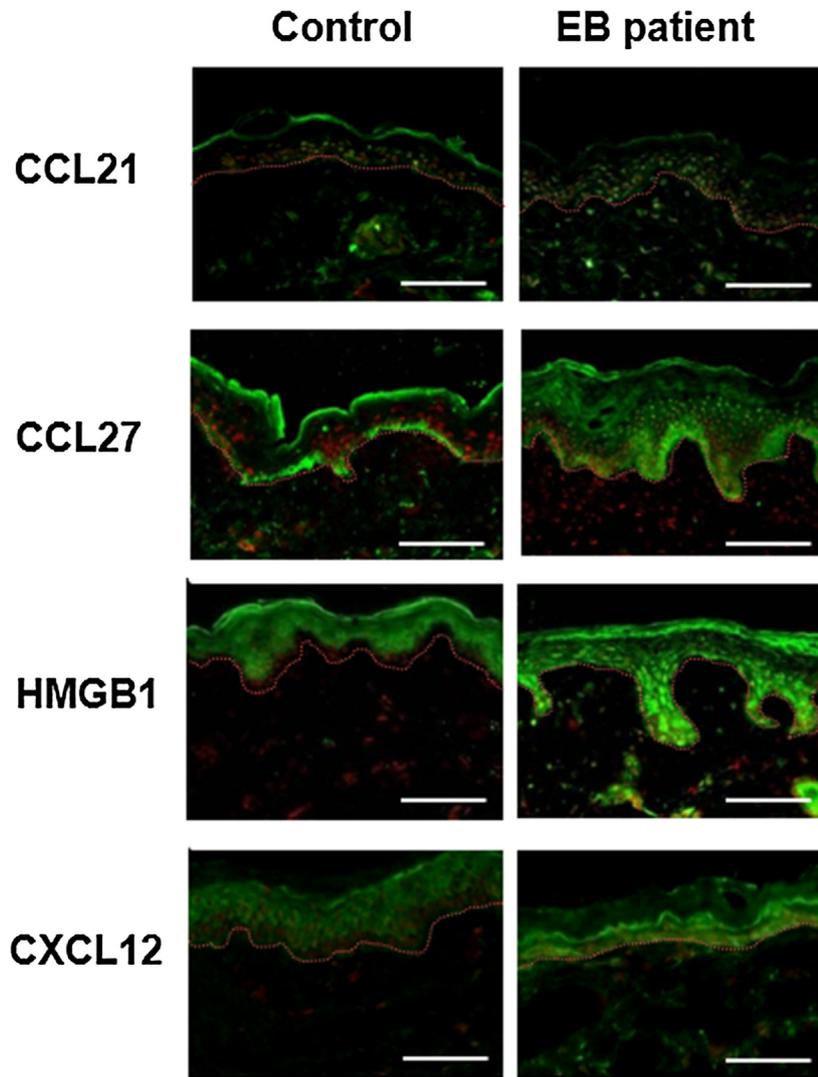


Fig. 4. Immunofluorescent staining of chemokine in skin sections of EB patients and controls. Representative images of CCL21, CCL27, HMGB1 and CXCL12 of EB patients and controls. Each target chemokine is indicated by green, and the propidium iodide (PI)-stained nuclei are indicated by red. The dotted line denotes the boundary between the epidermis and the upper dermis. Bar = 100 μ m.

or infectious disorders, ectopic CCL19 and CCL21 are also produced by endothelial cells, macrophages and keratinocytes [3,20–22].

In our study, serum levels of CCL21 were significantly lower in the EB patients than in the control subjects. However, CCL21 expression tended to be more evident in tissue samples from the EB patients than from the control subjects. These results suggest

the existence of a greater concentration gradient between the blood vessels and the skin tissue in EB patients than in the control subjects, a gradient that induces efficient MSC migration into the skin, as shown in the mechanism of lymphatic migration of dendritic cells [23].

Table 2
Immunofluorescent staining patterns of chemokine in skin sections of EB patients and controls.

Chemokine	Expression pattern	EB skin	Normal control
CCL21	Basal layer to the middle of epidermis	10/19	1/5
	No expression	9/19	4/5
CCL27	Entire epidermis	1/19	0/5
	Only basal layer	11/19	3/5
HMGB1	No expression	7/19	2/5
	Entire epidermis	6/19	0/5
CXCL12	Only upper epidermis above basal layer	9/19	5/5
	No expression	4/19	0/5
	Entire epidermis	2/10	0/5
	Only upper epidermis above basal layer	3/10	5/5
	No expression	5/10	0/5

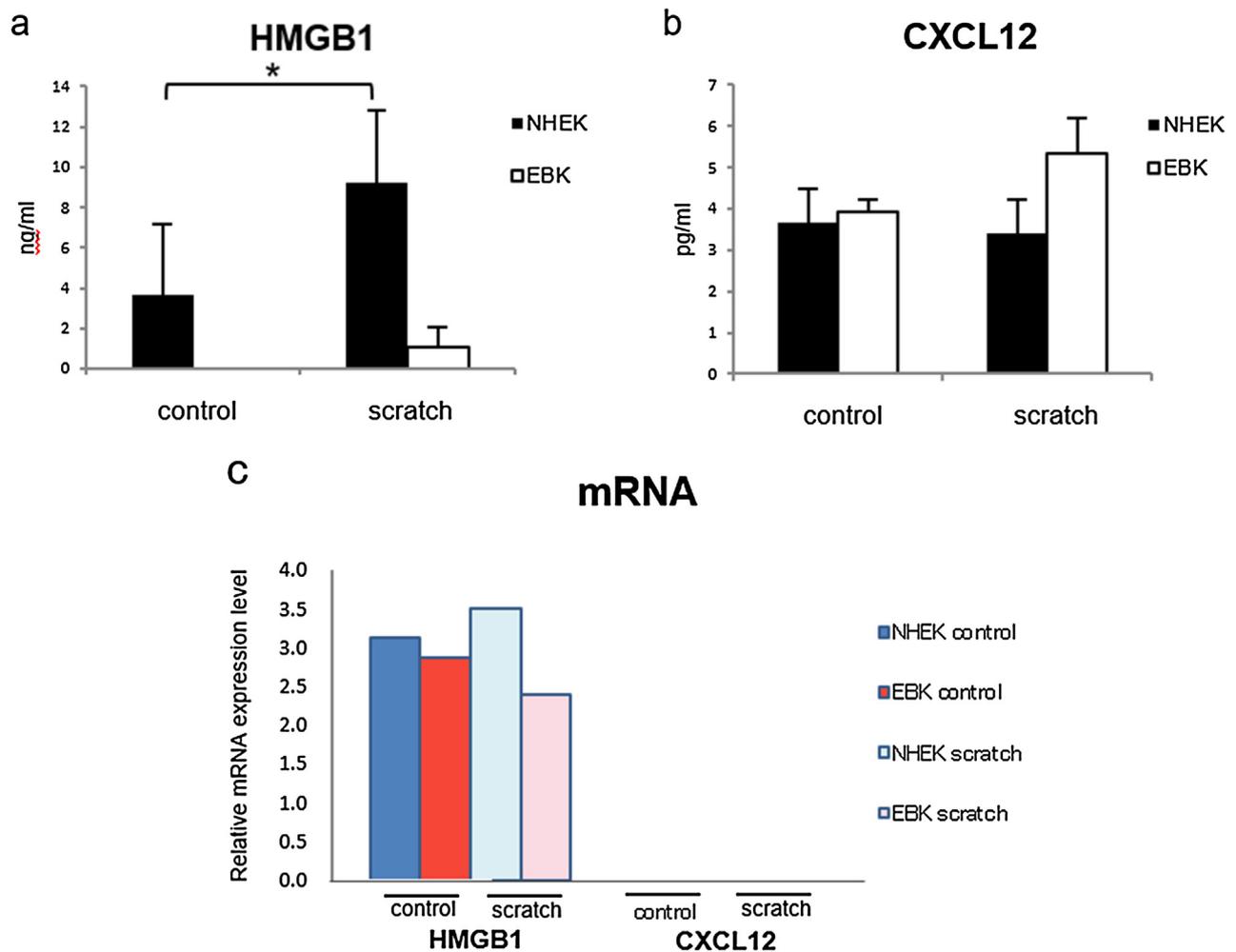


Fig. 5. Chemokine expression in keratinocytes.

(a and b) HMGB1 and CXCL12 protein levels of supernatant from cultured NHEKs (black bars; $n=2$) or EBKs (white bars; RDEB, $n=2$) with or without scratching were measured by ELISAs. The results are expressed as the mean \pm SEM. * $P < 0.05$. (c) qRT-PCR analysis of *HMGB1* and *CXCL12* mRNA. Data are mean \pm SEM of triplicate samples and are representative of three independent experiments. Each mRNA level is shown as an expression level relative to the mean mRNA level of NHEK. 18S rRNA was included as an internal control.

HMGB1 was initially reported as a pro-inflammatory cytokine in sepsis [24]. HMGB1 can be secreted by large variety of cell types when cells are severely stressed [25,26]. It promotes the migration and proliferation of regenerative cells towards inflammation and injury sites, and the accelerated regeneration of damaged tissues, including skin [10,27].

The elevation of HMGB1 levels in the sera of the EB patients in our study is consistent with previous reports [10,11]. Also, HMGB1 seemed to be expressed more broadly in the EB skin than in the control skin. However, contrary to the findings of Petrof and colleagues [11], we did not find a correlation between HMGB1 levels and the affected BSA of EB patients. In the scratch assay, the EBKs expressed less HMGB1 levels than that in the NHEKs in response to physical stimuli. Although the reason for the inconsistency between our results and the results of previous reports is unclear, we assume that these differences in responsiveness to mechanical stimuli and in wound conditions, such as the extent of inflammatory cell infiltrations, might affect the concentration of circulating HMGB1. Furthermore, some underlying general conditions, such as infectious diseases or chronic inflammatory ailments (e.g., atherosclerosis or diabetes), would

have influenced the fluctuations in circulating HMGB1 levels [28,29].

We also showed that CXCL12 levels in the sera of the EB patients were significantly higher than in those from the control subjects and tended to increase with an increase in affected BSA. As shown in HMGB1, it also seemed to be expressed more broadly in the EB skin than in the control skin. Numerous studies have demonstrated that CXCL12, which is also involved in the stress-induced recruitment of stem cells to damaged tissue, enhances bone marrow stem cells (BMSCs) migration into wounds and contributes to cutaneous tissue repair and regeneration [7,30–32]. It is reported that the HMGB1-induced recruitment of inflammatory cells depends on CXCL12 and that HMGB1 and CXCL12 form a heterocomplex [33]. Our results lend credence to the concept that CXCL12 released from damaged skin of EB patients would make BM-derived cells promote wound healing in coordination with HMGB1.

We also measured the levels of CCL27 and CCL28, which share the same receptor, CCR10. In humans, CCL27 is expressed specifically in epidermal keratinocytes [34]. CCL27 was reported to have a strong relation with migration of BM-derived cells to

damaged skin in mice experiments [3]. CCL28 is most closely homologous to CCL27 and is expressed in a wide range of human cell types and tissues, especially in epithelial cells of the mucosa.

Our results did not show significant differences in CCL27 and CCL28 levels between EB patients and control subjects, but we did find that CCL27 had a tendency to decrease with an increase in affected BSA. This may be due to the exclusive expression of CCL27 in keratinocytes, which led to the scarce live keratinocytes in the EB patients and resulted in fewer resources of CCL27. By contrast, CXCL12 secretion from extensively damaged tissues may contribute to a propensity for increases with an increase in the affected BSA.

In conclusion, this is the first report to elucidate the alterations in chemokine levels in EB patients, other than reports addressing alterations in HMGB1. These alterations can enhance the skin homing of therapeutic stem cells. Although the precise mechanisms behind the induction of BMSCs migration to wound lesions are not fully understood, chemokines such as HMGB1, CXCL12, CCL21 and CCL27 might relate to the wound-healing process in EB patients and could be key factors contributing to the efficient application of cell therapies such as allogeneic stem cell transplantation.

Conflicts of interest

The authors have no conflict of interest to declare.

Funding

This work was supported in part by Grants-in-Aid for Scientific Research (No. 24249062 to H.S. and No. 25713041 to Y.F.) from the Ministry of Education, Science, Sports, and Culture of Japan and by the Cosmetology Research Foundation 2012 (to Y.F.).

Acknowledgements

We are grateful to all the physicians at the hospitals and institutes who cooperated in this study and to the patients of Debra Japan who donated specimens. We also thank Ms. Yukiko Nakamura for her technical assistance.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jdermsci.2016.12.021>.

References

Due to addition of revised description, there are additional references.

- [1] J.E. Wagner, A. Ishida-Yamamoto, J.A. McGrath, M. Hordinsky, D.R. Keene, D.T. Woodley, et al., Bone marrow transplantation for recessive dystrophic epidermolysis bullosa, *N. Engl. J. Med.* 363 (2010) 629–639.
- [2] P. Conget, F. Rodriguez, S. Kramer, C. Allers, V. Simon, F. Palisson, et al., Replenishment of type VII collagen and re-epithelialization of chronically ulcerated skin after intradermal administration of allogeneic mesenchymal stromal cells in two patients with recessive dystrophic epidermolysis bullosa, *Cytotherapy* 12 (2010) 429–431.
- [3] D. Inokuma, R. Abe, Y. Fujita, M. Sasaki, A. Shibaki, H. Nakamura, et al., CTACK/CCL27 accelerates skin regeneration via accumulation of bone marrow-derived keratinocytes, *Stem Cells* 24 (2006) 2810–2816.
- [4] M. Sasaki, R. Abe, Y. Fujita, S. Ando, D. Inokuma, H. Shimizu, Mesenchymal stem cells are recruited into wounded skin and contribute to wound repair by transdifferentiation into multiple skin cell type, *J. Immunol.* 180 (2008) 2581–2587.
- [5] Y. Fujita, R. Abe, D. Inokuma, M. Sasaki, D. Hoshina, K. Natsuga, et al., Bone marrow transplantation restores epidermal basement membrane protein expression and rescues epidermolysis bullosa model mice, *Proc. Natl. Acad. Sci. U. S. A.* 107 (2010) 14345–14350.
- [6] J. Tolar, B.R. Blazar, J.E. Wagner, Concise review: transplantation of human hematopoietic cells for extracellular matrix protein deficiency in epidermolysis bullosa, *Stem Cells* 29 (2011) 900–906.
- [7] X. Xu, F. Zhu, M. Zhang, D. Zeng, D. Luo, G. Liu, et al., Stromal cell-derived factor-1 enhances wound healing through recruiting bone marrow-derived mesenchymal stem cells to the wound area and promoting neovascularization, *Cells Tissues Organs* 197 (2013) 103–113.
- [8] C. Hu, X. Yong, C. Li, M. Lü, D. Liu, L. Chen, et al., CXCL12/CXCR4 axis promotes mesenchymal stem cell mobilization to burn wounds and contributes to wound repair, *J. Surg. Res.* 183 (2013) 427–434.
- [9] S. Iinuma, E. Aikawa, K. Tamai, R. Fujita, Y. Kikuchi, T. Chino, et al., Transplanted bone marrow-derived circulating PDGFR α + cells restore type VII collagen in recessive dystrophic epidermolysis bullosa mouse skin graft, *J. Immunol.* 194 (2015) 1996–2003.
- [10] K. Tamai, T. Yamazaki, T. Chino, M. Ishii, S. Otsuru, Y. Kikuchi, et al., PDGFR α -positive cells in bone marrow are mobilized by high mobility group box 1 (HMGB1) to regenerate injured epithelia, *Proc. Natl. Acad. Sci. U. S. A.* 108 (2011) 6609–6614.
- [11] G. Petrof, A. Abdul-Wahab, L. Proudfoot, R. Pramanik, J.E. Mellerio, J.A. McGrath, Serum levels of high mobility group box 1 correlate with disease severity in recessive dystrophic epidermolysis bullosa, *Exp. Dermatol.* 22 (2013) 433–435.
- [12] J.D. Fine, L. Bruckner-Tuderman, R.A.J. Eady, E.A. Bauer, J.W. Bauer, C. Has, et al., Inherited epidermolysis bullosa: updated recommendations on diagnosis and classification, *J. Am. Acad. Dermatol.* 70 (2014) 1103–1126.
- [13] Statcel – The Usefull Add-in Software Forms on Excel, in: H. Yanai (Ed.), 2nd ed., OMS, Tokyo, Japan, 2004.
- [14] A.J. Singer, R.A. Clark, Cutaneous wound healing, *N. Engl. J. Med.* 341 (1999) 738–746.
- [15] R. Förster, A. Schubel, D. Breitfeld, E. Kremmer, I. Renner-Müller, E. Wolf, et al., CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs, *Cell* 99 (1999) 23–33.
- [16] A. Burman, O. Haworth, D.L. Hardie, E.N. Amft, C. Siewert, D.G. Jackson, et al., A chemokine-dependent stromal induction mechanism for aberrant lymphocyte accumulation and compromised lymphatic return in rheumatoid arthritis, *J. Immunol.* 174 (2005) 1693–1700.
- [17] P. Middel, D. Raddatz, B. Gunawan, F. Haller, H.J. Radzun, Increased number of mature dendritic cells in Crohn's disease: evidence for a chemokine mediated retention mechanism, *Gut* 55 (2006) 220–227.
- [18] J.K. Damás, L. Landrø, B. Fevang, L. Heggelund, S.S. Frøland, P. Aukrust, Enhanced levels of the CCR7 ligands CCL19 and CCL21 in HIV infection: correlation with viral load, disease progression and response to highly active antiretroviral therapy, *AIDS* 23 (2009) 135–138.
- [19] M. Weber, R. Hauschild, J. Schwarz, C. Mousson, I. de Vries, D.F. Legler, et al., Interstitial dendritic cell guidance by haptotactic chemokine gradients, *Science* 339 (2013) 328–332.
- [20] H. Wang, O. Bloom, M. Zhang, J.M. Vishnubhakat, M. Ombrellino, J. Che, et al., HMG-1 as a late mediator of endotoxin lethality in mice, *Science* 285 (1999) 248–251.
- [21] U. Andersson, K.J. Tracey, HMGB1 is a therapeutic target for sterile inflammation and infection, *Annu. Rev. Immunol.* 29 (2011) 139–162.
- [22] D. Tang, R. Kang, H.J. Zeh, M.T. Lotze, High-mobility group box 1, oxidative stress, and disease, *Antioxid. Redox Signal.* 14 (2011) 1315–1335.
- [23] S. Straino, A. Di Carlo, A. Mangoni, R. De Mori, L. Guerra, R. Maurelli, et al., High-mobility group box 1 Box 1 protein in human and murine skin: involvement in wound healing, *J. Invest. Dermatol.* 128 (2008) 1545–1553.
- [24] J.D. Abbott, Y. Huang, D. Liu, R. Hickey, D.S. Krause, F.J. Giordano, Stromal cell-derived factor-1 α plays a critical role in stem cell recruitment to the heart after myocardial infarction but is not sufficient to induce homing in the absence of injury, *Circulation* 110 (2004) 3300–3305.
- [25] Y. Landry, O. Le, K.A. Mace, T.E. Restivo, C.M. Beausejour, Secretion of SDF-1 α by bone marrow-derived stromal cells enhances skin wound healing of C57BL/6 mice exposed to ionizing radiation, *J. Cell Mol. Med.* 14 (2010) 1594–1604.
- [26] A. Jaerve, J. Schira, H.W. Müller, Concise review: the potential of stromal cell-derived factor 1 and its receptors to promote stem cell functions in spinal cord repair, *Stem Cells Transl. Med.* 1 (2012) 732–739.
- [27] M. Schiraldi, A. Raucci, L.M. Muñoz, E. Livoti, B. Celona, E. Venereau, et al., HMGB1 promotes recruitment of inflammatory cells to damaged tissues by forming a complex with CXCL12 and signaling via CXCR4, *J. Exp. Med.* 209 (2012) 551–563.
- [28] J. Morales, B. Homey, A.P. Vicari, S. Hudak, E. Oldham, J. Hedrick, et al., CTACK, a skin-associated chemokine that preferentially attracts skin-homing memory T cells, *Proc. Natl. Acad. Sci. U. S. A.* 96 (1999) 14470–14475.
- [29] D. Allonso, F.S. Belgrano, N. Calzada, M.G. Guzmán, S. Vázquez, R. Mohana-Borges, Elevated serum levels of high mobility group box 1 (HMGB1) protein in dengue-infected patients are associated with disease symptoms and secondary infection, *J. Clin. Virol.* 55 (2012) 214–219.
- [30] J.D. Abbott, Y. Huang, D. Liu, R. Hickey, D.S. Krause, F.J. Giordano, Stromal cell-derived factor-1 α plays a critical role in stem cell recruitment to the heart after myocardial infarction but is not sufficient to induce homing in the absence of injury, *Circulation* 110 (2004) 3300–3305.
- [31] Y. Landry, O. Le, K.A. Mace, T.E. Restivo, C.M. Beausejour, Secretion of SDF-1 α by bone marrow-derived stromal cells enhances skin wound healing of C57BL/6 mice exposed to ionizing radiation, *J. Cell Mol. Med.* 14 (2010) 1594–1604.

- [32] A. Jaerve, J. Schira, H.W. Müller, Concise review: the potential of stromal cell-derived factor 1 and its receptors to promote stem cell functions in spinal cord repair, *Stem Cells Transl. Med.* 1 (2012) 732–739.
- [33] M. Schiraldi, A. Raucci, L.M. Muñoz, E. Livoti, B. Celona, E. Venereau, et al., HMGB1 promotes recruitment of inflammatory cells to damaged tissues by forming a complex with CXCL12 and signaling via CXCR4, *J. Exp. Med.* 209 (2012) 551–563.
- [34] J. Morales, B. Homey, A.P. Vicari, S. Hudak, E. Oldham, J. Hedrick, et al., CTACK, a skin-associated chemokine that preferentially attracts skin-homing memory T cells, *Proc. Natl. Acad. Sci. U. S. A* 96 (1999) 14470–14475.