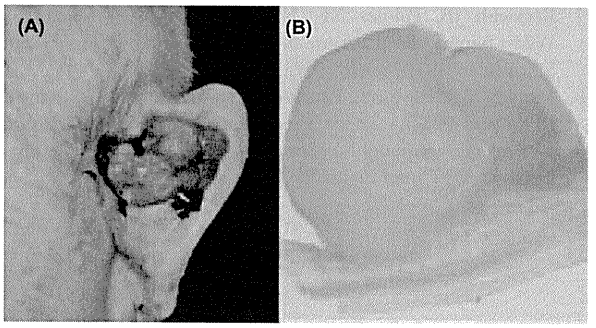

Fig. 1. (A) Clinical features of the tumor. An erosive, fresh-reddish colored, dome shaped nodule on the right post-auricular. (B) Scanning magnification demonstrates that the lesion was well demarcated and showed no evidence of invasion into the underlying cartilage (H&E).

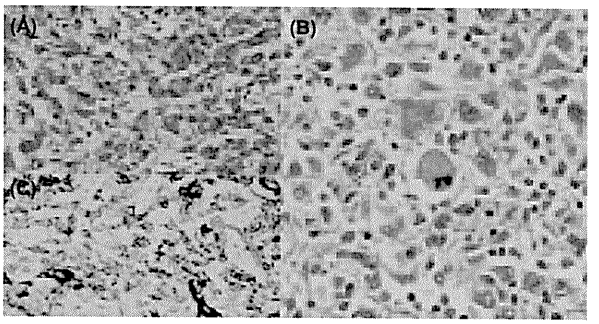

Fig. 2. (A) There were only small areas where the tumor cells formed solid sheets, especially in the center area of the nodule (H&E ×200). (B) Large portions of the tumor cells contained large, globular and cytoplasmic eosinophilic hyaline inclusions that displaced the nuclei peripherally (H&E ×400). (C) Tumor cells located in the center portion of the lesion were strongly positive for cytokeratin AE1/AE3 (×200).

their morphological resemblance to rhabdomyoblasts under a light microscope. However, an ultrastructural examination could not verify the evidence of rhabdomyoblastic differentiation. Later, the rhabdoid morphology thought to be a common endpoint in the origins, because it appeared to be associated with aggressive biological behavior, rapid growth and poor prognosis.[8] According to the close association among rhabdoid features, necrotic cells and debris, this phenotype could represent a phase of degeneration, or a preliminary stage of apoptosis or cell necrosis, rather than a specific type of differentiation.[6] This notion is consistent with the absence of immunohistological reactivity toward skeletal muscle markers in these tumor cells.

Rhabdoid SCC was first described in 1996 by Pai et al.[3] Additional cases have been reported since then, establishing this phenotype as a distinct morphologic variant of cutaneous SCC. Moreover, according to the literature, SCC with rhabdoid features shows a higher degree of recurrence and metastasis than other SCC variants.[6]

The histological examination of our initial biopsy specimen taken from a shallow region of the nodule identified few cellular atypia, numerous inflammation cells, and fibrotic and hemorrhagic tissue, but not neoplastic cells. The lesion was also tested negative for cytokeratin AE1/AE3. Only after the whole histological examination of the larger surgical specimen, the tumor cells were localized in the center portion of the lesion. Furthermore, the lesion was found positive for cytokeratin AE1/AE3, hence leading to a diagnosis as SCC. Therefore, shallow biopsies, as in the case of our first specimen, could increase the risk of misdiagnosing the lesions as a benign tumor.

The lesion was well-demarcated with no evidence of invasion into the underlying cartilage. The low degree of the invasiveness we observed is possibly consistent with the favorable prognosis in our case.

In conclusion, this report introduces characteristic histological patterns that can assist the diagnosis of cutaneous SCC with the rhabdoid features. A biopsy of the deep tissue should be considered crucial for reaching a correct diagnosis. Although our case did not show an aggressive biological behavior, but meaningful discussions on the prognosis of this histological SCC variant await additional investigations.

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Desmoglein1 and BP 180 ELISA indexes correlating with disease activity in a patient with coexisting pemphigus foliaceus and bullous pemphigoid

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An 88-year-old Japanese woman presented with erosions in the oral cavity. On physical examination, oral erosions, tense vesicles and erythema with partially crusted erosions were seen on the trunk. Acantholysis (Fig. 1a) and subepidermal blisters (Fig. 1b) were seen in skin biopsy specimens taken from the trunk. Direct immunofluorescence of the skin revealed *in vivo* deposition of IgG in both the cell surface and the basement membrane zone (BMZ) of the epidermis (Fig. 1c). Using indirect immunofluorescence using human skin section as substrate, IgG autoantibodies against cell surface and BMZ were detected at a titre of > 1 : 160 (Fig. 1d). Immunoblotting assays using normal human epidermal extracts and BP180 NC16a domain

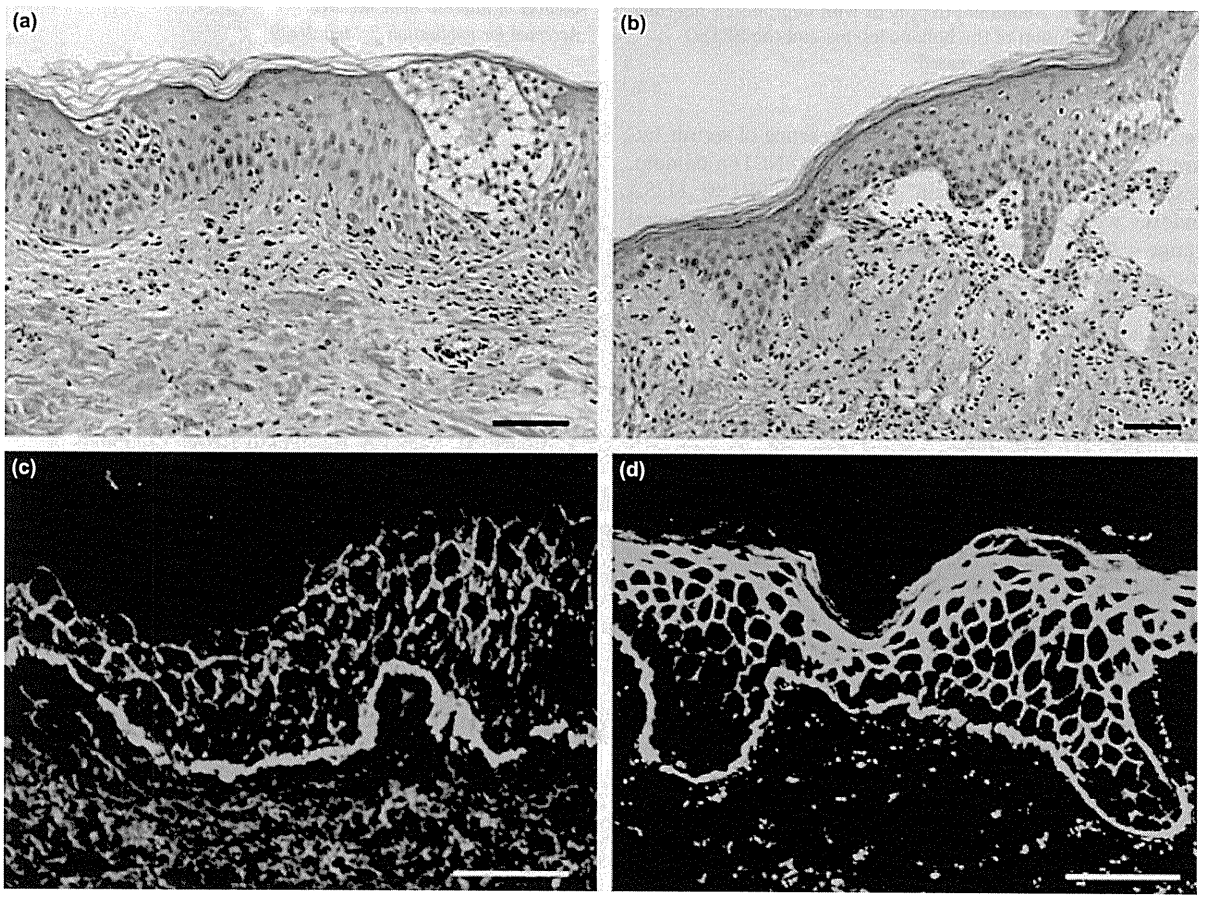

Figure 1 (a) Acantholysis and (b) subepidermal blisters (haematoxylin and eosin). Both (c) direct immunofluorescence of the skin and (d) indirect immunofluorescence using human skin sections confirmed IgG antibodies to the cell surface and basement membrane zone of the epidermis. Scale bar, 100 μm.

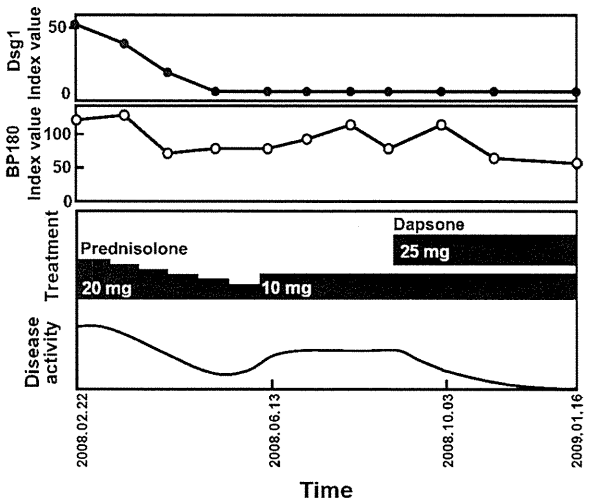

Figure 2 Desmoglein (Dsg)1 and BP180 ELISA indexes correlated with disease activity. With oral prednisolone 20 mg/day, the erosions and blisters disappeared, and the Dsg1 ELISA index became negative. Additional treatment with dapsone 25 mg/day, resulted in resolution of the bullous lesions and the BP180 ELISA index gradually decreased.

recombinant protein confirmed the presence of serum IgG antibodies against BP180 and the BP180 NC16a domain, respectively. The desmogein (Dsg)1 and BP180 ELISA indexes were 54 (normal range < 20) and 125 (normal range < 8), respectively. Oral prednisolone 20 mg/day and topical steroid application were effective, and the Dsg1 ELISA index decreased to 10. However, the BP180 ELISA index did not decrease, and it was still 88 when the patient had a flare while on oral prednisolone 5 mg/day. Oral dapsone 25 mg/day plus prednisolone 10 mg/day cleared the bullous skin lesions, and the BP180 ELISA index gradually decreased (Fig. 2).

In this case, the clinical features, histological findings, immunofluorescence and ELISA indexes confirmed the simultaneous coexistence of PF and BP. Previous reports[1–3] reported the simultaneous coexistence of PF and BP based on histological or immunofluorescence findings. None of the three cases had coexistence of anti-Dsg1 antibodies and anti-BP180 antibodies by ELISA. To our knowledge, our patient is the first case of coexistence of PF and BP confirmed by positive ELISA results for both anti-Dsg1 and anti-BP180 antibodies.

It was previously reported that ELISAs for Dsg1, Dsg3 and BP180 are more sensitive and specific than indirect immunofluorescence and that ELISA indexes tend to correlate with the disease activity.[4,5] In our case, Dsg1 and BP180 ELISA indexes correlated with disease activity along the time course. ELISA is a valuable tool for monitoring disease activity and provides the important information for determining treatments for various immunobullous diseases.

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Stem Cells, Tissue Engineering and Hematopoietic Elements

Bone Marrow-Derived Cells Are Not the Origin of the Cancer Stem Cells in Ultraviolet-Induced Skin Cancer

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Several lines of evidence have demonstrated that various cancers are derived from cancer stem cells (CSCs), which are thought to originate from either tissue stem or progenitor cells. However, recent studies have suggested that the origin of CSCs could be bone marrow-derived cells (BMDCs); for example, gastric cancer, which follows persistent gastric inflammation, appears to originate from BMDCs. Although our previous research showed the capability of BMDCs to differentiate into epidermal keratinocytes, it has yet to be determined whether skin CSCs originate from BMDCs. To assess the possibility that BMDCs could be the origin of CSCs in skin squamous cell carcinoma (SCC), we used a mouse model of UVB-induced skin SCC. We detected a low percentage of BMDCs in the lesions of epidermal dysplasia (0.59%), SCC *in situ* (0.15%), and SCC (0.03%). Furthermore, we could not find any evidence of clonal BMDC expansion. In SCC lesions, we also found that most of the BMDCs were tumor-infiltrating hematopoietic cells. In addition, BMDCs in the SCC lesions lacked characteristics of epidermal stem cells, including expression of stem cell markers (CD34, high α6 integrin) and the potential retention of BrdU label. These results indicate that BMDCs are not a major source of malignant keratinocytes in UVB-induced SCC. Therefore, we conclude that BMDCs are not the origin of CSCs in UVB-induced SCC. ***(Am J Pathol 2009, 174:595–601; DOI: 10.2353/ajpath.2009.080362)***

Stem cells, which have the capacity to self-renew and to differentiate into the various mature cells that constitute the tissue of organ, are found in many adult tissues including the skin.[1] Stem cells are critical for replenishing and maintaining the balance of cells (homeostasis) within the tissue and reconstituting tissue damaged during injury. Numerous studies have shown that the specific stem cell properties and the characteristics of stem-cell systems (populations of cells that derive from stem cells are organized in a hierarchical manner) are relevant to some forms of human cancer.[2,3] In cancers, cancer stem cells (CSCs) are thought to exist. CSCs, like tissue stem cells, would have a capacity for self-renewal and a proliferative ability with successive expansion potential promoting tumor structure organization Tumor-initiating cells, which are considered to be a population rich in CSCs, have been identified in cancers of the hematopoietic system[4,5] and various organs.[6–10]

Although several lines of evidence indicate that CSCs can arise from tissue stem cells[6,8,11,12] or mutated progenitor cells[13,14] current reports showed that gastric cancer, which follows persistent gastric inflammation because of the infection with *Helicobacter felis* (*H. felis*), appears to originate from bone marrow-derived cells (BMDCs).[15] Indeed, some populations of BMDCs have the potential to differentiate into mature cells of various nonhematopoietic organs including liver,[16] skeletal-muscle,[17] brain,[18] and skin.[19] We also showed that BMDCs and mesenchymal stem cells are able to transdifferentiate into keratinocytes.[20,21] BMDCs with this plasticity are frequently recruited to sites of injured or inflamed tissue, where they differentiate into mature tissue cells to contribute to tissue repair.[22] Results from *H. felis*-induced gastric cancer suggest that BMDCs with plasticity would differentiate into tissue stem or mature cells to reconstitute the damaged tissue, they then covert into CSCs, and contribute to carcinoma formation. Although recent investigations have demonstrated that BMDCs could contribute to cancers of small intestine, colon, lung,[23] larynx, and brain,[24] it is yet to be determined whether cancers originating from BMDCs certainly exist.

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Skin cancer is currently the most common malignancy in humans.[25] The skin has the role to protect our bodies from a wide range of environmental assaults including UVB irradiation, chemical carcinogens, and the entry of viruses and other pathogens. Therefore, epidermal keratinocytes have more opportunity to manifest maturation arrest. Particular epidemiological and scientific evidence has shown that UVB is one of the most important factors affecting skin carcinogenesis in the physical environment.[25,26]

As in the case of BMDC-originated gastric cancer after persistent inflammation with *H. felis* infection, it is presumed that BMDCs, which are recruited to the UVB-damaged epidermis and differentiate into epidermal keratinocytes to reconstitute the damaged skin, could then give rise to the maturation arrest during continuous UVB irradiation, convert into CSCs, and finally propagate to form bone marrow (BM)-derived skin cancer. Such a novel hypothesis, if true, would have profound implications for our present understanding of the pathogenesis of squamous cell carcinoma (SCC).

To investigate the possible role of BMDCs in skin cancer, we used a mouse model of UVB-induced skin SCC and evaluated the number and marker expressions of labeled BMDCs that differentiated into keratinocytes in skin SCC.

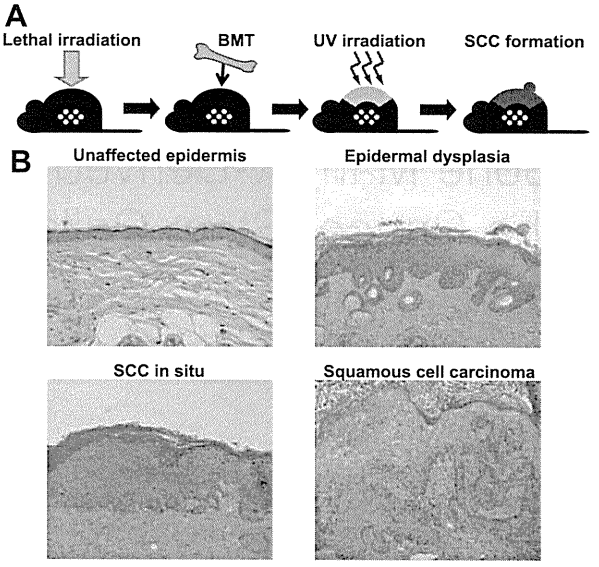

Figure 1. UVB-induced SCC model mice in which BMDCs are labeled with β-Gal enzyme or GFP. **A:** Lethally irradiated mice were transplanted with BM from ROSA26 mice expressing β-Gal enzyme or GFP mice expressing GFP. After confirmation of BM reconstitution, mice were UVB-irradiated. Intermittent UVB irradiation leads mice skin to form SCC. **B:** Tumors were histologically classified as unaffected, dysplasia, SCC *in situ*, or SCC based on tumor architecture, keratinocyte differentiation, and cytological atypia.

Materials and Methods

BM Transplantation

All animal procedures were conducted according to the guidelines of the Hokkaido University Institutional Animal Care and Use Committee under an approved protocol. BM was isolated from the femurs and tibias of male C57BL/6JGtrosa26 (ROSA26) or C57BL/6-TgN(ACTB-EGFP)1Osb/J (GFP) mice (The Jackson Laboratory, Bar Harbor, ME). After lethal irradiation (9 Gy), 1×10^6 BM cells from donor mice in a volume of 200 μl of sterile phosphate-buffered saline were transplanted to recipient C57BL/6 female mice via a single tail vein injection. Hematopoietic reconstitution was subsequently evaluated in peripheral blood 4 weeks after transplantation and more than 94% of BM cells were donor-derived cells.

Induction of UVB Radiation-Induced SCC

UVB-induced carcinogenesis was performed as previously reported (Figure 1A).[27] The UVB light source was a FL20SE30 fluorescent lamp (Clinical Supply, Tokyo, Japan). The UVB irradiation (180 mJ/cm^2) was continued daily for 10 days for tumor initiation to mice (n = 20). One week after the initiation, UVB exposure (180 mJ/cm^2) was performed twice a week until the end of the experiment at 10 months from the last UVB exposure. At 5 months, all irradiated mice (n = 8) had small papules (at least two papules) and erosion. At 10 months, all irradiated mice (n = 6) had tumors (at least three tumors), papules (at least five papules), and ulcer.

Histological Analysis

Mice were sacrificed and tissue was removed, embedded in OCT compound (Sakura, Torrance, CA), snap-frozen or fixed in 4% formalin, and embedded in paraffin. Tumor sections were visualized by routine staining with hematoxylin and eosin (H&E). All of the slides were reviewed twice in blinded manner by three dermatologists, and assessed for tumor architecture, keratinocyte differentiation, cytological atypia, and inflammation. Tumors were classified as dysplasia (typical papilloma), SCC *in situ*, or SCC based on tumor architecture and cytological atypia as described previously.[28] Some lesions exhibiting nonpapillomatous architecture and comprising one to three layers with well-differentiated keratinocytes were classified as normal. Ten samples were analyzed in each normal growth, dysplasia, SCC *in situ*, and SCC. Counts were averaged from eight or nine separate fields in each histological category.

Determination of Enzyme (X-Gal) Activity

Frozen sections (5 μm) were fixed for 30 minutes in 0.2% glutaraldehyde, washed in sodium phosphate buffer containing 0.01% sodium deoxycholate and 0.02% Nonidet P-40 and 1 mmol/L MgCl and incubated for 10 hours at 37°C in a 1-mg/ml X-Gal solution [5-bromo-4-chloro-3-indolyl-β-galactopyranoside: X-Gal, dissolved in dimethyl sulfoxide, 5 mmol/L $K_3Fe(CN)_6$, 5 mmol/L $K_4Fe(CN)_6$ $3H_2O$ in 0.1mol/L sodium phosphate buffer] and counterstained with H&E.

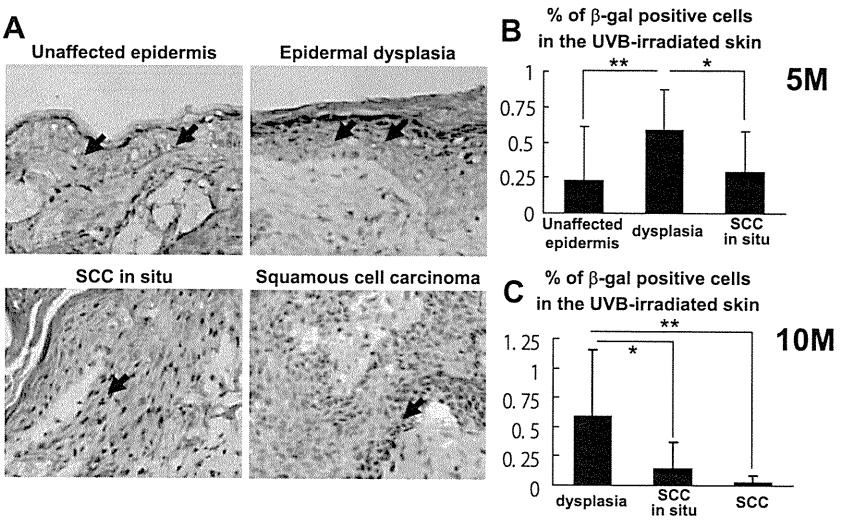

Figure 2. BMDCs in UVB-irradiated mouse skin. **A:** X-Gal-positive cells located within the basal layer in the unaffected epidermis lesions. In the epidermal dysplasia lesions, most X-Gal-positive cells (**arrows**) were found in the suprabasal layers. In the SCC *in situ* lesions, X-Gal-positive cells were found at the inner part of the tumor. In the SCC lesions, X-Gal-positive cells were also found at the inner part of the tumor. **B:** After 5 months of UVB irradiation, the percentage of X-Gal-positive cells was found at 0.15 ± 0.21% in the unaffected epidermis lesions, increased to 0.58 ± 0.25% in the epidermal dysplasia lesions, and decreased to 0.25 ± 0.20% in the SCC *in situ* lesions ($^{*}P < 0.05$, $^{**}P < 0.01$). **C:** After 10 months of UVB irradiation, the percentage of X-Gal-positive cells was 0.59 ± 0.57% in the epidermal dysplasia lesions and 0.15 ± 0.22% in the SCC *in situ* lesions. In the SCC lesions, the percentage of X-Gal-positive cells in the tumor was decreased to 0.03 ± 0.06% ($^{*}P < 0.05$, $^{**}P < 0.01$).

Immunofluorescence

Frozen blocks were prepared and sectioned as described above. Sections were fixed in 4% paraformaldehyde and analyzed for β-galactosidase-expressing cells by using polyclonal antibodies (Cappel, Aurora, OH) and fluorescent secondary antibodies (fluorescein isothiocyanate-labeled goat anti-rabbit antibody; Jackson ImmunoResearch, West Grove, PA). Sections fixed in 4% paraformaldehyde were also analyzed for GFP-expressing cells by using polyclonal antibodies (Molecular Probes, Carlsbad, CA). β-Galactosidase-expressing cells were also stained with antibodies to CD45 (BD Biosciences, San Diego, CA), pan cytokeratins (Progen, Heidelberg, Germany), CD34 (BD Biosciences), or α6 integrin (BD Biosciences). Sections ware viewed with a confocal laser-scanning fluorescence microscope (FV1000; Olympus, Tokyo, Japan).

BrdU Assay

The procedure for BrdU pulse labeling and the subsequent detection were performed as previously reported.[29] In brief, at the time of 9-month UVB irradiation, the tumor-bearing model mice were fed with water containing BrdU (1 mg/ml) for 10 days. Forty-five days after BrdU labeling, the tissues were removed. Frozen sections were fixed with 4% paraformaldehyde or 70% ethanol, stained with antibodies to BrdU (Roche, Penzberg, Germany) and fluorescent second antibodies (tetramethyl-rhodamine isothiocyanate-labeled goat anti-mouse antibody; Southern Biotechnology, Birmingham, AL).

Fluorescence in Situ *Hybridization*

X and Y chromosomes were detected on sections from the UVB-irradiated mice skin using a dual-color detection kit (Cambio, Cambridge, UK) according to the manufacturer's protocol (Cy5 for Y chromosomes and Cy3 for X chromosomes) and immediately viewed with a confocal microscope.

Results

Low Frequency of BMDCs in UVB-Irradiated Skin

To investigate the possible role of BMDCs in UVB-induced skin dysplasia/carcinoma progression, we used a model mouse whose BMDCs are labeled with β-galactosidase (β-Gal) or green fluorescent protein (GFP). Lethally irradiated mice were transplanted with BM from ROSA26 mice or GFP transgenic mice (Figure 1A). After the confirmation of BM reconstitution, mice were irradiated with UVB and developing tumors in mice skin were evaluated histologically. Each section was divided into four categories of unaffected, dysplasia, SCC *in situ*, and SCC (Figure 1B).[28] After 5 months of UVB irradiation, we found the dysplasia lesions and the SCC *in situ* lesions, whereas we found no SCC lesions in irradiated skin. After 10 months of UVB irradiation, the dysplasia lesions and the SCC *in situ* lesions were found to be continuous with the SCC lesions, whereas the unaffected epidermis lesions were completely absent.

To detect the presence of BMDCs in UVB-irradiated mouse skin, X-galactosidase (X-Gal) staining was performed. The numbers of BMDCs were quantified by counting the number of X-Gal-positive cells in the UVB-irradiated mouse skin (Figure 2A). After 5 months of UVB irradiation, even in the unaffected epidermis lesions, some X-Gal-positive cells, indicating BMDCs, were located within the basal layer. In the epidermal dysplasia lesions, some X-Gal-positive cells were also found within the basal layer, but most X-Gal-positive cells were found within the suprabasal layers. In the SCC *in situ* lesions, X-Gal-positive cells were found within the inner parts of the tumor. The percentage of occurrence of X-Gal-positive cells was 0.15% in the unaffected epidermis lesions. Since we previously showed that wounded skin contained BMDCs (0.03%),[20] repeated UVB irradiation might induce BMDC accumulation. The percentage of X-Gal-positive cells in the epidermal dysplasia lesions increased to 0.58%, whereas the percentage of X-Gal-

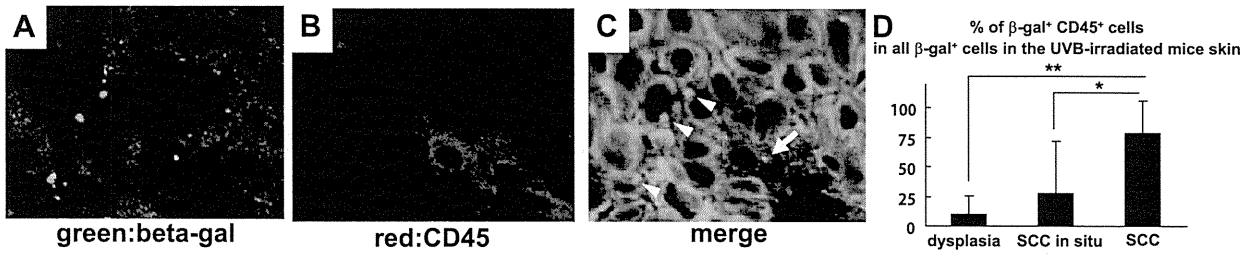

Figure 3. BM-derived infiltrating cells in the UVB-irradiated skin. Triple staining of β-Gal (green) (**A**), CD45 (red) (**B**), and pancytokeratin (cyan) was performed. **C:** Merged image showed β-Gal$^+$/CD45$^+$ (**arrow**) or β-Gal$^+$/CD45$^-$ (**arrowheads**) cells in the tumor. **D:** Percentage of CD45$^+$ of all β-Gal$^+$ cells in the UVB-irradiated mice skin. In all β-Gal$^+$ cells, 10.1 ± 15.3% was positive for CD45 in the epidermal dysplasia lesions, 27.3 ± 44.1% in the SCC *in situ* lesions, and at 78.7 ± 27.4% in the SCC lesions ($^*P < 0.05$, $^{**}P < 0.01$). Original magnifications, ×600.

positive cells in the SCC *in situ* lesions was decreased to 0.25% (Figure 2B). The number of β-Gal-positive cells and total epidermal cells of the UVB-irradiated skin were as follow; unaffected (6 of 2276), dysplasia (28 of 4804), SCC *in situ* (15 of 5445). We further confirmed that no X-Gal-positive cells were detected in untreated (unirradiated) mice. We failed to find any clusters of X-Gal-positive cells in either the unaffected epidermis or the tumor. These results indicate that BMDCs in the UVB-irradiated skin do not commonly give rise to a monoclonal expansion.

After 10 months of UVB irradiation, in the epidermal dysplasia lesions and SCC *in situ* lesions, we found X-Gal-positive cells in a similar location as mice skin that received 5 months of UVB irradiation. In the SCC lesions, X-Gal-positive cells were found within the inner part of the tumor (Figure 2A). X-Gal-positive cells were found at a percentage of 0.59% in the epidermal dysplasia lesions and 0.15% in the SCC *in situ* lesions. These percentages of X-Gal-positive cells in 10-month UVB-irradiated mouse skin were similar to the percentage in 5-month UVB-irradiated mouse skin. In the SCC lesions, the percentage of X-Gal-positive cells was at 0.03%, which decreased in comparison with the percentage in the SCC *in situ* lesions (Figure 2C). The number of β-Gal-positive cells and total epidermal cells of the UVB-irradiated skin were as follow; dysplasia (28 of 5141), SCC *in situ* (9 of 6559), SCC (4 of 13,701).

As an additional test for BM origin, we used a mouse model in which BMDCs were GFP$^+$ using BMT from GFP transgenic mice. Although we evaluated the percentages of BMDCs in UVB-irradiated skin, the GFP$^+$/pancytokeratin$^+$ cells were found at an extremely low percentage, ~0.12% in the epidermal dysplasia lesions and 0% in the SCC *in situ* lesions (data not shown). Previous reports about the *H. felis* gastric cancer also showed a similar tendency that the percentages of malignant cells with the marker of BMDCs was much lower in GFP-labeled model mice than in β-Gal-labeled model mice.[15] Therefore we used an UVB-irradiated mouse model with labeled BMDCs with β-Gal in the following experiments.

Most BMDCs in the SCC Are Inflammatory Hematopoietic Cells

We considered that some X-Gal-positive cells in the UVB-irradiated skin were likely to be the tumor-infiltrating hematopoietic cells. To investigate the presence of these cells, triple staining for β-Gal, CD45 (hematopoietic marker), and a pancytokeratin (cytokeratin marker) was performed (Figure 3, A–C). The number of β-Gal$^+$/CD45$^+$ of all β-Gal$^+$ cells per field was counted in UVB-irradiated mouse skin. In all β-Gal$^+$ cells, 10.1% were positive for CD45 in the epidermal dysplasia lesions. Percentages of CD45$^+$ cells of all β-Gal$^+$ cells were 27.3% and 78.7% in the SCC *in situ* lesions and in the SCC lesions, respectively (Figure 3D). Some of the CD45$^+$ cells were fused with carcinoma cells. Indeed, CD45 has been found to be expressed by cancer cells.[30–32] However, we were unable to find X-Gal-positive cells that co-expressed CD45 and pancytokeratin. The result of our experiments clearly shows that some β-Gal$^+$ cells are tumor-infiltrating hematopoietic cells, whereas other β-Gal$^+$/CD45$^-$ cells might be BMDCs that differentiated into tumor keratinocytes. However, the percentage of β-Gal$^+$/CD45$^+$ cells (indicating tumor-infiltrating hematopoietic cells) is increased in the SCC lesions. This observation would indicate that the actual occurrence rate of BM-derived keratinocytes is lower than our counting of BMDCs that were detected with X-Gal staining.

Small Number of BMDCs in the SCC Exhibited Donor XY Chromosomes

To further confirm BM origin, we analyzed UVB-induced skin SCC cells from female hosts (XX chromosomes) transplanted with male donor BM (XY chromosomes) using fluorescence *in situ* hybridization technique. We counted more than 10,000 cells and detected some donor-derived keratinocytes with XY chromosome expression, indicating BM origin (less than 0.05%) (Figure 4A).

In various organs, BMDCs contribute to the tissue reconstitution by either fusion[22] or transdifferentiation.[19] To determine whether BMDC engraftment into the specific tissue cells was because of differentiation or somatic cell fusion, fluorescence *in situ* hybridization was used because the fused cells would be expected to possess XXXY chromosomes. Although we observed keratinocytes with Y chromosomes in the tumor, none of them expressed an XXXY chromosome. However, fusion hybrids notoriously lose chromosomes and the absence of tetraploid cells does not rule out fusion.[33–35] Therefore, we could not exclude the possibility of cell fusion with the present data.

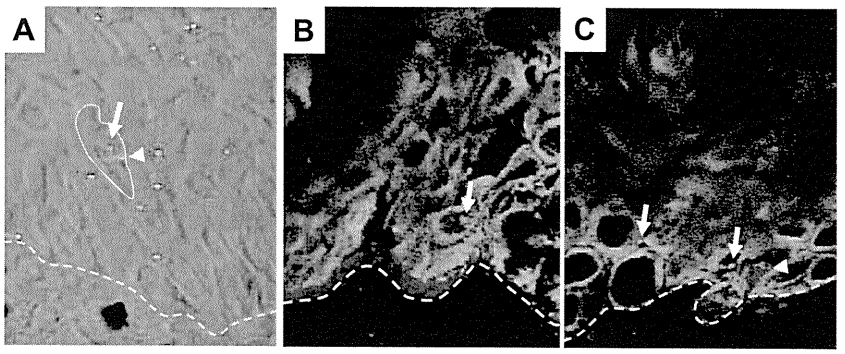

Figure 4. XY chromosome expressions and epidermal stem cell markers of the BMDCs in the UVB-irradiated skin. **A:** Fluorescence *in situ* hybridization showed cells with single X chromosome (red, **arrow**) and single Y chromosome (cyan, **arrowhead**) in the UVB-induced skin SCC. XY chromosome cells, indicating BMDCs were indicated. **B:** Triple staining of β-Gal (green), α6 integrin (red), and pancytokeratin (cyan) was performed. **Arrow** shows β-Gal$^+$ tumor keratinocytes. Although α6 integrin was positive within the edge of the tumor, we could not find any significant overexpression of α6 integrin of β-Gal$^+$/cytokeratin$^+$ cells. **C:** Triple staining of β-Gal (green), BrdU (red), pancytokeratin (cyan). **Arrows** show β-Gal$^+$ tumor keratinocytes. **Arrowhead** shows a BrdU$^+$ tumor keratinocyte. We found no β-Gal$^+$/BrdU$^+$ tumor keratinocytes.

BMDCs in the SCC Failed to Express Epidermal Stem Cell Marker

Although the CSC markers of skin SCC have yet to be defined, published studies suggest that tumor-initiating cells might be positive for the stem cell marker of the original organs.[6,8] To investigate the possibility that BMDCs in the UVB-irradiated skin could share some characteristics of CSCs of skin SCC, we assayed the location of these presumptive CSCs that are positive for epidermal stem cell markers in the UVB-induced skin SCC.

Although CD34 is an established marker of skin epithelial stem cells,[36] none of the keratinocytes (including BM-derived keratinocytes) in the UVB-induced skin SCC expressed CD34 (data not shown). Furthermore, skin epithelial stem cells express elevated levels of α6 integrin compared with differentiated keratinocytes.[37] Although some keratinocytes in the edge of SCC showed α6 integrin expression, β-Gal$^+$/pancytokeratin$^+$ cells (indicating BM-derived keratinocytes) did not show significantly upregulated α6 integrin expression compared with non-BM-derived keratinocytes (Figure 4B). In addition, tissue stem cells can be distinguished from transit-amplifying cells by their ability to incorporate and retain 5-bromo-2′-deoxyuridine (BrdU) throughout a long period of time. Therefore, tissue stem cells can be identified as label-retaining cells (LRCs).[29] To determine whether BMDCs in the UVB-irradiated mouse skin exhibit any LRC characteristics, the tumor-bearing mice were fed water containing BrdU. In the UVB-irradiated mice skin, no LRCs expressed β-Gal (Figure 4C). These results indicate that BMDCs in the UVB-induced skin SCC did not share any of these characters of the presumptive CSCs of the skin SCC.

Discussion

Based on recent investigations that suggest the possibility for BMDCs to be the origin of cancers,[15,38] we used a labeled BMDC mouse model and investigated the role of BMDCs during UVB-induced carcinogenesis. With intermittent UVB irradiation, the epidermal morphology in mouse skin changed from the normal state through dysplasia, SCC *in situ*, and finally to SCC. These histological changes are analogous to the natural phenomenon observed in UVB-induced human skin carcinogenesis. We certainly found BMDCs in UVB-irradiated mouse skin. Our data further suggests that BMDCs are recruited to the UVB-damaged skin and transdifferentiate into epidermal keratinocytes to reconstitute the skin, as we previously reported in wound repair.[20] We show the accelerated recruitment of BMDCs in the epidermal dysplasia lesions and the decreased rate of BMDCs in the SCC lesions. We propose this is attributable to the propagation of non-BM-derived malignant keratinocytes. Although BMDCs are recruited to the UVB-damaged skin and transdifferentiate into unaffected epidermal keratinocytes, BMDCs do not convert into malignant keratinocytes so that the rate of BMDCs relatively decreases as non-BM-derived tumor keratinocytes propagate to form skin SCC.

As a result, we found very few instances of BM-derived keratinocytes in the UVB-irradiated mouse skin. This observation strongly suggests that BMDCs are unlikely to be the origin of UVB-induced skin SCC. The objection will no doubt be raised that BMDCs might lose the expression of BM markers during the continuous UVB irradiation. Therefore we were careful to examine BM-derived keratinocytes in skin SCC with three different BMDC markers (β-Gal, GFP, Y chromosome analysis). Our conclusion is exactly the opposite of the *H. felis*-induced murine gastric carcinoma study.[15] It is reasonable to suppose that the difference in the results between *H. felis*-induced gastric carcinoma study and our UVB-induced skin carcinoma study is partially attributable to the process of carcinogenesis including the type of genetic damage and degree of inflammation. In *H. felis*-induced gastric carcinoma, the pathogenic factor, namely CagA, increases the proliferation of host cells or inhibits cell apoptosis, stimulating the malignant transformation of host cells.[39,40] These processes would be important for cancer progression from BMDCs. In humans, previous reports showed that solid cancers contain BM-derived cancer cells at a low level of 0 to 6% except for lung carcinoma that contains ~20% of BM-derived cancer cells.[23,24] These data further showed that BMDCs do not contribute to skin cancers.[23] Our results are consistent with these observations.

The epidermis is continuously supplied with keratinocytes from the hair follicle bulge stem cells throughout adult life.[41] Most epidermal keratinocytes that acquire

oncogenic mutations are lost during differentiation. Therefore, only long-term resident cells, such as stem cells, have the capacity to accumulate the required number of genetic hits necessary for tumor development. For this reason, it is not unreasonable to assume that these epidermal stem cells in the bulge could acquire oncogenic mutations, transdifferentiate into CSCs, and proliferate as malignant cells in the skin cancer. Although a previous report showed that BMDCs were more frequently found in the bulge area,[42] we could not find such a tendency in our experiments in UVB-induced carcinogenesis. Our previous research in the damaged skin also showed no tendency of BMDC accumulation at specific skin sites.[20] Furthermore, we failed to find any evidence of BMDC clonal expansion in the UVB-irradiated mice skin. We also showed that BMDCs express no epidermal stem cell markers and fail to behave as LRCs, one of the main characteristics of tissue stem cells. Although the existence of the CSCs in the skin cancer has yet to be properly defined, we suggest that the CSCs in the UVB-induced skin SCC, if present, do not commonly originate from BMDCs.

It is important to determine the origin of the CSCs for the elucidation of carcinogenic mechanisms or for the treatment of cancer. Because of the recent reports that showed sarcoma derived from mesenchymal stem cells,[43,44] an objection against transferring cells with the potential to have properties of stem or progenitor cells has arisen in regenerative medicine. However we can conclude from the results of our experiments that cancer cells in the UVB-induced skin SCC do not originate from BMDCs. Therefore we consider that in adopting or using BMDCs for regenerative medicine, the possibility of unexpected carcinogenesis can primarily be excluded and that BMDCs should be further tested and adapted for use in regenerative medicine, especially for skin.

We demonstrated the existence of BM-derived keratinocytes in the UVB-irradiated skin. These BM-derived keratinocytes were considered to be the result of transdifferentiation, not fusion. However, the number of BM-derived keratinocytes was extremely few, with no clonal expansion. Furthermore, BM-derived keratinocytes failed to express the epidermal stem cell markers (CD34, high α6 integrin and LRCs). Through our laboratory experiments, the possibility that BMDCs are the origin of UVB-induced skin SCC is extremely low.

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A randomized double-blind trial of intravenous immunoglobulin for pemphigus

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Background: Pemphigus is a rare life-threatening intractable autoimmune blistering disease caused by IgG autoantibodies to desmogleins. It has been difficult to conduct a double-blind clinical study for pemphigus partly because, in a placebo group, appropriate treatment often must be provided when the disease flares.

Objective: A multicenter, randomized, placebo-controlled, double-blind trial was conducted to investigate the therapeutic effect of a single cycle of high-dose intravenous immunoglobulin (400, 200, or 0 mg/kg/d) administered over 5 consecutive days in patients relatively resistant to systemic steroids.

Methods: We evaluated efficacy with time to escape from the protocol as a novel primary end point, and pemphigus activity score, antidesmoglein enzyme-linked immunosorbent assay scores, and safety as secondary end points.

Results: We enrolled 61 patients with pemphigus vulgaris or pemphigus foliaceus who did not respond to prednisolone (≥20 mg/d). Time to escape from the protocol was significantly prolonged in the 400-mg group compared with the placebo group ($P < .001$), and a dose-response relationship among the 3 treatment groups was observed ($P < .001$). Disease activity and enzyme-linked immunosorbent assay scores were significantly lower in the 400-mg group than in the other groups ($P < .05$ on day 43, $P < .01$ on day 85). There was no significant difference in the safety end point among the 3 treatment groups.

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Other investigators in the Pemphigus Study Group are listed in the Appendix.

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Limitation: Prednisolone at 20 mg/d or more may not be high enough to define steroid resistance.

Conclusion: Intravenous immunoglobulin (400 mg/kg/d for 5 d) in a single cycle is an effective and safe treatment for patients with pemphigus who are relatively resistant to systemic steroids. Time to escape from the protocol is a useful indicator for evaluation in randomized, placebo-controlled, double-blind studies of rare and serious diseases. (J Am Acad Dermatol 2009;60:595-603.)

Pemphigus is a life-threatening, rare intractable autoimmune blistering disease caused by IgG autoantibodies to desmoglein (Dsg) (epidermal adhesion factor). It is characterized by the development of blisters and erosions of the skin and mucosa.[1] Currently, oral steroids are the drugs of first choice for pemphigus, and may be used in combination with immunosuppressants or plasma exchange. However, many patients with pemphigus experience cycles of remission and recurrence, and accordingly become unresponsive to conventional therapy. On the other hand, patients with complications such as diabetes mellitus, gastrointestinal disease, osteoporosis, infection, or immunodeficiency are relatively contraindicated for use of high-dose (HD) steroids. For such patients, an alternative effective treatment strategy is required.

Although several reports suggesting the effectiveness of HD intravenous immunoglobulin (IVIG) in the treatment of pemphigus have been published since its introduction as monotherapy in 1989, most are case reports with a low evidence level or involved clinical research with a limited number of patients using multiple treatment cycles.[2] No well-controlled, double-blind clinical study to demonstrate the efficacy of HD-IVIG has been conducted.[3-13] This is because: (1) pemphigus is a rare intractable disease; (2) appropriate treatment must be provided in a timely manner if symptoms are aggravated or unchanged for a certain period of time; (3) inclusion of a placebo group compromises compliance with the study protocol; and (4) it is not ethical to treat patients with pemphigus using placebo because mortality is high.

We developed a novel evaluation end point to solve these problems and verified the usefulness of HD-IVIG in a single treatment cycle for this rare intractable disease.

Abbreviations used:

ADRs: adverse drug reactions
Dsg: desmoglein
HD: high dose
IVIG: intravenous immunoglobulin
PAS: pemphigus activity score
PF: pemphigus foliaceus
PV: pemphigus vulgaris
TEP: time to escape from the protocol

METHODS

Patients

This study was conducted in 27 medical institutions in Japan with affiliated dermatologists specialized in autoimmune blistering disease. Patients were given the diagnosis of pemphigus vulgaris (PV) or pemphigus foliaceus (PF) as confirmed based on our national diagnostic criteria as follows: pemphigus was diagnosed when at least one item from every 3 findings, or two items from clinical findings and one item from immunologic findings were satisfied.

1. Clinical findings
   - Multiple, easily rupturing, flaccid blisters of the skin
   - Subsequent progressive, refractory erosions or crust after blisters
   - Noninfectious blisters or erosions of visible mucosa including oral mucosa
   - Nikolsky sign
2. Histologic findings
   - Intraepidermal blisters caused by loss of adhesion between epidermal cells (acantholysis)
3. Immunologic findings
   - IgG (or complement) deposition in the intercellular spaces of the lesional or normal-appearing skin and mucosa as detected by direct fluorescent antibody assay
   - Antiepidermal intercellular IgG autoantibody (anti-Dsg IgG autoantibody) identified by indirect fluorescent antibody assay or enzyme-linked immunosorbent assay

The study patients had to meet all the following inclusion criteria and none of the exclusion criteria.

1. Inclusion criteria: patients aged 20 years or older who provided written informed consent to participate in the study and met all of the following criteria.

Table I. Criteria for pemphigus activity score

Variable score	Skin lesion area*	No. of new blisters/d	Oral mucosal lesions†
3	≥15%	≥5	≥30%
2	≥5% and <15%	1 to 4	≥5% and <30%
1	<5%	Occasionally‡	<5%
0	None	None	None

*Percentage of entire surface area.
†Score is doubled for patients who have only oral mucosal lesions at time of study enrollment.
‡Blisters sometimes newly develop within 1 week but not every day.

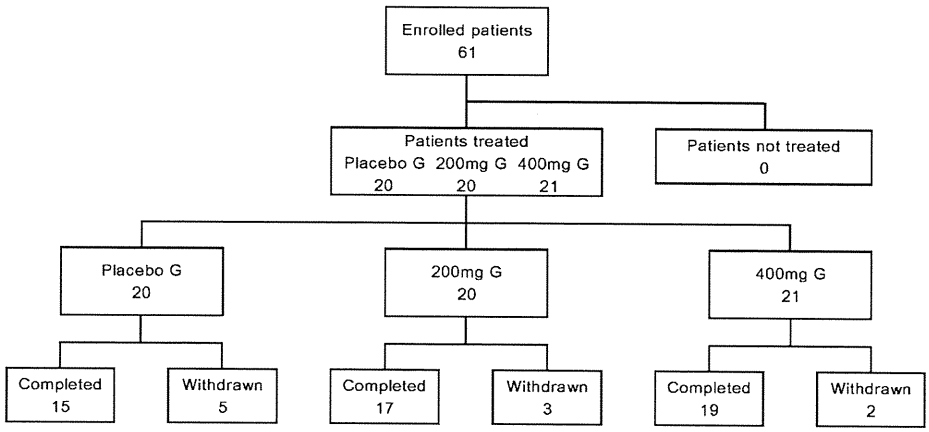

Fig 1. Disposition of patients. *G*, Group.

- Treatment with any steroid at greater than or equal to 20 mg/d (prednisolone equivalent)
- Symptoms (total pemphigus activity score [PAS] [Table I]) did not respond to steroid therapy

2. Exclusion criteria: patients who met any of the following criteria were excluded from the study because efficacy evaluation of the test drug might be affected and to assure the safety of patients.
   - Patients treated with plasma exchange therapy, steroid pulse therapy, or HD-IVIG within 30, 14, or 42 days, respectively, before informed consent and the start of study treatment
   - Patients with a history of shock or hypersensitivity to the test drug
   - Patients with IgA deficiency, hepatic disorder, renal disorder, or hemolytic or blood loss anemia
   - Patients with any previous or existing cerebrovascular or cardiovascular disorder

Study design

This was a multicenter, randomized, placebo-controlled, double-blind, parallel-group study. The study protocol and written informed consent form approved by the institutional review board at each study institution were used in the study. Observation of the first patient was started on November 4, 2004, and that of the last patient was completed on September 25, 2006.

Treatment groups

The IVIG group received IV drip infusion at 200 or 400 mg/kg/d administered in divided dose over 5 consecutive days. The placebo group received IV drip infusion of physiologic saline for 5 consecutive days.

Investigational drugs manufactured by Nihon Pharmaceutical Co Ltd (Higashikanda, Tokyo, Japan) were used in the study.

Methods of allocation

Patients were randomized by a central enrollment system to the treatment groups according to a dynamic allocation scheme to ensure that there were no between-group differences in the dose of prior steroid, total PAS, or disease type.

Blinding

Because the investigational drugs were distinguishable in terms of appearance and viscosity after

Table II. Demographic and other baseline characteristics

Characteristic	Category	Dose: Placebo n = 20	Dose: 200 mg n = 20	Dose: 400 mg n = 21	Between-group comparison
Sex	Male	9	10	8	NS* (*P* = .766)
	Female	11	10	13	
Age, y	Mean ± SD	53.1 ± 10.9	57.0 ± 14.6	50.1 ± 11.7	NS† (*P* = .225)
Body weight, kg	Mean ± SD	57.8 ± 11.6	58.0 ± 10.4	57.7 ± 9.1	NS* (*P* = .686)
Disease type	PV	13	14	13	NS* (*P* = .942)
	PF	7	6	8	
Disease duration, mo	Mean ± SD	16.1 ± 13.6	28.6 ± 32.3	28.5 ± 46.9	NS† (*P* = .414)
Baseline PAS	Mean ± SD	3.3 ± 1.4	3.6 ± 1.8	3.7 ± 1.1	NS† (*P* = .660)
Steroid dose, mg	Mean ± SD	27.6 ± 9.7	23.9 ± 11.1	27.4 ± 11.1	NS† (*P* = .461)
Immunosuppressants	No. of patients (%)	2 (10.0)	7 (35.0)	5 (23.8)	NS* (*P* = .179)

NS, Not significant difference; *PAS*, pemphigus activity score; *PF*, pemphigus foliaceus; *PV*, pemphigus vulgaris.
Two-sided test for both analyses.
*Fisher exact test.
†One-way analysis of variance.

reconstitution, independent staff at each study institution separately prepared and administered the dosing solution, and evaluated efficacy and safety in each patient to maintain blinding. The bottles of the investigational drugs were covered with a masking cover and provided to the independent staff member in charge of administration. Each independent staff member involved signed a blinding confirmation form at the end of the study to assure that blinding was maintained.

End points

Time to escape from the protocol (TEP) was used as the primary efficacy end point. TEP was defined as the length of the period until a patient stayed on the protocol without any additional treatment. When symptoms were unchanged for 2 weeks or aggravated, the treatment given was considered to be ineffective and additional treatment was required such as increase in steroid dose, change in steroid type, use of additional immunosuppressive agents, or plasma exchange; these patients were considered escaped from the protocol. This methods allow doctors in charge to have flexibility to rescue patients with other treatment when needed.

The secondary end points used in the study included: (1) PAS over time (scores [0-3 point] for skin lesion area, number of new blisters/d, and oral mucosal lesions, and their total scores [Table I]); and (2) the titers of pemphigus autoantibodies over time (anti-Dsg1 autoantibody titer and anti-Dsg3 autoantibody titer). Titers of pemphigus autoantibodies were determined by enzyme-linked immunosorbent assay.[14,15] As a safety end point, the occurrence of adverse events by 85 days after the start of the study treatment (day 85) was investigated. Adverse events were recorded up to day 43 if patients escaped from the protocol by day 43 or up to TEP if patients escaped from the protocol after day 44.

Statistical analysis

The cumulative rate of TEP, which was estimated by evaluation of the dose-response relationship of TEP and by analysis using the Kaplan-Meier method, was compared among the treatment groups by log rank test. Scores for skin lesion area, number of new blisters/d, and oral mucosal lesions, and total score, the secondary end point, up to day 85 were compared with baseline data by the paired *t* test for each treatment group. The data after TEP were imputed from the data at the TEP (last observation carried forward). Adverse events occurring up to day 85 for which the causal relationship with HD-IVIG or placebo was judged to be other than "not related" were handled as adverse drug reactions (ADRs). A two-sided significance level of .05 was used for analyses.

RESULTS

Disposition of patients

The disposition of patients enrolled in the study is shown in Fig 1. A total of 61 patients were treated with the investigational drug (placebo, 20; 200 mg, 20; and 400 mg, 21). All the enrolled patients including 10 patients (placebo, 5; 200 mg, 3; and 400 mg, 2) who were withdrawn from the study according to the requirements in the protocol were included in the analyses. The main reasons for study withdrawal were the evaluator's decision to withdraw the patient and the occurrence of adverse events. The demographic and other baseline

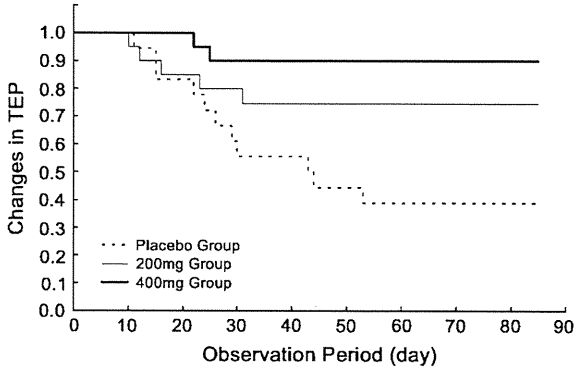

Fig 2. Changes in time to escape from protocol (*TEP*). TEP was significantly prolonged in 400-mg group compared with placebo group with dose-dependent fashion. Cumulative TEP on day 85 was 10.0% in 400-mg group, 25.0% in 200-mg group, and 61.0% in placebo group (log rank test). Between-group comparison demonstrated significant prolongation of TEP in 400-mg group compared with placebo group ($P < .001$, log rank test). In contrast, difference between 200-mg and placebo groups was not significant ($P = .052$). In addition, dose-response relationship was observed in TEP ($P < .001$). Data are stated using TEP ratio.

characteristics are presented in Table II. There were no significant between-group differences in the distribution of baseline characteristics. The average disease durations of 200- and 400-mg groups are longer than in the placebo group, but this is because the former group happened to contain patients with extremely long duration (116 months in 200 mg; 142 and 169 months in 400 mg) and the difference was not statistically significant.

Efficacy (primary end point): TEP

TEP was evaluated as the primary end point (Fig 2). In the 400-mg group, 19 of 21 patients stayed on the protocol during the observation period. Two patients escaped from the protocol with TEPs of 22 and 25 days. In the 200-mg group, 15 of 20 patients stayed on the protocol and the shortest TEP was as early as 10 days among the 5 escaped patients. In the placebo group, only 9 patients stayed on the protocol, and the shortest TEP was as early as 11 days. TEP was within 30 days for 8 patients.

TEP in the active treatment groups was compared with that in the placebo group (log rank test). The TEP in the 400-mg group was significantly longer than that in the placebo group ($P < .001$), whereas the difference between the 200-mg and placebo groups was not significant ($P = .052$). Log rank test of TEP for the 61 patients indicated a dose-response relationship for this parameter ($P < .001$).

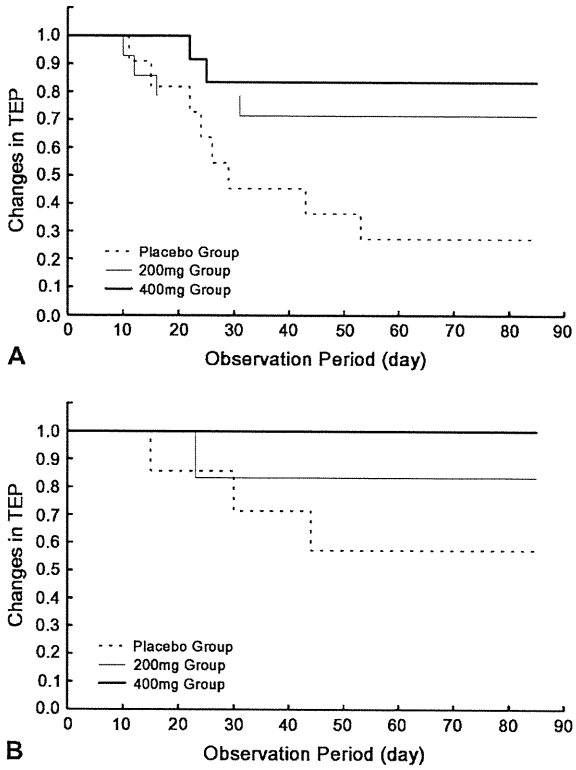

Fig 3. Cumulative time to escape from protocol (*TEP*) shown by pemphigus subtype. Cumulative TEP estimated by Kaplan-Meier method was divided in disease subtype of pemphigus vulgaris (PV) (**A**, n = 13 in 400-mg group, n = 14 in 200-mg group, n = 13 in placebo group) and pemphigus foliaceus (PF) (**B**, n = 8 in 400-mg group, n = 6 in 200-mg group, n = 7 in placebo group). Cumulative TEP in patients with PV on day 85 was 15.0% in 400-mg group, 29.0% in 200-mg group, and 73.0% in placebo group, whereas that of patients with PF was 0.0% in 400-mg group, 17.0% in 200-mg group, and 43.0% in placebo group. Between-group comparison demonstrated significant prolongation of TEP in 400-mg group compared with placebo group (PV, $P = .007$; PF, $P = .044$; log rank test). In contrast, difference between 200-mg and placebo groups was not significant (PV, $P = .055$; PF, $P = .416$). In addition, dose-response relationship was observed in TEP (PV, $P = .007$; PF, $P = .043$).

Analyses stratified by baseline characteristics (disease type and PAS) also demonstrated dose-response relationships and significant differences between the 400-mg and placebo groups, as in the overall analyses (Figs 3 and 4).

Efficacy (secondary end point)

Pemphigus activity score. Efficacy was also evaluated based on the changes in clinical symptoms, ie, changes in PAS determined based on skin lesion area, number of new blisters/d, and oral mucosal lesions. In the 400-mg group, total PAS

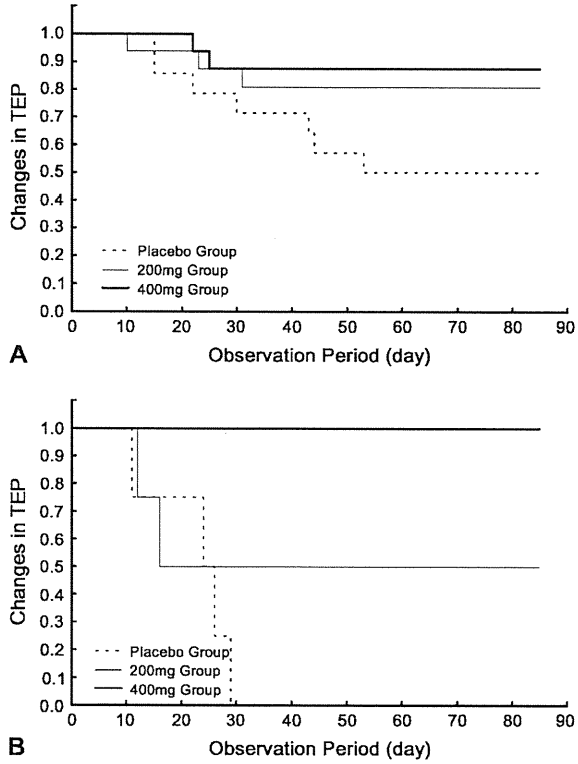

Fig 4. Cumulative time to escape from protocol (*TEP*) shown in different pemphigus activity score (PAS). Data were divided by PAS into two groups: total PAS of 0 to 4 (**A**, n = 17 in 400-mg group, n = 16 in 200-mg group, n = 16 in placebo group) and total PAS of 5 to 9 (**B**, n = 4 in 400-mg group, n = 4 in 200-mg group, n = 4 in placebo group). Cumulative TEP in patients with total PAS of 0 to 4 on day 85 was 12.0% in 400-mg group, 19.0% in 200-mg group, and 50.0% in placebo group, whereas those of patients with total PAS of 5 to 9 was 0.0% in 400-mg group, 50.0% in 200-mg group, and 100.0% in placebo group. Between-group comparison demonstrated significant prolongation of TEP in 400-mg group compared with placebo group (total score 0-4, P = .028; total score 5-9, P = .006). In contrast, difference between 200-mg and placebo groups was not significant (total score 0-4, P = .109; total score 5-9, P = .345). In addition, dose-response relationship was observed in TEP (total score 0-4, P = .024; total score 5-9, P = .012).

was significantly decreased from the baseline score at all points of observation (day 8, P = .05; after day 15, $P < .01$). It was decreased from 3.7 on day 1 to 2.0 on day 85 (by 46.8%) (Fig 5). In the 200-mg group, total PAS was significantly decreased from the baseline score at all points of observation after day 15 (day 15-43, $P < .05$; day 57-85, $P < .01$). It was decreased from 3.7 on day 1 to 2.3 on day 85 (by 36.6%). On the other hand, in the placebo group, no significant decrease from baseline score was observed at any of the points of observation. Each PAS

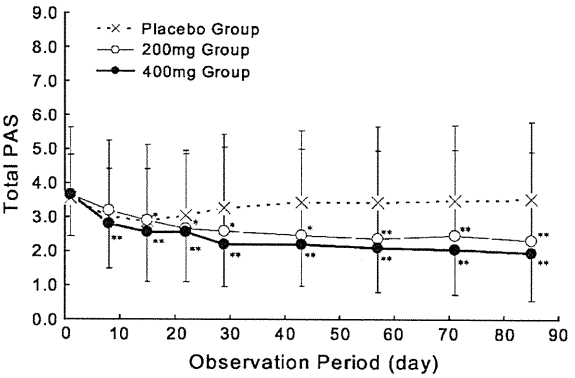

Fig 5. Changes of pemphigus activity score (*PAS*) over time. Total PAS was significantly lower in 400- and 200-mg groups than in placebo group. Significant difference from day 1 at hazard ratio of *0.05 and **0.01.

(skin lesion area, number of new blisters/d, and oral mucosal lesions) also exhibited a significant change from baseline in the 400-mg group ($P < .01$) but not in the placebo group (data not shown).

Titers of anti-Dsg IgG autoantibodies

It has been reported that levels of IgG autoantibodies to Dsg1 and Dsg3 in patients with pemphigus correlate with disease activity.[14,15] Accordingly, efficacy was also evaluated based on the changes in anti-Dsg1 IgG autoantibody titer for patients with PF and PV or in anti-Dsg3 IgG autoantibody titer for patients with PV (Fig 6). In the 400-mg group, anti-Dsg1 and −Dsg3 IgG antibody titers were significantly decreased from baseline on days 43 and 85 (day 43 and 85, $P < .01$). In the 200-mg group, anti-Dsg1 and −Dsg3 IgG antibody titers also exhibited significant decreases on day 85 but not day 43 (day 43, $P < .05$; day 85, $P < .01$). On the other hand, in the placebo group, no significant decrease from baseline was observed in either anti-Dsg1 or −Dsg3 IgG antibody titer.

Safety

The incidence of ADRs was 28.6% (n = 6/21) in the 400-mg group, 35.0% (n = 7/20) in the 200-mg group, and 25.0% (n = 5/20) in the placebo group. No significant difference was observed between the placebo and 200- or 400-mg groups. ADRs reported in the study included: headache in two patients, aggravated chronic hepatitis C, decreased lymphocytes, palpitations, abdominal discomfort, constipation, nausea, pain at the injection site, increased creatinine, increased blood pressure, and decreased platelet count in one patient each in the 400-mg group; and increased alanine aminotransferase in 3 patients; increased γ-glutamyltranspeptitase, hepatic dysfunction, and increased bilirubin in two patients each; and common cold, muscle pain, increased

aspartate aminotransferase, increased blood pressure, decreased lymphocytes, increased neutrophils, decreased white blood cell count, bleeding tendency, anorexia, hypoalbuminemia, hepatic encephalopathy, gastrointestinal bleeding, malaise, fever, increased ammonium, increased C-reactive protein, decreased hematocrit, decreased hemoglobin, decreased platelet count, decreased red blood cell count, and decreased urine volume in one patient each in the 200-mg group. All these ADRs were consistent with the information displayed on the Food and Drug Administration Web site (http://www.fda.gov/cber/gdlns/igivimmuno.htm).

One patient in the 200-mg group died of hepatic failure as a result of aggravation of hepatitis C, which was an underlying complication reported before the start of the study.

This event was judged as probably related to the investigational drug in the evaluator's opinion.

DISCUSSION

Most clinical research involving a rare disease is based on case reports or data from limited samples obtained in open-label studies. In particular, in life-threatening, serious, and intractable diseases, such as pemphigus, appropriate treatment must be provided in a timely fashion if symptoms are aggravated or unchanged for days. This makes performance of a placebo-controlled, double-blind comparison study infeasible. On the other hand, the efficacy of new drugs for malignant tumors or for patients requiring pain relief is evaluated based on the time to recurrence of tumor or the number of patients requiring rescue analgesia.[16-21] Based on these considerations, we developed a novel efficacy indicator (ie, TEP) with reference to the end points used for efficacy evaluation of drugs for malignant tumors or for patients requiring pain relief, to conduct a placebo-controlled, double-blind comparison study in patients with pemphigus who were relatively resistant to systemic steroids. This new efficacy end point provides flexibility for physicians to rescue patients when required and proved to be useful to evaluate the efficacy of a single cycle of HD-IVIG in a double-blind comparison design. However, some concerns remain regarding the rigidity: a period of 3 to 7 days before the start of study treatment was required to confirm the unresponsiveness of patients to steroids, and switching to other treatments was prohibited during the first 5-day treatment period.

The mode of action of HD-IVIG is complex. It is found to exert its effect through modulation of expression and function of Fc receptors, interference with complement activation and the cytokine

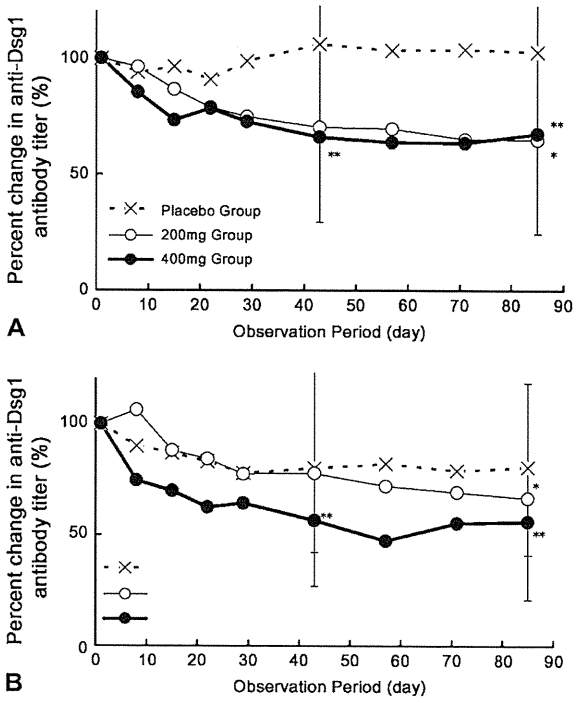

Fig 6. Changes of anti-desmoglein (*Dsg*) IgG titers. Anti-Dsg IgG titers were significantly lower in 400-mg intravenous immunoglobulin group than in placebo group over time. Changes of titers in anti-Dsg1 IgG autoantibodies (**A**) in patients with pemphigus vulgaris (PV) and pemphigus foliaceus and in anti-Dsg3 IgG autoantibodies (**B**) in patients with PV were shown (mean ± SD). Significant difference from day 1 at hazard ratio of *0.05 and **0.01.

network, provision of anti-idiotypic antibodies, modulation of dendritic cell, T- and B-cell activation, differentiation, and their effector functions.[22,23] Thus, HD-IVIG has multiple modes of action and is thought to act synergistically. HD-IVIG exerts immunomodulatory effects in autoimmune and inflammatory disorders without suppressing the immune system, which provides a distinctive advantage over conventional treatment.

Most of the previous studies suggesting efficacy of HD-IVIG for treatment of pemphigus involved multiple treatment cycles. However, our study demonstrated that a single cycle with HD-IVIG for 5 days has a therapeutic benefit to suppress the disease activity of pemphigus. Like rituximab, for which efficacy was recently reported in a single cycle,[24] IVIG is expensive and should be considered for patients who show difficulty with or resistance to conventional treatments.

In conclusion, our study suggests that TEP is a useful indicator for evaluation for rare intractable diseases such as pemphigus, and that a single cycle of HD-IVIG appears to be an effective treatment for

patients with pemphigus who are relatively resistant to systemic steroids.

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APPENDIX

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