impact of the TLR endogenous ligands underlying IL-6 induction in pathological tissue fibrosis is awaited.

Previous studies have shown the role of Th2 and Th17 responses in fibrotic diseases including SSc (42). SSc has a distinct Th2 polarization, especially in early stage of dcSSc. Th2 cells produce key soluble pro-fibrotic mediators including IL-4, IL-6, and IL-13. Knockdown of T-bet, master regulator of Th1, results in severer skin sclerosis with BLM treatment, and it is almost completely alleviated by IL-13 blockade. Thus, T-bet might serve as a repressor of dermal sclerosis through an IL-13-dependent pathway (8). These findings are consistent with our data that T-bet upregulation was significantly suppressed in more fibrotic BLM-treated WT mice compared with their less fibrotic TLR4$^{-/-}$ counterparts. In contrast, IL-13, which was upregulated with BLM treatment, was significantly reduced by TLR4 knockout. IL-13 is another important Th2 cytokine, and activates tissue fibrosis (43). Furthermore, IL-17A and Th17 cells are the fundamental mediators of autoimmune diseases (44). IL-17A promotes autoimmunity by triggering a positive-feedback loop via IL-6 production (45). BLM-induced lung fibrosis is IL-17A dependent (46), and IL-17A abrogation leads to decreased dermal fibrosis in the SSc murine models (13). Our experiments showed decreased Th2/Th17 response towards BLM treatment in TLR4$^{-/-}$ mice, which is compatible with these reports. Since IL-6 mediates the Th17 phenotype (45), it is plausible that decreased IL-6 production in the tissue resulted in less ROR-γt and IL-17A expression in CD4^{+} T cells. Taken together, TLR4 activation might be critically involved in the differential polarization in Th1/Th2/Th17 balance towards Th2/Th17 and play important roles in the induction of fibrosis.

Finally, our results revealed significantly decreased hypodermal fibrosis in

TLR4$^{-/-}$;TSK/+ mice compared with TSK/+ mice. TSK/+ mice model is a genetic SSc murine model, which was originally identified with a spontaneous mutation in *Fbn1* gene coding fibrillin-1 that results in increased synthesis and excessive accumulation of collagen in the skin and visceral organs (30). These mice exhibit elevated TGF-β1 production and their primary fibroblasts show enhanced collagen production with hypersensitivity to TGF-β1 stimulation (47, 48). This model is thus primarily characterized by endogenous activation of fibroblasts, while on the other hand, it is known to present with a skewed humoral response and produce anti-DNA topoisomerase I antibody (49). CD19 loss significantly attenuates fibrosis in TSK/+ mice, suggesting the important role of B cells in its pathogenesis (49). Furthermore, IL-4 gene disruption rescues TSK/+ mice from skin fibrosis while not from lung emphysema (50), and IL-17A deficiency in TSK/+ mice attenuates skin thickness (13). Considering that our results with BLM-induced model indicate the pivotal role of TLR4 in B cell activation and in Th2/Th17 skew which is characterized by IL-4 and IL-17A secretion by T cells, respectively, it is possible that TLR4 deficiency alleviated the fibrosis in TSK/+ mice at least partly through attenuation of these immunologically pathogenic mechanisms. At the same time, given that TLR4 signaling augments TGF-β responses and is crucial for fibroblast activation (22), it is also probable that disruption of this signaling attenuated the fibrosis in TSK/+ mice directly through decreased collagen production by fibroblasts with defective TGF-β responsiveness. Further studies are needed to fully elucidate the mechanisms by which TLR4$^{-/-}$;TSK/+ mice exhibit reduced fibrosis.

There might be a complex interplay of mechanisms leading to the fibrosis in SSc, but it became clear that TLR4 activation is one of the pivotal mechanisms of

fibrosis in its murine models. Further investigation is needed to clarify the pathogenesis underlying SSc and the contribution of TLRs.

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FIGURE LEGENDS

Figure 1. TLR4 and HA expressions are enhanced by BLM treatment.

A, Lesional skin and lung of WT mice treated with PBS or BLM for 3 weeks were stained for TLR4. Representative images are shown (scale bar = 100 μm). Insets depict TLR4 positive fibroblasts (arrows), infiltrating cells (arrowheads), and endothelial cells (dotted arrow) in the skin (scale bar = 10 μm). **B,** Data were quantified by counting

positive cells under magnification of ×200 high power field (HPF) (n = 4-5 mice per group; *P < 0.05). **C**, Representative immunofluorescence images for α-SMA/TLR4, CD31/TLR4, and HA/α-SMA double staining in skin samples from each group (scale bar = 50 μm). Arrows indicate double positive cells for respective staining. **D,** Mononuclear cells from inguinal and axillary lymph nodes from WT mice treated with PBS or BLM were gated and costained for B220, CD3, F4/80, CD11c, and TLR4. TLR4 fluorescence intensities in each positive cell are shown in the X-axis. Data shown are representative of experiments repeated three times.

Figure 2. Fibrosis, inflammatory cell infiltration, angiogenesis and inflammatory cytokine expression in the lesional skin induced by BLM treatment are attenuated by TLR4 deletion.

A, Representative skin sections with H&E and Masson's trichrome staining are shown (scale bar = 100 μm). Dermal thickness and collagen content of each group are summarized. Relative ratio of collagen content of each group is shown with PBS-treated WT mice set at 1. In addition, sections were stained for α-SMA and positive cells were counted (n = 4 - 8 mice per group; *P < 0.05, **P < 0.01). **B,** Skin sections from mice treated with PBS/BLM for 1 week were stained for B220, CD3, F4/80, toluidine blue, and CD31, and positive cells were counted (n = 4 mice per each group; *P < 0.05). **C,** mRNA levels in the lesional skin were assessed by qRT-PCR (n = 4 - 8 mice per group; *P < 0.05, **P < 0.01). **D,** The lysates of homogenized lesional skin were subject to ELISA to assess cytokine expression (n = 4-8 mice per group; *P < 0.05). One additional independent experiment using another group of mice provided similar results.

Figure 3. Inflammatory and fibrotic changes in lungs induced by BLM are also alleviated by TLR4 knockout.

A, Representative lung sections of WT and TLR4$^{-/-}$ mice injected with PBS or BLM are shown. H&E and Masson's trichrome staining (scale bar = 100 μm). **B,** Lung fibrosis score and collagen content of each group is summarized (n = 4-8 mice per each group; $*P < 0.05$, $**P < 0.01$). Relative ratio of collagen content of each group is shown with PBS-treated WT mice set at 1. **C,** mRNA levels in the right lung of each mice were assessed by qRT-PCR (n = 4-8 mice per group; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$). One additional independent experiment using another group of mice provided similar results.

Figure 4. Serum total IgG and anti-DNA topoisomerase I antibody levels are decreased with attenuated expression of IL-6 by TLR4 deletion.

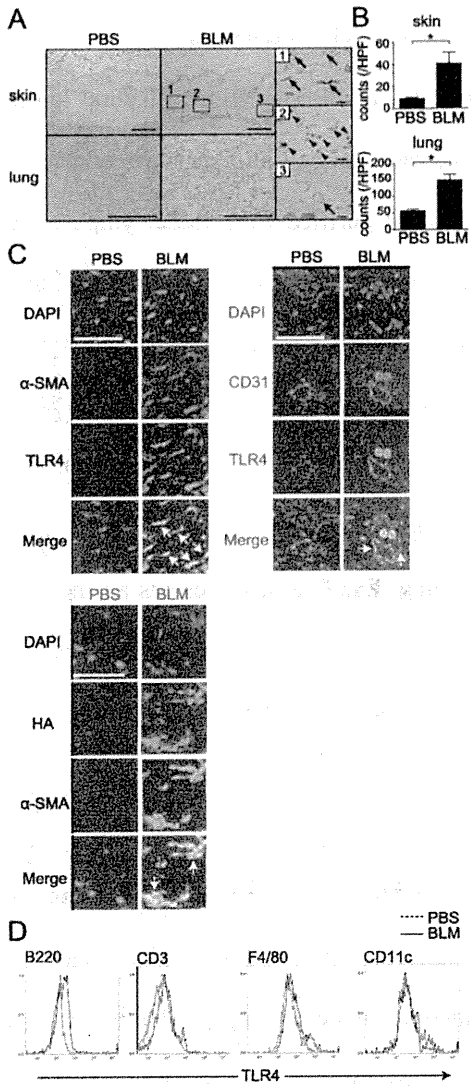
A, Sera from mice treated with PBS or BLM were collected and subject to the measurement of total IgG, IgM, anti-DNA topoisomerase I antibody, and IL-6 by ELISA (n = 4-6 per each group; $*P < 0.05$, $**P < 0.01$). **B,** Lesional skin sections were stained with anti-IL-6 antibody. Representative sections are shown (scale bar = 50 μm). IL-6 positive fibroblasts (arrows), infiltrating cells (arrowheads), and endothelial cells (dotted arrow) are indicated. **C,** Fibroblasts, endothelial cells, macrophages, B cells, and T cells were isolated, and stimulated with LPS or left untreated. Cell culture supernatant was collected, stored, and used for IL-6 measurement by ELISA (n = 4-6 per each group; $*P < 0.05$). One additional independent experiment using another group of mice provided similar results.

Figure 5. BLM treatment induces B cell activation and skew towards Th2/Th17 milieu, which are alleviated by TLR4 deletion.

A, After 1-week treatment with PBS or BLM, bilateral axillary and inguinal lymph nodes were harvested and the total cell number was counted (n = 4-8 per each group; $^{**}P < 0.01$). **B,** B cells were purified from these lymph nodes and cultured without stimulation. Cell culture supernatant was collected and used for IL-4, IL-6 and TGF-β1 measurement by ELISA (n = 4-6 per each group; $^{*}P < 0.05$). **C,** Representative FACS plots of intracellular IL-4, IL-17A, and IFN-γ staining in $CD4^+$ T cells from these lymph nodes of BLM-treated WT and $TLR4^{-/-}$ mice are shown. **D,** Combined data from three independent experiments showing positivity of IL-4, IL-17A, and IFN-γ among $CD4^+$ T cells by intracellular staining. Each point represents an individual mouse (n = 5-8 per each group; $^{*}P < 0.05$). Transcription factor T-bet, ROR-γt, and Foxp3 expression by $CD4^+$ T cells is also shown. Each symbol represents an individual mouse (n = 5-10 per each group; $^{*}P < 0.05$).

Figure 6. TLR4 deletion in TSK/+ mice attenuates hypodermal fibrosis.

A, Representative skin sections of WT, $TLR4^{-/-}$, TSK/+, and $TLR4^{-/-}$;TSK/+ 8-week-old female mice with hypodermal tissue are shown (H&E staining; scale bar = 200 μm). Hypodermal thickness of each group is summarized (n = 4 mice per each group; $^{*}P < 0.05$). **B,** Representative lung sections of above mice are shown (H&E staining; scale bar = 100 μm). One additional independent experiment using another group of mice provided similar results.



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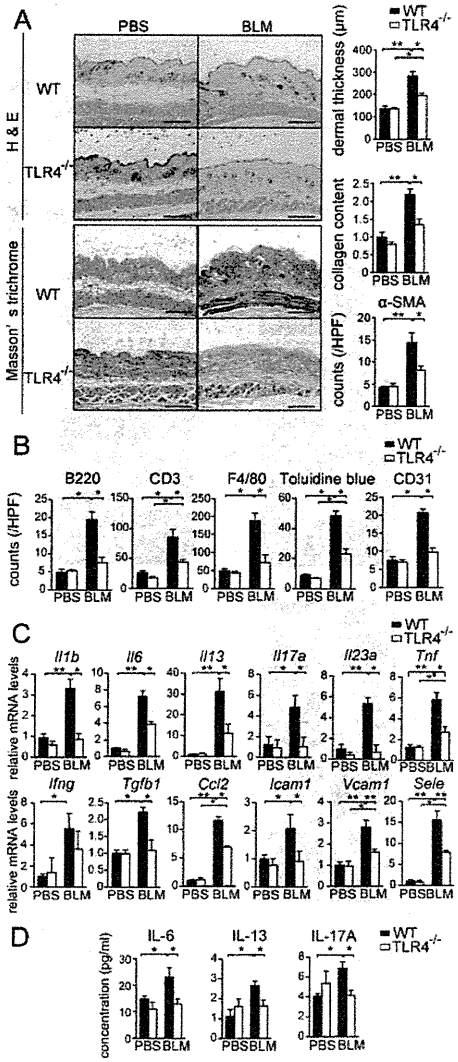

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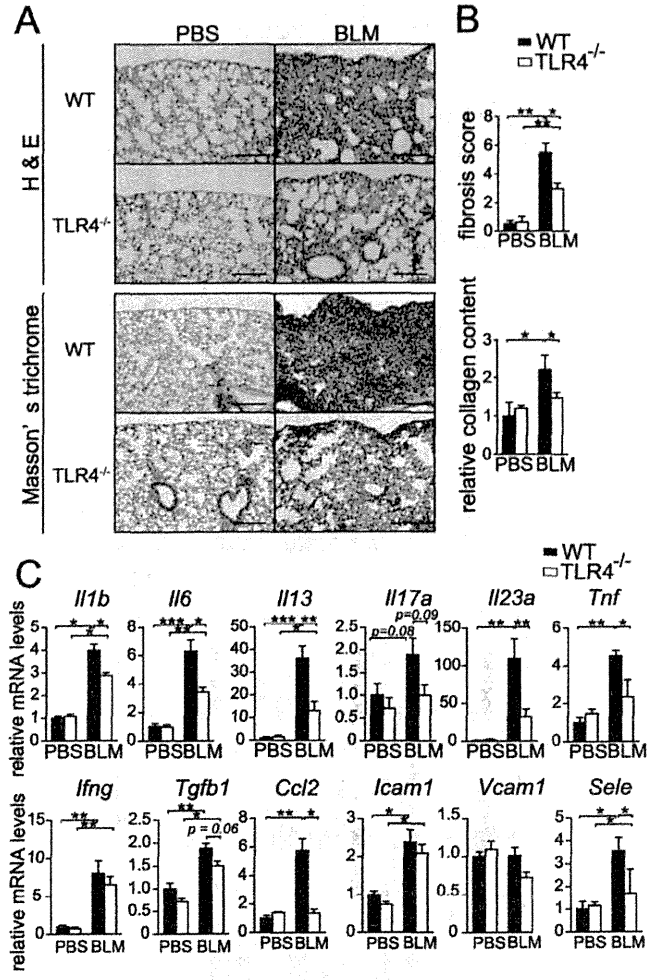

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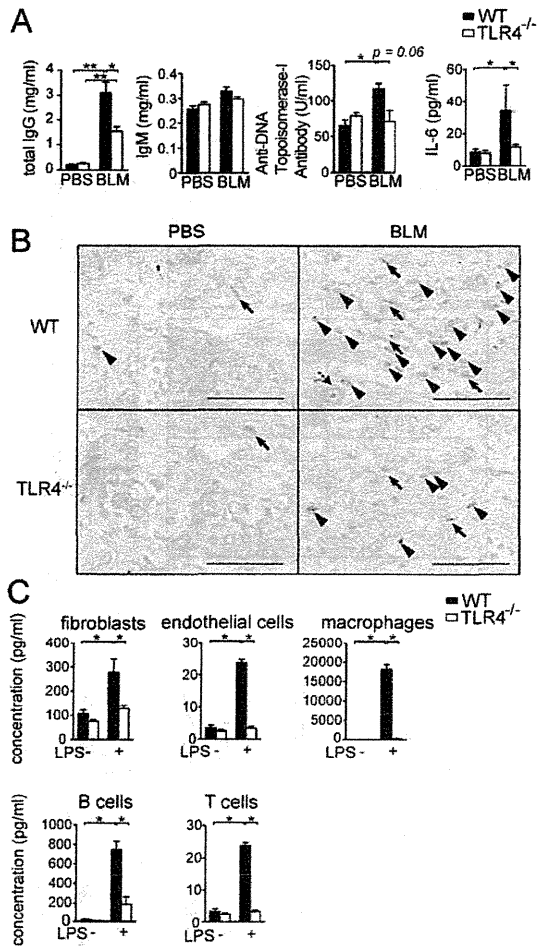

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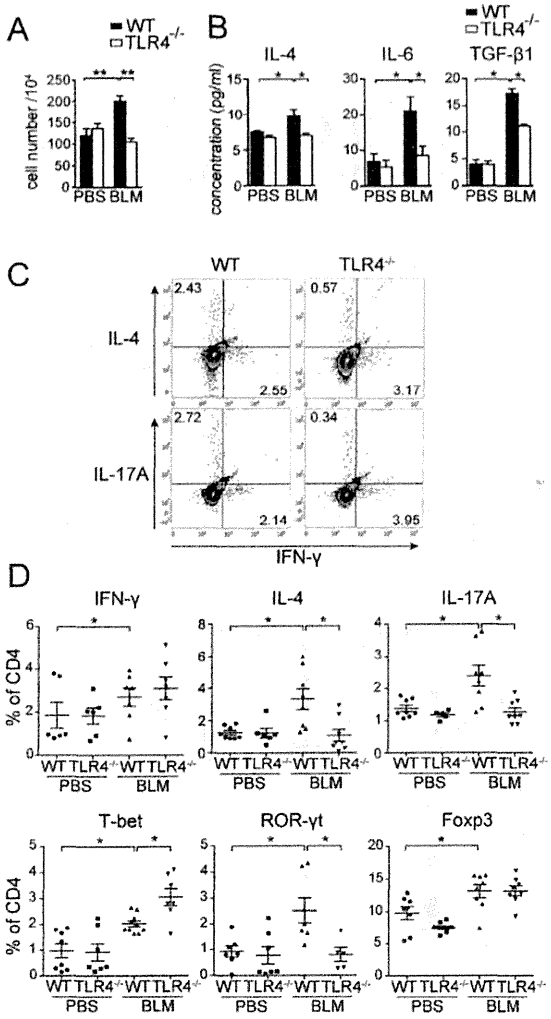

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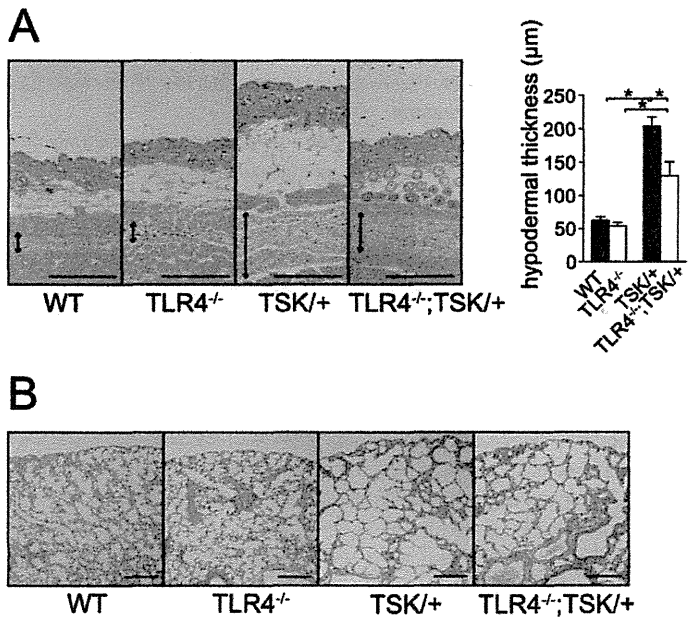

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SHORT REPORT

Histological features of localized scleroderma '*en coup de sabre*': a study of 16 cases

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Abstract

Background Early lesions of localized scleroderma are histologically characterized by perivascular lymphocytic infiltrate in the reticular dermis and swollen endothelial cells. However, there have been few information regarding histological features other than these findings in localized scleroderma.

Objective Since *en coup de sabre* (ECDS) is a certain subset of localized scleroderma with a relatively uniform clinical manifestation, we focused on this disease subset and evaluated its histopathological features.

Methods A total of 16 patients with ECDS were retrospectively evaluated on the basis of clinical and histological findings.

Results Regardless of clinical manifestations, vacuolar degeneration was found in all of the ECDS patients. Importantly, keratinocyte necroses were restricted to early and active ECDS lesions. In early ECDS patients (disease duration of <3 years), moderate to severe perivascular and/or periappendageal lymphocytic infiltrate and vacuolar changes in follicular epithelium were more prominent, whereas epidermal atrophy was less frequently observed, than in late ECDS patients (disease duration of ≥6 years).

Conclusion Vacuolar degeneration at the dermoepidermal junction is a common histological feature in ECDS and perivascular and/or periappendageal lymphocytic infiltrate and vacuolar degeneration of follicular epithelium are characteristic especially in early ECDS, further supporting a canonical idea that the elimination of mutated epidermal cells by immune surveillance contributes to tissue damage and resultant fibrosis in localized scleroderma.

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Conflicts of interest

None declared.

Funding sources

None declared.

Introduction

Scleroderma is a chronic disease of unknown aetiology characterized by skin fibrosis and is divided into two clinical entities: localized scleroderma and systemic sclerosis.[1] Localized scleroderma differs from systemic sclerosis in that it is not accompanied by Raynaud's phenomenon, acrosclerosis and internal organ involvement and the life prognosis of patients with localized scleroderma is good.[1] Early lesions of localized scleroderma are histologically characterized by perivascular lymphocytic infiltrate in the reticular dermis and swollen endothelial cells.[1–3] However, there have been few information regarding histological features of localized scleroderma other than these findings. Since *en coup de sabre* (ECDS) is a certain subset of localized scleroderma with a relatively uniform clinical manifestation,[4] we retrospectively investigated the histological characteristics and their clinical association in ECDS. We also discussed the significance of our findings to speculate the pathogenesis of localized scleroderma.

Materials and methods

Patients

Sixteen patients (three men and 13 women) with lesions clinically and histologically diagnosed as ECDS, who presented to the dermatology department of Tokyo University hospital between 2001 and 2011, were retrospectively included in this study. None was complicated with Parry–Romberg syndrome nor treated with immunomodulating agents, including systemic corticosteroids and immunosuppressants, before presentation. For each patient, age, disease duration and clinicopathologic data were obtained. Skin biopsies were evaluated for epidermal atrophy, spongiosis, vacuolar degeneration of basal cell layer, satellite cell

necrosis, basal pigmentation, melanin incontinence, perivascular infiltrate, perineural infiltrate, periappendageal infiltrate, vacuolar changes of follicular epithelium and dermal fibrosis. Each histological feature was evaluated semiquantitatively and classified into four grades: –, absent; +, mild; ++, moderate and +++, severe.

Statistical analysis

Statistical analysis was carried out with Fisher's exact probability test for the analysis of frequency. Statistical significance was defined as a *P*-value of <0.05.

Results

Clinical manifestations

Patients' information and the clinical features are summarized in Table 1. At presentation, the age of the patients ranged from 9 to 59 years (median, 22 years). The age when the first clinical manifestations appeared ranged from 8 to 59 years (median, 21 years). The disease duration ranged from 2 months to 8 years (median, 1.25 years). The clinical features at presentation were erythema (Case 12 and 14), hypopigmentation (Case 9), erythematous plaque with atrophy, depression and/or hair loss (Case 1, 2, 3 and 7), brownish and/or greyish plaque with atrophy, sclerosis, depression and/or hair loss (Case 4, 6, 8, 11, 13 and 15), and hypopigmented plaque with atrophy, sclerosis, depression and/or hair loss (Case 5, 10, and 16). All patients but one showed disease progression at presentation. Regarding central nervous system involvement, headaches (Case 4 and 5), intracranial calcifications (Case 6 and 15) and abnormal electroencephalogram (Case 6, 8, and 15) were seen.

Histological features of ECDS lesions

Patients' histological data are summarized in Table 2. The most prominent change in epidermis was interface dermatitis. Epidermal lymphocytic infiltrate accompanied with spongiosis, tagging of lymphocytes along the dermoepidermal junction and vacuolar changes were found in all specimens, regardless of clinical presentation and disease duration (Fig. 1a–d). Furthermore, melanin incontinence was seen in 11 (69%) patients (Fig. 1a,b). Moreover, keratinocyte necrosis, which is frequently accompanied with moderate interface dermatitis (Fig. 1a), was seen in two patients with early and active lesions, which are characterized by a hypopigmented plaque without sclerotic change and rapidly enlarging erythema respectively. These results suggest that interface dermatitis is a common histological feature of ECDS. Importantly, vacuolar changes accompanied with spongiosis in hair follicular epithelium were also seen in 10 (62.5%) patients (Fig. 1e,f), but there was no correlation in the degree of these changes between epidermis and hair follicular epithelium.

Regarding the histological features in the dermis, dermal fibrosis was found in all patients, but the degree of fibrosis did not correlate with disease duration. Perivascular and/or periappendageal lymphocytic infiltrate with scattered plasma cells was observed in all patients. As reported before,[5] perineural lymphoplasmacytic infiltrate was also observed in 11 (69%) patients (Fig. 1g,h).

Histological differences between early and late ECDS lesions

As described above, moderate interface dermatitis may be useful as a histological marker of early and active ECDS lesions. We further evaluate if any histological features other than moderate interface dermatitis may characterize early ECDS lesions. Since sclerotic plaques spontaneously regress up to 3.8 years after disease onset in 50% of ECDS patients,[6] we divided the ECDS patients into two subgroups according to their disease duration, such as ECDS patients with disease duration of <3 years (early ECDS; 10 patients) and ECDS patients with disease duration of ≥6 years (late ECDS; six patients), and compared their histopathological features. Epidermal atrophy was more frequently seen in late ECDS patients than in early ECDS patients (83% vs. 20%, $P < 0.05$). On the other hand, 'perivascular and/or periappendageal infiltrate', 'vacuolar changes of follicular epithelium' and 'perineural infiltrate' were more prominent in early ECDS patients. These histological features ranging from moderate to severe were completely restricted to early ECDS lesions and, especially in the former two histological features, the difference in their prevalence between early and late ECDS lesions reached a statistical significance [100% vs. 0% ($P < 0.01$) for perivascular and/or periappendageal infiltrate and 80% vs. 0% ($P < 0.01$) for vacuolar changes of follicular epithelium]. Importantly, the intensity of vacuolar changes in epidermis was comparable between early and late ECDS patients. Regarding perineural infiltrate, neither its presence nor intensity predicts the coexistence of central nervous system involvement.

Discussion

This study was undertaken to clarify the histopathological features of ECDS lesions. Contrary to the previous report showing no evidence of interface dermatitis in patients with localized scleroderma,[2] all ECDS patients demonstrated epidermal lymphocytic infiltrate, tagging of lymphocytes along the dermoepidermal junction and vacuolar changes, regardless of disease duration, clinical presentation and the intensity of perivascular lymphocytic infiltrate. Notably, two ECDS patients in early and active stage revealed moderate vacuolar changes accompanied with keratinocyte necrosis in epidermis, suggesting that moderate interface dermatitis serves as a histological marker of early and active ECDS lesions. Furthermore, when we defined early and late ECDS as ECDS patients with disease duration of <3 years and of ≥6 years, respectively, the degrees of perivascular and/or periappendageal infiltrate and vacuolar changes of follicular epithelium were much greater, whereas epidermal