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LETTER TO THE EDITOR

Mixed tumor of the skin arising on the auricle

Dear Editor,

A-63-year-old Japanese woman was referred to us for evaluation of a 6-year history of a nodule on the left auricle. It gradually became larger with mild tenderness. The patient was otherwise in excellent health, and her family history was unremarkable. On examination, she had a slightly reddish, well-demarcated, dome-shaped tumor, 7.5 mm in diameter, on the crus of helix of the left auricle. The nodule was located in the subcutis without any change of the overlying skin. A biopsy specimen was taken from the margin of the tumor, as observed with a remaining thread and crust in Figure 1. It was tentatively diagnosed as a benign sweat apparatus neoplasm.

The tumor was excised and there was no recurrence after 2 years. Histological examination of the excised specimen revealed that the tumor was surgically removed with free margin (Fig. 2a). The overlying epidermis was unremarkable. There was a well-circumscribed unencapsulated neoplasm, arranged in nests within the dermis, and not connected with the epidermis. The deeper part of the tumor extended to the cartilage, but did not invade it. The individual tumor islands were composed of tubular lumina, which showed marked variation in the size and shape, and some of them were branching and cystically dilated (Fig. 2b,c). The tubular lumina were lined by two layers of epithelial cells and were embedded in a stroma. Decapitation secretion of the inner epithelial cells into the lumen was unremarkable. Mucin was deposited in some areas, as assessed by Alcian blue staining (Fig. 2d). In addition to the luminal formation, aggregates of epithelial cells without lumina were also observed. Occasional mitotic figures were observed, but atypical forms were not detected.

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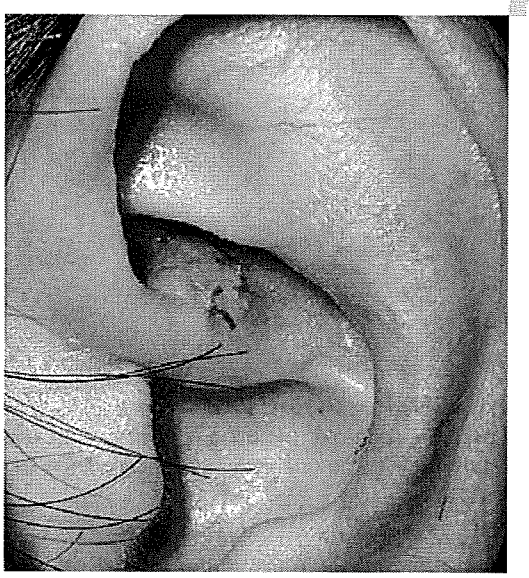

Figure 1. Tumor on the crus of helix on the left auricle. Note that a thread remained after biopsy.

An immunohistochemical study revealed that the tumor cells were positive for S-100 protein (Fig. 2e), epithelial membrane antigen (EMA), carcinoembryonic antigen (CEA) (Fig. 2f) and 34βE12. The cells lining the ductal lumen and aggregating without lumina demonstrated nuclear staining of S-100 protein. The cytoplasm of cells lining the lumen was positive for EMA. The lumen was outlined with CEA-positive material. Neoplastic cells positive for 34βE12 (cytokeratin 1/5/10/14) were focally detected.

This tumor consisted of epithelial cells forming variously-sized ductal lumina and abundant myoepithelial cells positive for S-100 protein[1] as usually seen in mixed tumor of the skin or pleomorphic adenoma. Adenomas arising on the auricle have been rarely reported, and there has been a case documented as pleomorphic adenoma of the auricle.[2] Because our tumor exhibited the proliferation of variously-sized glands with myxoid change of the stroma, we diagnosed the tumor as a mixed tumor of the skin, which differentiates presumably towards sweat glands. On the other hand, there have been reported a considerable number of patients with adenoma or mixed tumor occurring in the external auditory canal.[3,4] These tumors exhibit a dual cell population of basal

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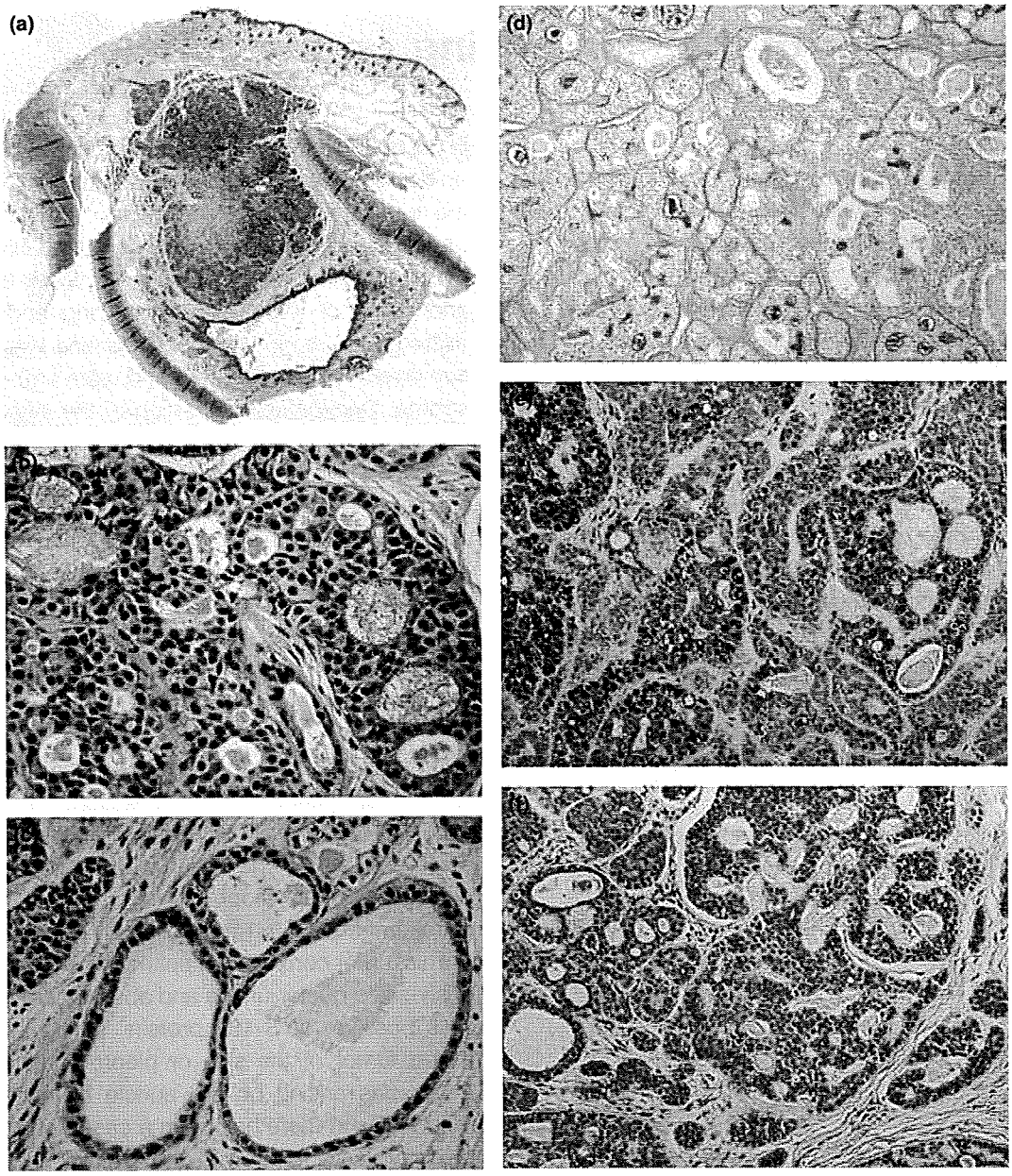

Figure 2. Photomicrograph of excision specimens. (a) Low magnification of whole tumor (hematoxylin–eosin). (b) High magnification, showing tumor nests forming ductal structures (hematoxylin–eosin). (c) High magnification, showing small and large tubular lumina in the tumor island (hematoxylin–eosin). (d) Alcian blue staining. (e,f) Immunohistochemical stainings for S-100 protein (e) and carcinoembryonic antigen (f).

myoepithelial-type cells and luminal cells.[5] They may originate from the ceruminous glands, which are modified sweat glands, confined to the skin lining of the cartilaginous part of the external auditory meatus. S-100 protein as well as cytokeratin highlights the tumor cells,[5] as seen in our tumor. Given that the crus of auricular helix is an extension of the external meatus, we cannot negate the possibility that our tumor is a unique ceruminous adenoma of the external auditory canal,[4,5] which shares features with mixed tumor of the skin.[5,6] Nevertheless, for our neoplasm, we prefer the diagnosis of mixed tumor of the skin occurring on the unique location. It is possible that mixed tumor of the skin, when arising on the

auricle, exhibits various extents of monomorphic histological property presumably because of the local environmental conditions of the auricle for tumor development.

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Original Article

Induction of eosinophil- and Th2-attracting epidermal chemokines and cutaneous late-phase reaction in tape-stripped skin

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Abstract: Skin barrier damage induces various harmful or even protective reactions in the skin, as represented by enhancement of keratinocyte cytokine production. To investigate whether acute removal of stratum corneum modulates the production of chemokines by epidermal cells, we treated ears of BALB/c and C57BL/6 mice by tape-stripping, or acetone-rubbing as a control of acute barrier disruption procedure. There was no difference between the tape-stripped and acetone-rubbed skin sites in the increased and recovered levels of transepidermal water loss. The mRNA expression levels of all the chemokines tested, including Th1 chemokines (CXCL10, CXCL9 and CXCL11), Th2 chemokines (CCL17 and CCL22) and eosinophil chemoattractant (CCL5), were higher in the epidermal cells from BALB/c than in those of C57BL/6 mice. In particular, CCL17, CCL22 and CCL5 were remarkably elevated in BALB/c mice and augmented by tape-stripping more markedly than acetone-rubbing, whereas Th1 chemokines were enhanced by acetone-rubbing more remarkably. Tape-stripping induced dermal infiltration of eosinophils in BALB/c but not C57BL/6 mice. In a contact hypersensitivity model, where BALB/c mice were sensitized on the abdomen and challenged on the ears with fluorescein isothiocyanate, mice exhibited higher ear swelling responses at the late-phase as well as delayed-type reactions, when challenged *via* the tape-stripped skin. The challenge *via* tape-stripped skin augmented the expression of IL-4 and CCR4 in the skin homogenated samples, indicating infiltration of Th2 cells. These findings suggest that acute barrier removal induces the expression of Th2 and eosinophil chemokines by epidermal cells and easily evokes the late phase reaction upon challenge with antigen.

Key words: barrier disruption – chemokines – eosinophil – late-phase reaction – tape stripping

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Introduction

Stratum corneum, which is the outermost, cornified layer of the epidermis, serves as skin barrier and protects from external micro-organisms and chemicals and even sunlight radiation (1). When this barrier is destroyed or removed, these hazardous invaders penetrate through the skin. Upon exposure to the agents, however, the epidermis produces or expresses various protective molecules such as cytokines (2), anti-bacterial peptides (3) and cornification-promoting molecules (4).

There are both the acute and chronic disruption procedures for experimental impairment of the skin barrier in rodents (5,6). Furthermore, the acute barrier disruption has been performed by two different procedures in mice, stripping with scotch tape and rubbing with acetone cotton (2). These two treatment modalities stimulate keratinocytes to produce cytokines, such as interleukin-1α, tumor necrosis factor-α and granulocyte/macrophage colony stimulating factor and enhance contact hypersensitivity when a hapten is applied on the barrier-disrupted skin at either of the sensitization or challenge phase (2). However, the pathophysiological significance of barrier disruption in skin infiltration of inflammatory cells remains to be clarified. Given that barrier damage induces the release of chemokines from the epidermis, it is possible that certain inflammatory cells are present or prone to infiltrate in the barrier-disrupted skin.

Although both tape-stripping and acetone-rubbing have been categorized as the procedure of acute barrier disruption, it remains unknown whether these two treatments exert the same effect on the production of chemokines by epidermal cells and the resultant infiltration of leucocytes in the skin. While tape-stripping mechanically removes the cornified layer of the epidermis, acetone-rubbing chemically deletes sphingolipids, such as ceramide, existing

between the layers of corneocytes (2). Therefore, it seems that tape-stripping resembles scratching and more likely reflects clinical conditions. For example, the tape-stripped skin may share the chemokine production status with the skin of pruritic disorders such as atopic dermatitis.

This study was aimed to investigate whether tape-stripping modulates the production of chemokines by epidermal cells in a comparison with acetone-rubbing. We treated the ears of mice by tape-stripping or acetone-rubbing and examined the expression/production of chemokines by epidermal cells (ECs), the infiltration of inflammatory cells and the late-phase and delayed-type hypersensitivities. In addition, it was necessary that Th2- and Th1-preponderant mouse strains, BALB/c and C57BL/6 (B6) mice respectively, were compared in the effects of tape-stripping. Results suggest that tape-stripping induces the production of Th2 cell- and eosinophil-associated chemokines more markedly than acetone-rubbing in BALB/c mice and this skewed elaboration of chemokines determines the infiltration of Th2 cells and eosinophils and the cutaneous hypersensitivity responses.

Materials and methods

Animals and chemicals

Seven to 10 week-old female BALB/c and B6 mice were purchased from Japan SLC (Hamamatsu, Japan). These mice were maintained in the Laboratory Animal Research Center in University of Occupational and Environmental Health under specific pathogen-free conditions. All animal experiments were performed according to the guidelines for the care and use of animals approved by our university. Fluorescein isothiocyanate (FITC) was obtained from Sigma Chemical Co. (St Louis, MO).

Acute barrier disruption procedures

The procedures were reported previously (2). Mechanical barrier disruption was achieved by stripping both sides of the earlobe with cellophane tape (Nichiban, Tokyo, Japan) seven times. This manipulation effectively removed stratum corneum without hazardous haemorrhagic change. For chemical disruption, both sides of the earlobe were gently rubbed for 30 s with cotton ball dipped in absolute acetone. These two disruption procedures were performed in different mice.

Transepidermal water loss (TEWL)

Immediately (time 0) and at various times after barrier disruption with tape-stripping and acetone-rubbing, TEWL was measured in the treated and untreated earlobes with Vapa Scan AS-VT100RS (Asahi Biomed, Yokohama, Japan) in the measurement room which had a temperature of 23–25°C and a relative humidity of 49–54%.

Preparation of epidermal cells (ECs)

Skin sheets from earlobes untreated or treated with tape or acetone 6, 12 and 24 h before were floated in 0.2% trypsin (Difico Laboratories, Detroit, MI, USA), dissolved in phosphate-buffered saline (PBS; pH 7.4) for 1 h at 37°C (7). Epidermis was then separated from dermis with forceps in PBS supplemented with 10% of fetal calf serum (Gibco, Carlsbad, CA, USA). EC suspensions were prepared by pipetting and filtration through nylon mesh (pore size, 77 mm) and included 97% keratinocytes and 2% Langerhans cells as assessed using flow cytometric analysis with anti-I-A antibody (8).

Real-time quantitative PCR analysis of ECs

For detection of epidermal chemokines, EC suspensions were used as samples. Total cellular RNA of ECs was extracted with the RNA extraction kit (Promega, Madison, WI, USA) from freshly prepared skin samples. There was no remarkable difference between the acetone-rubbed and tape-stripped samples in the amounts of extracted RNA. RNA was then reverse transcribed and amplified by random hexamer in single tube assay using the TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, USA) with gene-specific sense and antisense primers and a detection probe labelled on the 5′ end with the reporter dye 6-FAM. Primers and probes were obtained from TaqMan Gene Expression Assays Inventories (Applied Biosystems) for CXCL10/IP-10, CXCL9/Mig, CXCL11/I-TAC, CCL17/TARC, CCL22/MDC and CCL5/RANTES. Using the ABI Prism 7000 sequence detection systems (Applied Biosystems), duplicate samples were reverse transcribed and amplified under the following consecutive steps: 2 min at 50°C, 10 min at 95°C, followed by 50 amplification cycles of 15 s at 95°C and 1 min at 60°C. Sequence-specific amplification was detected as an increased fluorescent signal of 6-FAM exceeding the threshold limit during the amplification cycle. Quantification of gene-specific message levels was determined by comparing fluorescence intensity from unknown RNA samples to the fluorescence intensity of standard curve generated from control mRNA levels. Amplification of the gene for mouse β-actin was performed on all samples to control interspecimen variations in RNA amounts. The result for each gene was normalized to the quantity of mouse β-actin detected in the sample. Levels of gene-specific message were graphed as normalized message units as determined from standard curve.

Enzyme-linked immunosorbent assay (ELISA)

Chemokine levels were studied using ELISA method. Dispersed ECs from barrier-disrupted or untreated earlobes were cultured at 1×10^6 per ml in Eagle's minimal essential medium containing 10% fetal calf serum, 1% streptomycin

and 1% penicillin, without any additional stimulant, for 48 h at 37°C in humidified 5% CO_2 in air. When dispersed ECs are cultured for different culture periods, the amount of cytokines in the culture supernatants are markedly increased between 24 and 48 h and reached maximum at 72 h (9). As the 72-h culture possibly masks the influence of barrier disruption, we used the 48-h culture for the assessment. The Quantikine (R & D Systems, Minneapolis, MN) protocol for sandwich ELISA was used to quantify total amount of CCL17, CCL22 and CCL5 in the culture supernatants.

Histological assessment

Skin specimens were obtained from earlobes and fixed in 20% buffered formalin and embedded in paraffin. Multiple 3 mm sections were stained with haematoxylin and eosin (H&E) for eosinophil and lymphocyte counting. The numbers of eosinophils and lymphocytes in the dermis were enumerated in three high power fields of microscopy and expressed per one section (0.25 mm^2) at 400×. Each section was assessed in random order by two observers of us without the knowledge of patient identification.

Preparation of skin homogenized samples and real-time quantitative PCR analysis

To examine the cytokines and chemokine receptors of T cells, homogenized samples of whole ears were used. BALB/c mice were sensitized on the shaved abdomen with 200 μl of 1% FITC in acetone/dibutyl phthalate (1:1 ratio) three times a week for 2 weeks and earlobes were provoked by painting of 40 μl of 1% FITC 24 h after tape-stripping. At 3, 6 and 12 h after challenge, the earlobes were prepared and homogenized using a T 10 basic Ultra-Turrax (Ika-Werke, Staufen, Germany) with the Trizol reagent (Invitrogen Inc., Carlsbad, CA, USA). Total cellular RNA was extracted with the PureLink Micro-to-Midi Total RNA Purification System (Invitrogen Inc.) from Trizol samples. Primers and probes were obtained from TaqMan Gene Expression Assays Inventories (Applied Biosystems) interferon-γ (IFN-γ), interleukin-4 (IL-4), CXCR3, and CCR4. Quantitative PCR was performed as described above.

Contact hypersensitivity (CHS)

Mice were sensitized with FITC by painting of the shaved abdomen with 200 μl of 1% FITC. Five days after sensitization, the earlobes were barrier disrupted or untreated. After 24 h, mice were elicited by painting of both sides of earlobes with 40 μl of 0.5% FITC and the increase in ear thickness was measured immediately before and 1, 4, 8 and 24 h after painting using a dial thickness gauge (Ozaki Co, Tokyo, Japan). Ear swelling was calculated as (ear thickness after challenge) – (ear thickness before challenge).

Statistical analysis

Data were analysed using an unpaired two-tailed *t*-test. $P < 0.05$ was considered to be significant.

Results

Absence of differences in TEWL following treatment between tape-stripping and acetone-rubbing and between BALB/c and B6

In advance of testing the actions on the epidermal chemokine production and cell infiltration, we compared tape-stripping and acetone-rubbing in their effects on TEWL, a representative marker for the barrier function. We used two strains of mice, Th2-preponderant BALB/c and Th1-preponderant B6 mice. Earlobes of mice were stripped with cellophane tape or rubbed with acetone and TEWL was monitored after treatment. TEWL was elevated immediately after either of the treatments and declined thereafter at comparable levels (Fig. 1). Therefore, there was no difference between tape-stripped and acetone-rubbed skin sites in the increment and recovery of TEWL. Furthermore, BALB/c (Fig. 1a) and B6 mice (Fig. 1b) had virtually the same TEWL values following the treatments.

Higher expression of Th2 and eosinophil chemokine mRNAs by tape-stripping than acetone-rubbing in ECs of BALB/c mice

Earlobes of mice were stripped with cellophane tape or rubbed with acetone and EC suspensions were prepared from the ears at 6, 12 or 24 h after treatment and subjected to real-time PCR analysis. As shown in Fig. 2, the two barrier disruption procedures differentially induced the expression of mRNA for Th1 chemokines (CXCL10, CXCL9 and CXCL11), Th2 chemokines (CCL17 and CCL22) and eosinophil-chemoattracting chemokine (CCL5) (10,11), depending on the mouse strains and the timing after treatment. BALB/c mice exhibited higher expression levels of all the chemokines than did B6 mice. In BALB/c mice, the increased expression was discernible at 12 h and remarkable at 24 h after treatment. CCL17, CCL22 and CCL5 were more strongly induced by tape-stripping than acetone-rubbing, but inversely, CXCL10, CXCL9 and CXCL11 were expressed more remarkably by acetone-rubbing than tape-stripping. Thus, tape-stripping is capable of inducing the production of Th2 chemokines by ECs in BALB/c mice.

To confirm the expression of Th2 chemokines and CCL5 promoted by tape-stripping, EC suspensions were prepared from BALB/c mice at 6, 12 or 24 h after the treatment and cultured for 48 h. The chemokine concentration in the culture supernatants was measured using ELISA. ECs from the treated mice produced higher levels of CCL17, CCL22 and

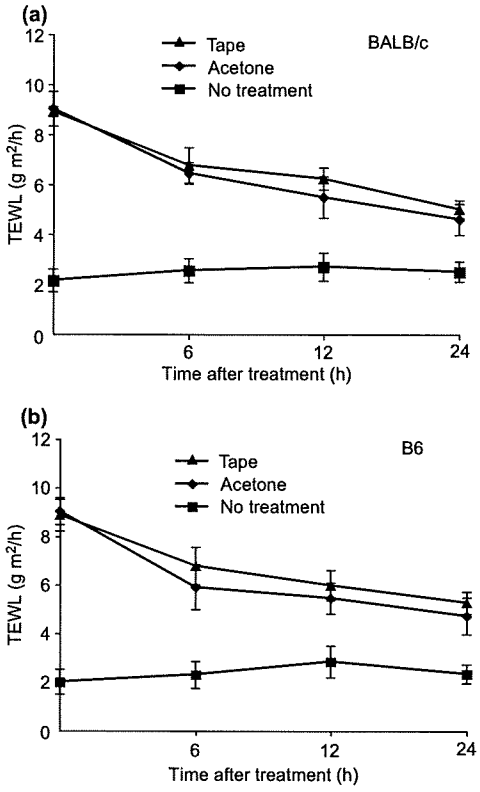

Figure 1. TEWL following tape-stripping or acetone-rubbing. Earlobes of BALB/c (a) and B6 mice (b) were stripped with tape or rubbed with acetone. Immediately after treatment (time 0), or 6, 12 or 24 h after treatment, TEWL was measured. Data are expressed as the mean ± SD of five mice.

CCL5 than those of untreated mice (Fig. 3). Again, tape-stripping induced the production of these chemokines, particularly CCL17, at significantly higher levels than acetone-rubbing.

Infiltration of eosinophils in tape-stripped ears of BALB/c mice

As tape-stripping stimulated ECs to produce chemokines, we monitored the infiltrate of inflammatory cells in the tape-stripped earlobes of BALB/c and B6 mice. Interestingly, we found that the tape-stripped ears of BALB/c mice showed infiltration of eosinophils in the dermis (Fig. 4a,b). Eosinophils appeared in the dermis at 8 h after treatment and increased in number at 24 h (Fig. 4c). Acetone-rubbing did not induce such an infiltrate of eosinophils. In B6 mice, the tape-stripping-provoked infiltrate of eosinophils was barely perceptible (Fig. 4d). Thus, the enhanced expression of CCL5 seems to be significant *in vivo*. In both strains, lymphocytes also infiltrated after either treatment with acetone or tape (Fig. 4e,f).

Augmented expression of IL-4 and CCR4 by challenge *via* tape-stripped skin in repeatedly sensitized mice

To examine the effect of tape-stripping on the induction of Th2 cells, we used the repeated sensitization method. As even the repeated sensitization with a hapten does not exclusively induce Th2 cells, Th1 cytokines are simultaneously increased by the challenge to some extent. BALB/c mice were sensitized with 1% FITC three times a week for 2 weeks on the abdomen and challenged with 1% FITC on the earlobes untreated or stripped with tape 24 h before. The ears were taken at 3, 6 or 12 h after challenge, homogenized and subjected to real-time PCR analysis for the expression of IFN-γ, IL-4, CXCR3 and CCR4. IFN-γ and IL-4 are representative Th1 and Th2 cytokines respectively, and CXCR3 and CCR4 are Th1 and Th2 chemokine receptors respectively (11). The expression of IFN-γ was increased at 3–12 h after challenge and the challenge *via* tape-stripped skin elevated its expression compared with the challenge *via* untreated skin (Fig. 5a). IL-4 expression was also enhanced by the challenge *via* tape-stripped skin at 3, 6 and 12 h (Fig. 5b). As to the chemokine receptors, while CXCR3 was not affected by challenge through the tape-stripped skin (Fig. 5c), CCR4 was augmented by the challenge (Fig. 5d). Thus, the expression of Th2 cytokine as well as IFN-γ and Th2 chemokine receptor was augmented in the tape-stripped and challenged skin, suggesting promoted accumulation of Th2 cells by tape-stripping.

Enhancement of both late-phase and delayed-type hypersensitivities in tape-stripped mice

It has been reported that the delayed-type contact hypersensitivity is enhanced through the barrier-disrupted skin (2). We further explored the *in vivo* significance of tape-stripping-augmented chemokine production in cutaneous hypersensitivities. BALB/c mice were sensitized with 1% FITC and challenged with 0.5% FITC on the tape-stripped or untreated earlobes. At 8 and 24 h after challenge, higher ear swelling responses were observed in mice challenged *via* tape-stripped ears than those challenged *via* untreated skin (Fig. 6). Acetone-rubbing, instead of tape-stripping, did not enhance the ear swelling responses at 8 h after challenge, while the treatment augmented the response 24 h after challenge (data not shown). Therefore, elicitation through the tape-stripped skin augmented not only the delayed-type but also the late-phase reactions of contact hypersensitivity where Th2 cells and eosinophils are involved (12).

Discussion

Our study showed that acute barrier disruption upregulates the production/expression of chemokines by ECs, depend-

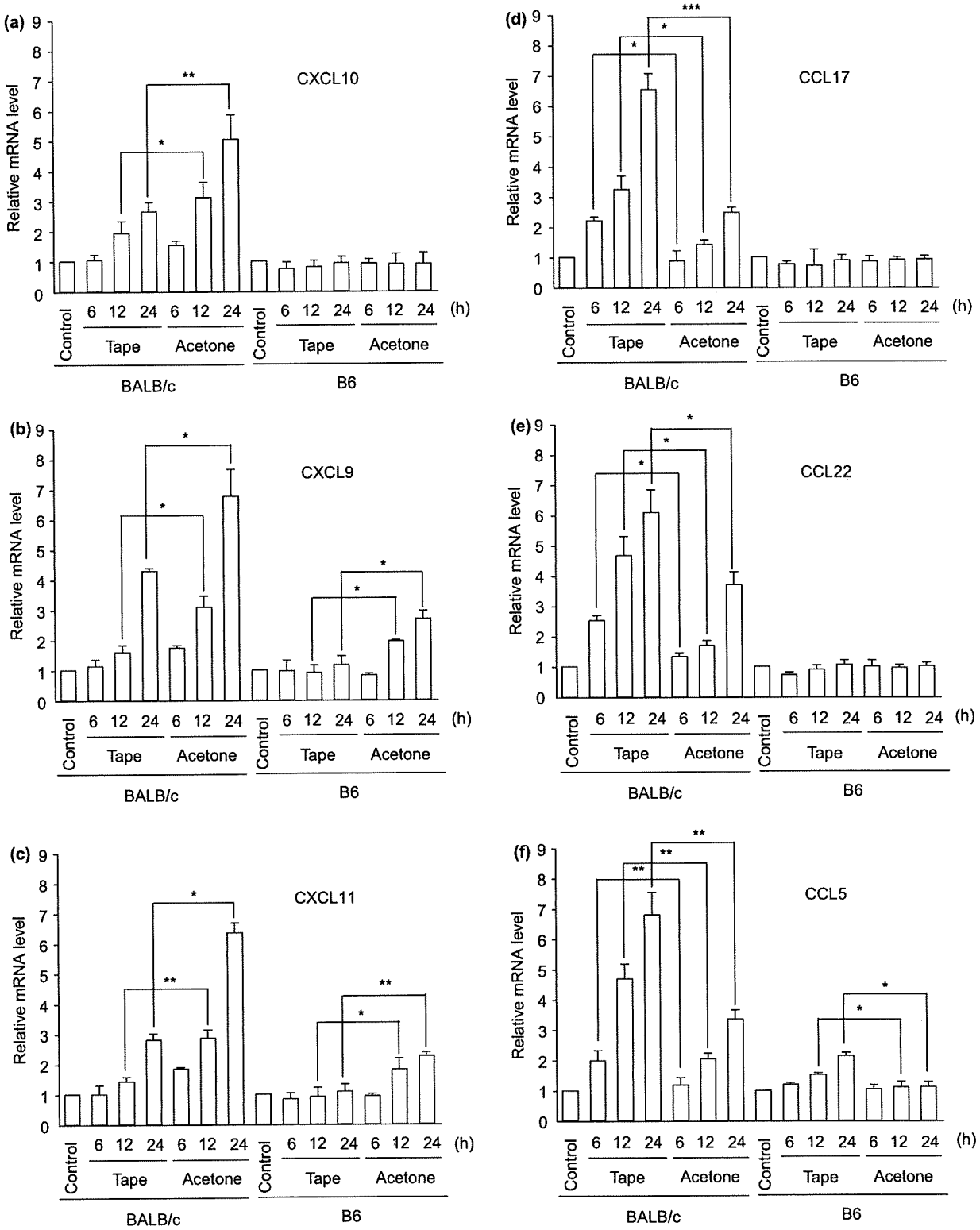

Figure 2. Real-time PCR analysis of mRNA expression for chemokines in ECs from barrier-disrupted earlobes. Earlobes of BALB/c and B6 mice were stripped with tape or rubbed with acetone. At 6, 12 and 24 h after treatment, EC suspensions were prepared and subjected to real-time PCR analysis for chemokines, including Th1 chemokines (CXCL10, CXCL9 and CXCL11), Th2 chemokine (CCL17 and CCL22) and CCL5. The expression of mRNA is represented as fold increase (2^{-DDC_t}), where $DDC_t = [DC_t \text{ (sample)}] - [DC_t \text{ (ECs without treatment)}]$ and $DC_t = [C_t \text{ (sample)}] - [C_t \text{ (b-actin)}]$. Data are expressed as the mean ± SD of five mice (*$P < 0.05$, **$P < 0.005$, ***$P < 0.0001$).

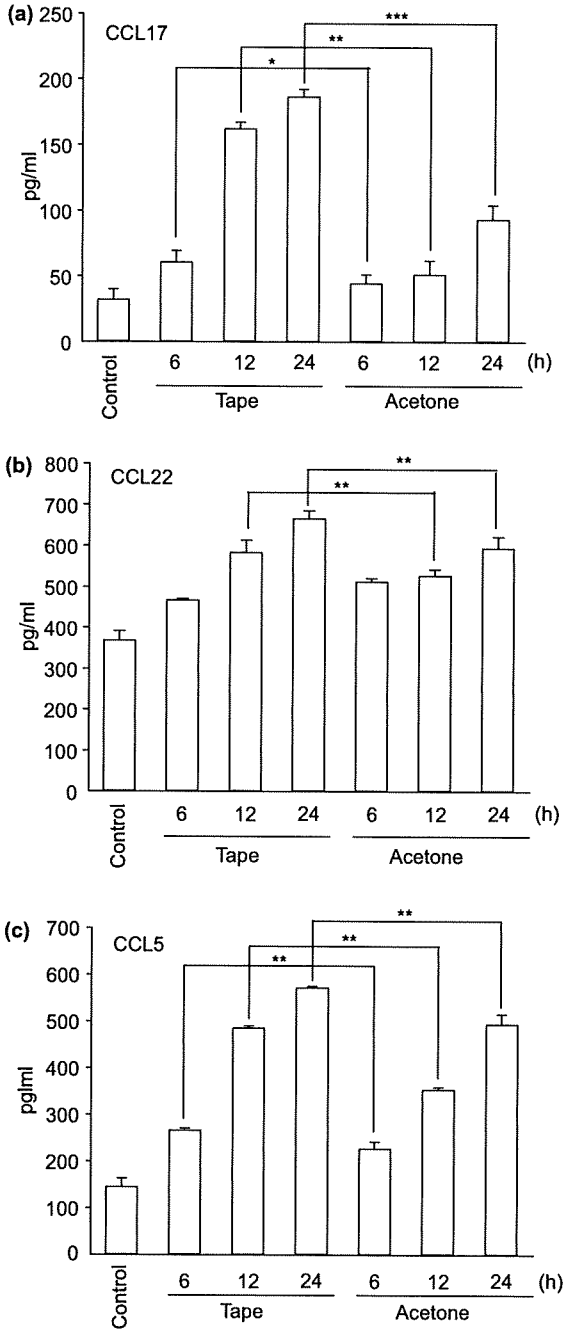

Figure 3. ELISA for chemokines in culture supernatants from ECs obtained from barrier-disrupted earlobes in BALB/c mice. Earlobes of BALB/c mice were stripped with tape or rubbed with acetone. At 6, 12 and 24 h after treatment, EC suspensions were prepared and cultured for 48 h. The concentration of CCL17, CCL22 and CCL5 in the culture supernatants was measured using ELISA. Data are expressed as the mean ± SD of five mice (*$P < 0.05$, **$P < 0.005$, ***$P < 0.0001$).

ing on the procedure of disruption, type of chemokines and strain of mice. Th2 chemokines CCL17 and CCL22 and eosinophil chemoatrractant CCL5 (10,11) were augmented by tape-stripping more markedly than acetone-rubbing, while Th1 chemokines CXCL10, CXCL9 and CXCL11 (10,11) were enhanced by acetone-rubbing. The increased production of CCL17, CCL22 and CCL5 was clearly observed in Th2-polarized BALB/c mice but not in Th1-dominant B6 mice. It should be stressed that, in accordance with this observation, tape-stripping allowed eosinophils to infiltrate in the dermis of BALB/c mice. In addition, FITC challenge *via* tape-stripped ears of sensitized BALB/c mice induced the expression of IL-4 and CCR4, indicating accumulation of Th2 cells in the tape-stripped and hapten-challenged skin. Accordingly, the tape-stripped mice showed increased responses at 8 h as well as 24 h when they were challenged *via* the treated ears. These findings suggest that tape-stripping stimulates ECs to express/produce Th2 chemokines and eosinophil chemoattractant and hapten application *via* the tape-stripped skin evokes the late phase reaction.

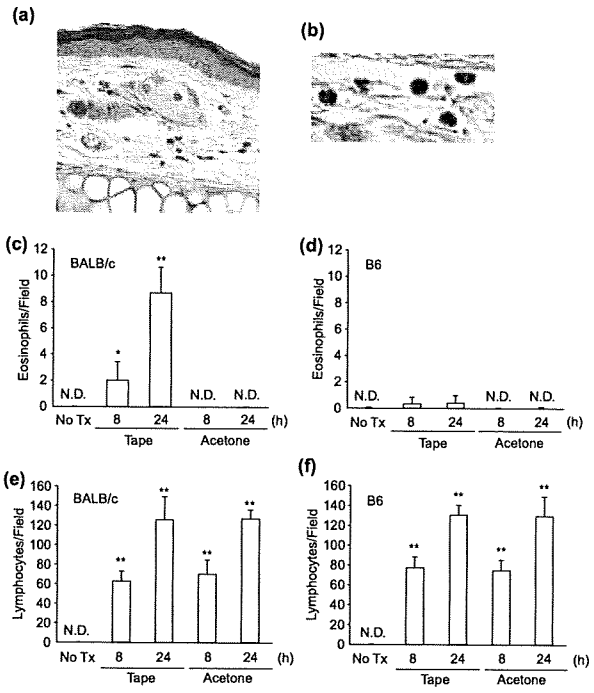

Figure 4. Histological pictures and numbers of infiltrating eosinophils and lymphocytes in tape-stripped earlobes of BALB/c mice. Earlobes of BALB/c and B6 mice were tape-stripped and acetone-rubbed and histological sections (H&E) were prepared 8 and 24 h later. Control sections were obtained from non-treated earlobes. (a, b) Histological picture of BALB/c mice at 24 h after tape-stripping (original magnification 200× and 400×). The numbers of eosinophils and lymphocytes were counted per one section (0.25 mm^2) at 400×. (c) Eosinophil counts in BALB/c mice treated with acetone-rubbing or tape-stripping. (d) Eosinophil counts in B6 mice treated with acetone-rubbing or tape-stripping. (e) Lymphocyte counts in BALB/c mice treated with acetone-rubbing or tape-stripping. (f) Lymphocyte counts in B6 mice treated with acetone-rubbing or tape-stripping. Data are expressed as the mean ± SD of five mice (*$P = 0.0061$, **$P < 1.0 \times 10^{-6}$). No Tx, no treatment; and N.D., not detected.

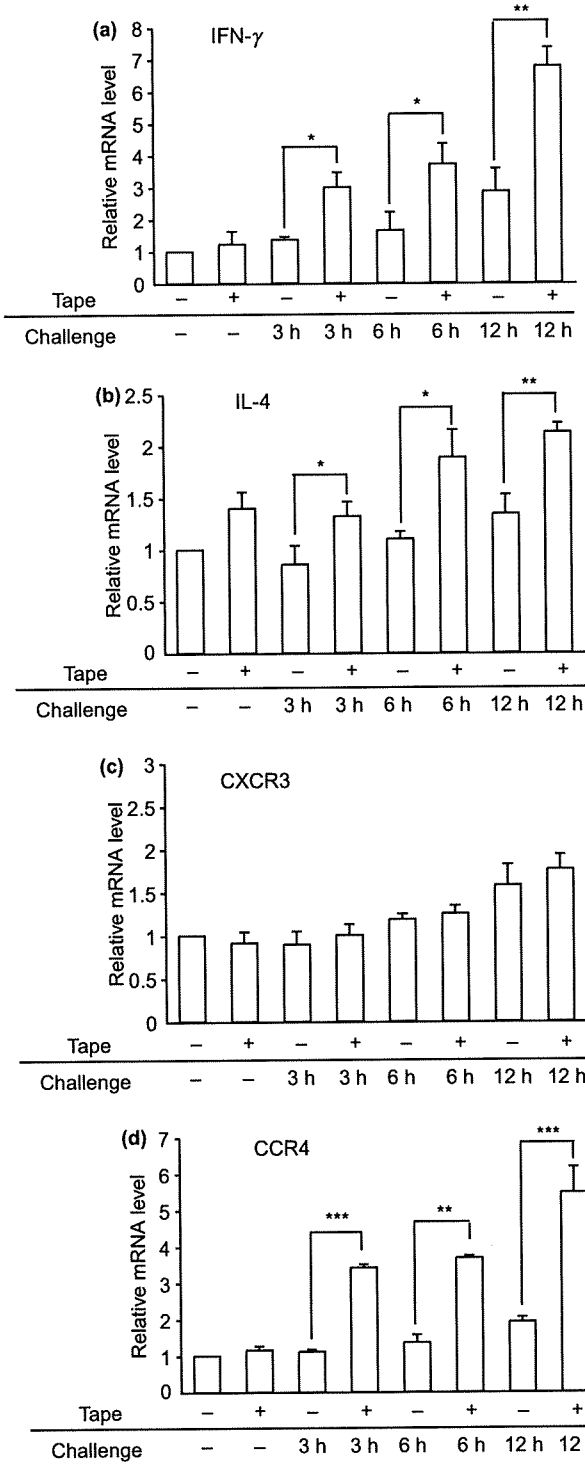

Figure 5. Real-time PCR analysis of mRNA expression for IFN-γ, IL-4 and CCR4 in the challenged skin. BALB/c mice were sensitized on the shaved abdomen with 1% FITC three times a week for 2 weeks and earlobes were provoked by painting of 1% FITC 24 h after tape stripping. At 3, 6 and 12 h after challenge, the earlobes were prepared and homogenized and subjected to real-time PCR analysis to assess the expression of IFN-γ, IL-4 and CCR4. Data are expressed as the mean ± SD of five mice (*P < 0.05, **P < 0.005, ***P < 0.0001).

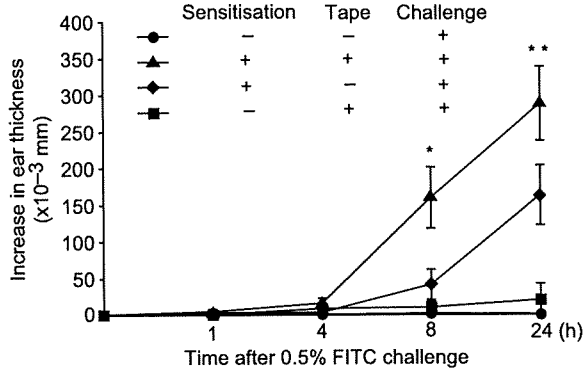

Figure 6. Augmentation of CHS to FITC n barrier-disrupted mice. BALB/c mice were repeatedly sensitized on the shaved abdomen with 1% FITC, and earlobes were provoked by painting of 0.5% FITC after tape stripping. Ear swelling responses were measured 1, 4, 8 and 24 h after challenge. Data represent Δ ear swelling from the basal ear thickness and are expressed as the mean ± SD of five mice (*P = 0.00046, **P = 0.0026).

Both tape-stripping and acetone-rubbing are known as a procedure for the acute barrier disruption, and in fact, the TEWL values of the treated skin were comparable in our study. However, the two treatments had different capacities to stimulate ECs to produce Th1 and Th2 chemokines. As keratinocytes can produce both Th1 and Th2 chemokines (11), this observation might be interpreted as an indication that tape-stripping and acetone-rubbing preferentially stimulate keratinocytes to produce Th2 and Th1 chemokines respectively. However, our recent study suggests that the main sources of Th1 (CXCL10, CXCL9 and CXCL11) and Th2 chemokines (CCL17 and CCL22) are keratinocytes and Langerhans cells respectively (13). We found that repeated application of hapten induces Th2 chemokine production by Langerhans cells. This raises an alternative possibility that tape-stripping stimulates Langerhans cells to produce Th2 chemokines and does keratinocytes to produce Th1 chemokines. In this scenario, acetone-rubbing possibly induces keratinocyte Th1 chemokine production without stimulating Langerhans cells to produce Th2 chemokines. Langerhans cells are also known to release CCL5 (14) and the observed increment of this eosinophil attractant might be derived from Langerhans cells. Tape-stripping has been shown to activate keratinocytes to produce Langerhans cell-maturating cytokines including interleukin-1α, tumor necrosis factor-α and granulocyte/macrophage colony stimulating factor (2). In parallel with this maturation, Langerhans cells might also release Th2 and eosinophil-chemoattracting chemokines.

The differences in the chemokine expression between the two stains were clearly seen with tape-stripping. BALB/c mice were more susceptible to tape-stripping than B6 mice in the

expression of all the chemokines examined. In particular, the expression of the Th2 chemokines and eosinophil chemoatrractant was markedly promoted in BALB/c mice. Although the increased expression of the Th2 and eosinophil chemokines in BALB/c mice is in accordance with the Th2-skewing property of this mouse strain, the exact mechanism underlying this preponderant expression remains unknown. To address this issue, we cultured keratinocytes from BALB/c and B6 mice, examined the production of Th2 chemokines after stimulation with interferon-γ and/or tumor necrosis factor-α and found no difference in the chemokine production between the two strains of mice. Therefore, the keratinocytes themselves are considered not to differ from each other. Given that interferon-γ suppresses Langerhans production of Th2 chemokines (13), the difference in the Th2 chemokine production might be attributable to the different Th1 or Th2 cytokine dominancy in each strain.

The tape-stripped skin exhibited enhanced degrees of the late-phase as well as delayed-type reactions upon challenge with hapten. This is thought to be a reflection of the infiltrates of eosinophils and Th2 cells (15). It is well known that patients with atopic dermatitis have skin barrier impaired by loss of filaggrin (16), decreased amounts of ceramide (8) and secondary damage following inflammation (17). Scratching caused by itch further exaggerates the barrier damage in atopic patients (18). Both the delayed-type and late-phase reactions have been put forward for the mechanisms underlying skin lesions of atopic dermatitis (19). The delayed-type reaction is clinically represented by eczematous dermatitis and mediated by Th1 and Tc1 cells (20). On the other hand, the late-phase reaction is clinically recognized by edematous erythema and mediated by Th2 and eosinophils (21). The late-phase reaction is prone to occur in the stratum corneum-removed skin and scratching exacerbates certain skin disorders such as atopic dermatitis by inducing Th2 and eosinophil-attracting chemokines. Moreover, it is tempting to speculate from our study that scratching could induce eosinophil infiltration in healthy skin and yield a late phase reaction without application of an allergen. Studies in human skin may clarify these important issues.

Acknowledgement

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Conflict of interest statement

The authors state no conflict of interest.

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ORIGINAL ARTICLE

Effects of oral antibiotic roxithromycin on quality of life in acne patients

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ABSTRACT

Macrolides are effective for inflammatory acne, but there are not many studies on roxithromycin. In this study, patients with acne were surveyed for improvement of their quality of life after treatment with roxithromycin. Patients were orally given roxithromycin 300 mg daily for 2–4 weeks. At the time of pre- and post-treatment, the dermatologists graded the severity of acne symptoms, and the patients answered questionnaires. In 123 half faces of 76 patients, 80 half faces were improved, 42 half faces were not changed, and one half face was deteriorated. The score of "symptom and feeling" and "leisure" in DLQI-J and "emotions" and "symptoms" in Skindex-29-J were significantly decreased after roxithromycin treatment. Roxithromycin has a therapeutic effect on inflammatory acne and leads to improvement of quality of life in the patients.

Key words: acne, macrolide, roxithromycin, quality of life.

INTRODUCTION

Facial acne is one of the most common skin diseases and the severity varies from mild comedones or small inflammatory papules to cystic or conglobated lesions. There are various therapies for acne, including oral and topical antibiotics, topical retinoids, hormonal agents and miscellaneous therapies.[1] Facial acne affects the daily lives of both teenagers and those in early adulthood.[2,3] Mallon *et al.*[4] compared the quality of life (QOL) of acne patients to that of patients with asthma, epilepsy, diabetes, back pain, arthritis or coronary heart disease, and found that the impact of acne on QOL can be as great as those of severe and even life-threatening diseases. On the other hand, the severity of acne varies among races. For instance, acne lesions of Japanese patients are less severe than those of Western patients. Therefore, it is an interesting issue to evaluate QOL of Japanese patients and its change by treatment modalities.

For this assessment, we used questionnaires including Japanese versions of the Skindex-16,[5] Skindex-29-J and DLQI-J,[6] which were developed as QOL questionnaires of global standards translated into Japanese. Furthermore, a recent careful study by Japanese multi-dermatological centers successfully determined the classification of acne severity in Japanese patients.[7] These questionnaires and assessment of severity have enabled us to investigate the QOL of Japanese acne patients.

Systemic antibiotics are also standard treatments of inflammatory acne in Japan and there is evidence to support the use of tetracyclines, macrolides and trimethoprim-sulfamethoxazole.[1] Especially, macrolides, including erythromycin, clarithromycin, azithromycin and roxithromycin (RXM), are widely used as antibacterial agents and are known to have an anti-inflammatory effect.[8] RXM also has various modulatory bioactivities to immunocompetent cells that are involved in allergy and inflammation.[9] It down-modulates the antigen-presenting function of professional antigen-presenting Langerhans cells[10] and the cytokine/chemokine production of epidermal keratinocytes.[11] In accordance with these experimental

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findings, RXM exerts beneficial therapeutic effects on various inflammatory or immunological skin disorders.[12–15] Moreover, RXM has unique actions such as anti-androgenic activity of the skin[16] and suppression of certain T-cell populations.[17] These various actions of RXM seem to stem from certain inhibition of transcription.[18,19] Therefore, in this study, we chose RXM among macrolides and examined the change of QOL before and after RXM treatment along with the alteration of acne severity. This investigation was performed by 14 dermatologists at 12 dermatology departments of general hospitals or private offices as an open trial. Results show that QOL was impaired even in Japanese patients and was significantly improved by RXM treatment.

METHODS

Patients and setting

A total of 76 patients (aged 16–38 years; 11 men and 65 women) seen for treatment of acne as outpatients at the departments or sections of dermatology of two general hospitals (University of Occupational and Environmental Health Japan, Kyushu Kosei Nenkin Hospital [Dr K. Izu]) and 10 private clinics (Drs K. Eguchi, T. Hirano, M. Hisanaga, T. Maruyama, K. Masaki, C. Minami, T. Nomiyama, T. Ohta, N. Shiraishi and K. Yanagisawa) were enrolled in this study. We explained the purpose and method of the research to the patients with inflammatory eruptions, and informed consent was obtained. For the treatment of acne, the patients were orally given antibiotic RXM 300 mg daily for 2–4 weeks, without topical medications or physical therapies.

Measures

Questionnaires

The survey was performed with two different self-administered questionnaires, DLQI-J and Skindex29-J,[6] along with investigation on demographic and clinical characteristics including perceived severity of acne symptoms. The patients were asked to immediately fill out the questionnaires at pre- and post-treatment.

Objective assessment of acne severity

The index of the severity of acne symptoms was decided based on Japanese Grading Criteria for Acne Severity that has been advocated by Japanese Acne Study Group.[7] The severity was classified into four groups by the number of inflammatory eruptions in each half face: 0–5 for mild (I); 6–20 for moderate (II); 21–50 for severe (III); and more than 51 for very severe (IV).

Table 1. Background of the patients

	Men (n = 11)	Women (n = 65)
Age (years)	22.2 ± 1.65	24.2 (± 0.89)
Duration of disease (months)	19.5 (± 6.04)	29.8 (± 5.22)

Severity	Right side	Left side
Very severe	1	1
Severe	16	14
Moderate	55	53
Mild	4	4

Statistical analysis

Changes in severity and QOL scores were analyzed using Wilcoxon rank sum test. $P < 0.05$ was considered statistically significant. The score of the QOL is presented as mean ± standard deviation.

RESULTS

Changes in severity of acne between pre- and post-treatment with RXM

The clinical data of 76 patients were obtained from 14 dermatologists participating in this study (Table 1). The patients were female-predominant, and mainly consisted of moderate-to-severe levels of acne. In 76 patients, 123 parts (right half side of the face, 61; left half side of the face, 62) of the face could be accurately judged for change of severity before and after RXM administration. The severity was designated as I–IV (from mild to very severe) based on the number of acne lesions.[7] As shown in Figure 1, 80 sides were improved, 42 sides were not changed and one side was deteriorated. From this evaluation, 31 patients were improved on both sides and 12 patients were unchanged or deteriorated on both sides.

Evaluation of QOL

DLQI-J and Skindex-29-J questionnaires were obtained from the patients and the data are summarized in Table 2. Because there were no-answer blanks, the total number of each item varied. In DLQI-J, the

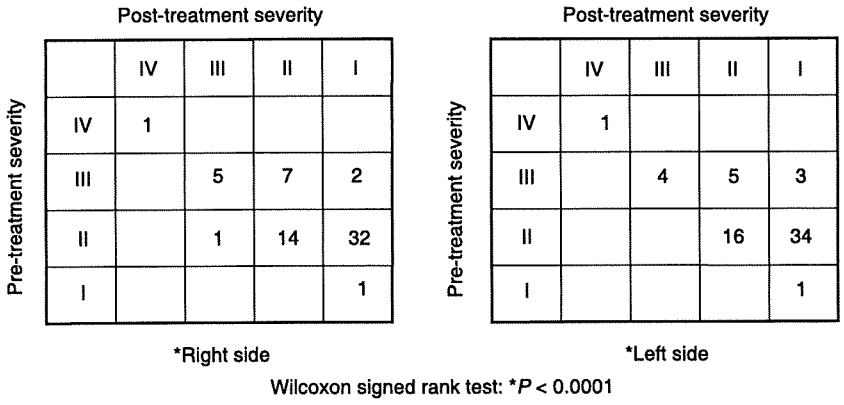

Figure 1. Change in severity by roxithromycin (RXM) treatment. Dermatologists categorized each half side of the faces of acne patients as mild (I), moderate (II), severe (III) and very severe (IV) based on Japanese Grading Criteria for Acne Severity. *$P <$ 0.0001, difference in severity of pre-treatment vs post-treatment, assessed by Wilcoxon rank sum test.

Table 2. Changes in each category of DLQI-J and Skindex-29-J between pre- and post-treatment with roxithromycin

	Scale	*n*	Pre-treatment score	Post-treatment score	Wilcoxon matched pairs singled-rank test
DLQI-J	Symptom and feeling	50	3.00 ± 1.616	1.96 ± 1.068	$P < 0.0001$
	Daily activities	45	0.78 ± 1.295	0.58 ± 0.812	$P = 0.3552$
	Leisure	39	0.87 ± 0.978	0.49 ± 0.644	$P = 0.0083$
	Work and School	44	0.50 ± 0.762	0.32 ± 0.639	$P = 0.1083$
	Personal relationship	38	0.24 ± 0.590	0.24 ± 0.590	$P = 1.0000$
	Treatment	50	0.60 ± 0.808	0.50 ± 0.763	$P = 0.4518$
	Total	42	5.74 ± 4.067	3.83 ± 3.011	$P = 0.0005$
Skindex-29-J	Emotions	46	60.89 ± 21.174	48.87 ± 22.911	$P < 0.0001$
	Symptoms	46	39.31 ± 17.409	33.31 ± 18.433	$P = 0.0042$
	Functioning	46	22.16 ± 17.478	19.69 ± 16.954	$P = 0.0501$
	Total	46	39.61 ± 16.357	33.06 ± 17.249	$P = 0.0002$

scores of "symptom and feeling" and "leisure" were significantly decreased after RXM treatment, but the other items were not improved. In Skindex-29-J, the scores of "emotions" and "symptoms" were decreased after RXM treatment, and "functioning" was marginally alleviated. In both assessments, the total scores were significantly reduced by RXM treatment.

We analyzed these items in relation to treatment efficacy, severity, age and disease duration. When the clinically improved group (31 patients) and the unchanged or deteriorated group (12 patients) were compared, the former group showed marked reductions of all scores of DLQI-J (Fig. 2a) and Skindex-29-J (Fig. 2b). This suggested that the QOL was improved accordingly with the objective clinical improvement. As to the severity of acne, the QOL values of the patients with mild or moderate lesions were more markedly reduced by RXM than those with severe or very severe lesions (Fig. 3). The patients aged 23 years or more showed significant decreases of the QOL values in all items compared to those aged less than 23 years (Fig. 4). We determined to divide the patients into the two groups of below 22 years and 23 years or more because this grouping yielded a comparable number of patients and similar values of QOL before therapy. Finally, the cases of shorter duration of the disease (less than 6 months) exhibited a higher reduction of the QOL values (Fig. 5).

DISCUSSION

In the patients enrolled in this study, the QOL values before the start of therapy (total DLQI-J score of 5.7 ± 4.06 and Skindex-29-J score of 39.6 ± 16.35) were higher than those of normal subjects and comparable to the scores of acne patients reported previously.[6,20,21] Both scores of DLQI-J and Skindex-29-J were significantly lowered by RXM treatment along with the clinical improvement. The evaluation of clinical symptoms was performed by the dermatologists based on the

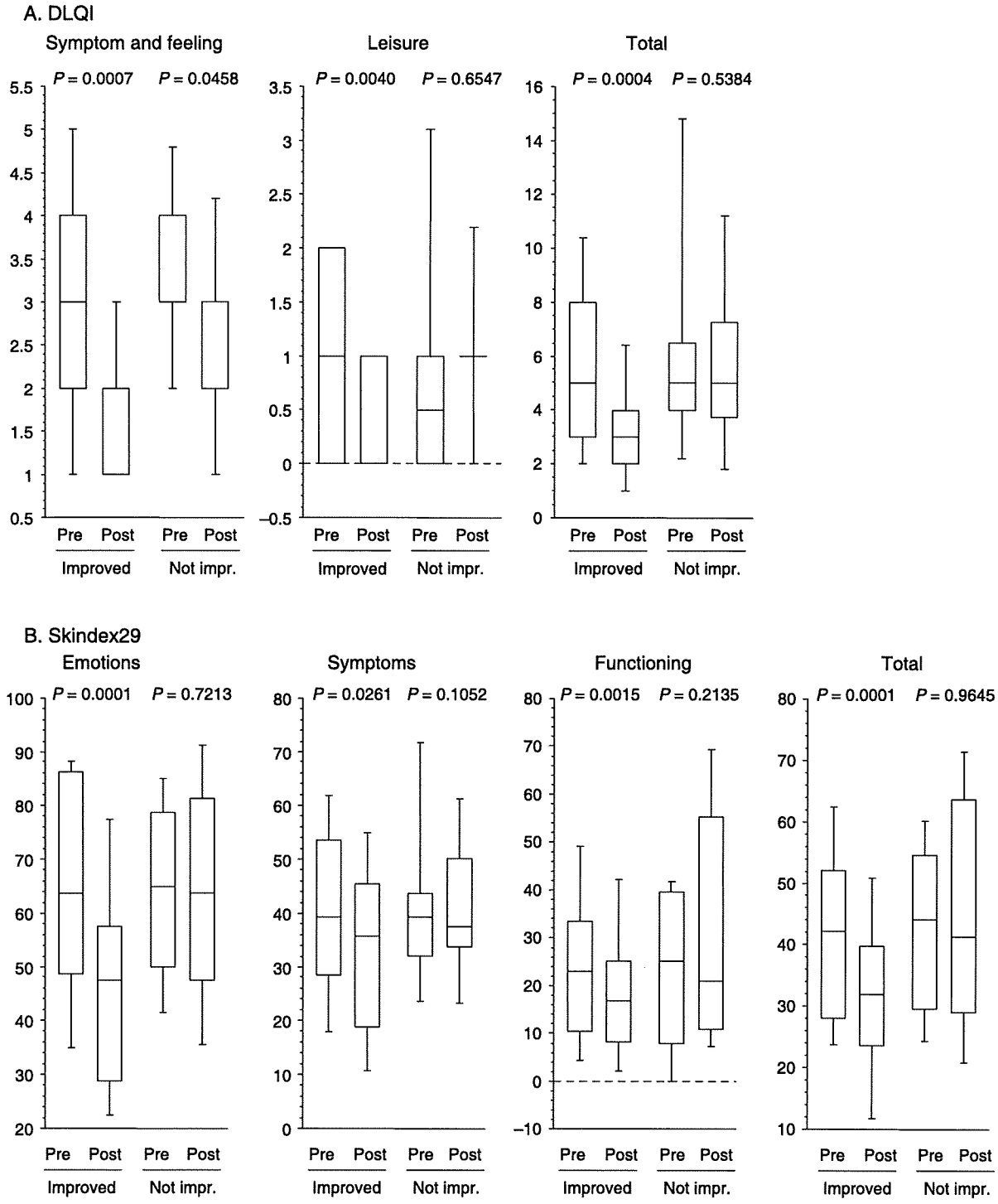

Figure 2. Roxithromycin (RXM) efficacy dependency of quality of life scores pre- and post-treatment: improved patients, $n = 24$–31; "Not impr." represents unchanged or deteriorated patients, $n = 10$–12.

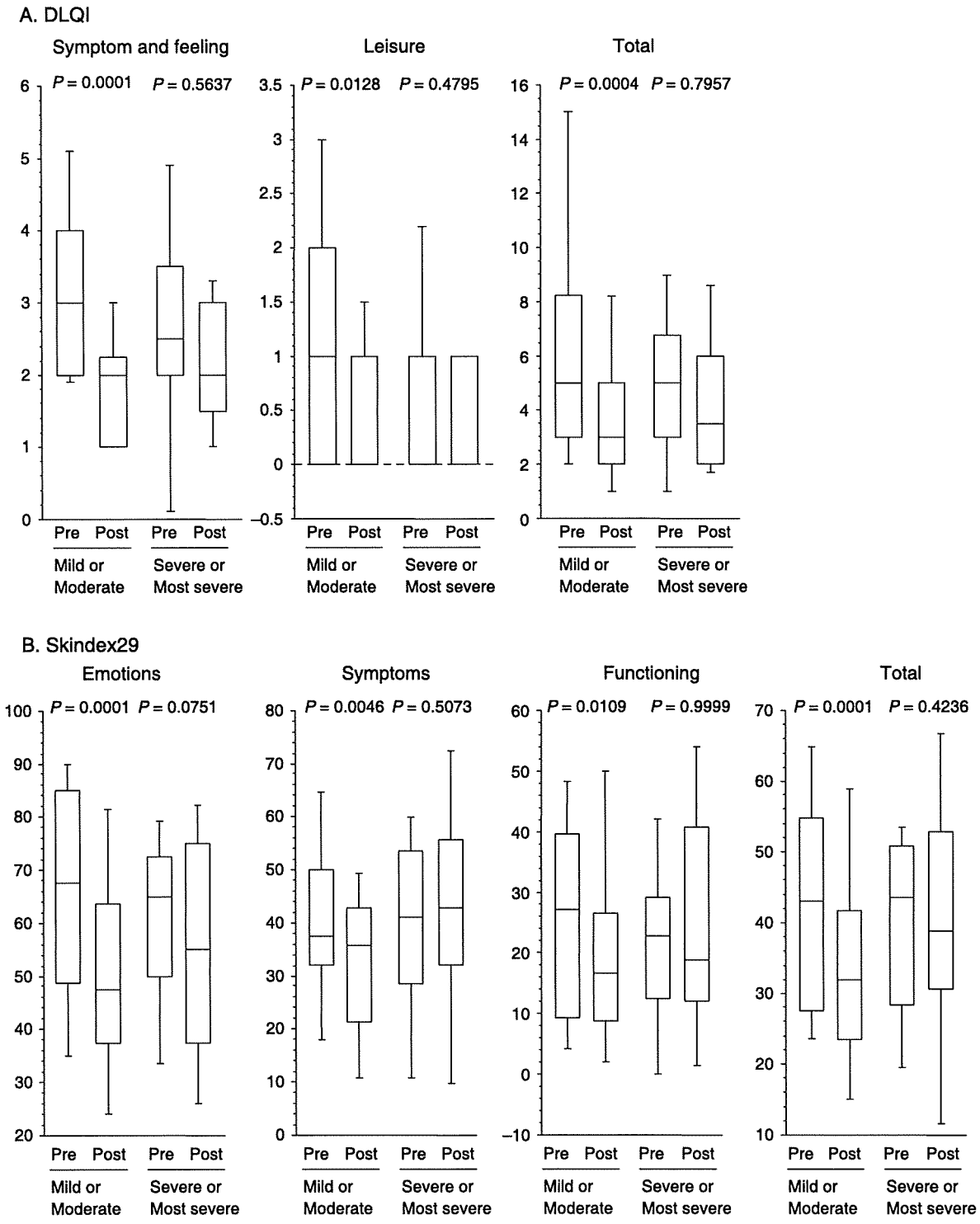

Figure 3. Severity in pre-treatment dependency of quality of life scores pre- and post-treatment: mild or moderate, $n = 25$–32; severe or very severe, $n = 9$–12.

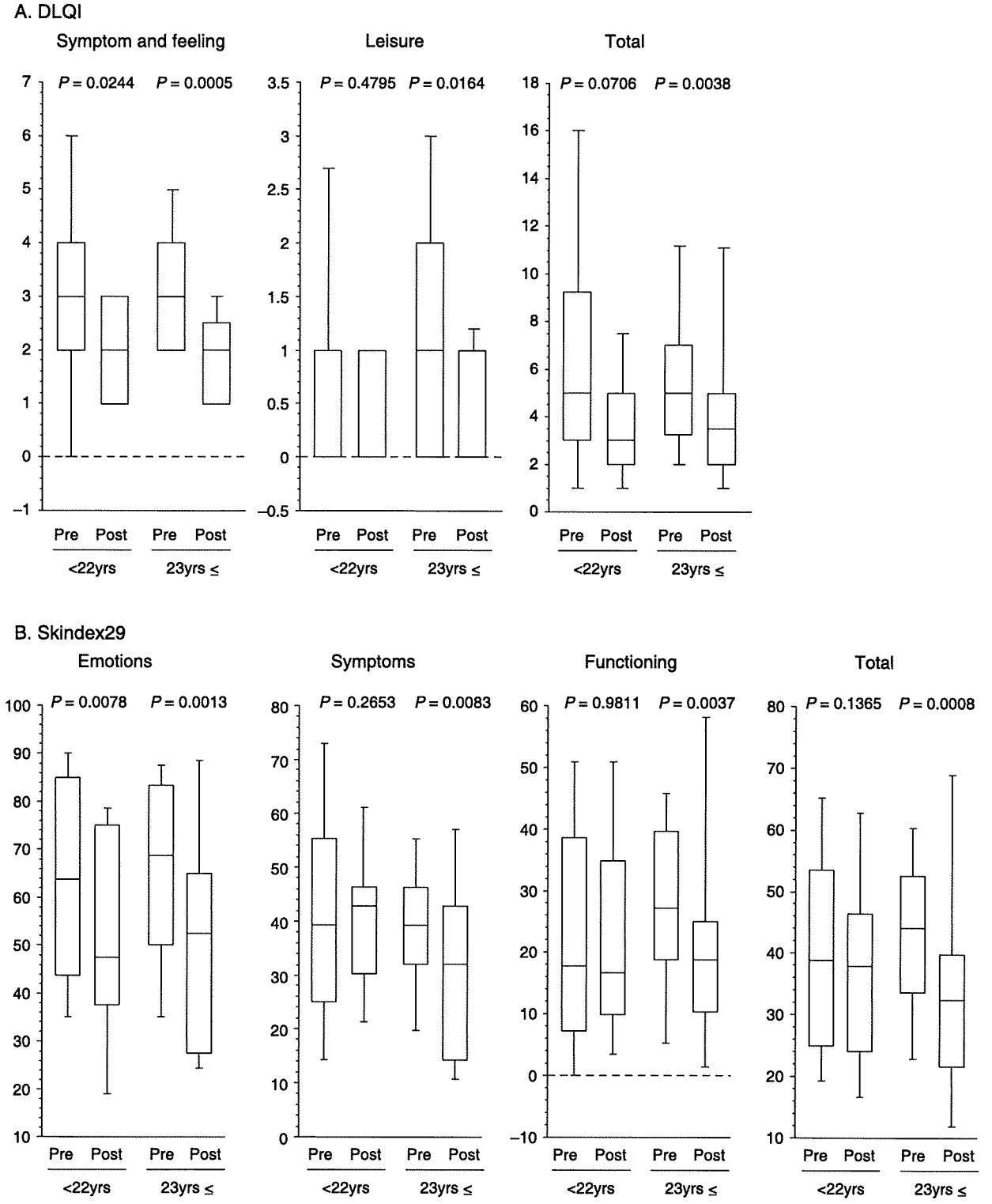

Figure 4. Age dependency of quality of life scores pre- and post-treatment: <23 years, $n = 15–20$; ≥23 years, $n = 19–24$.

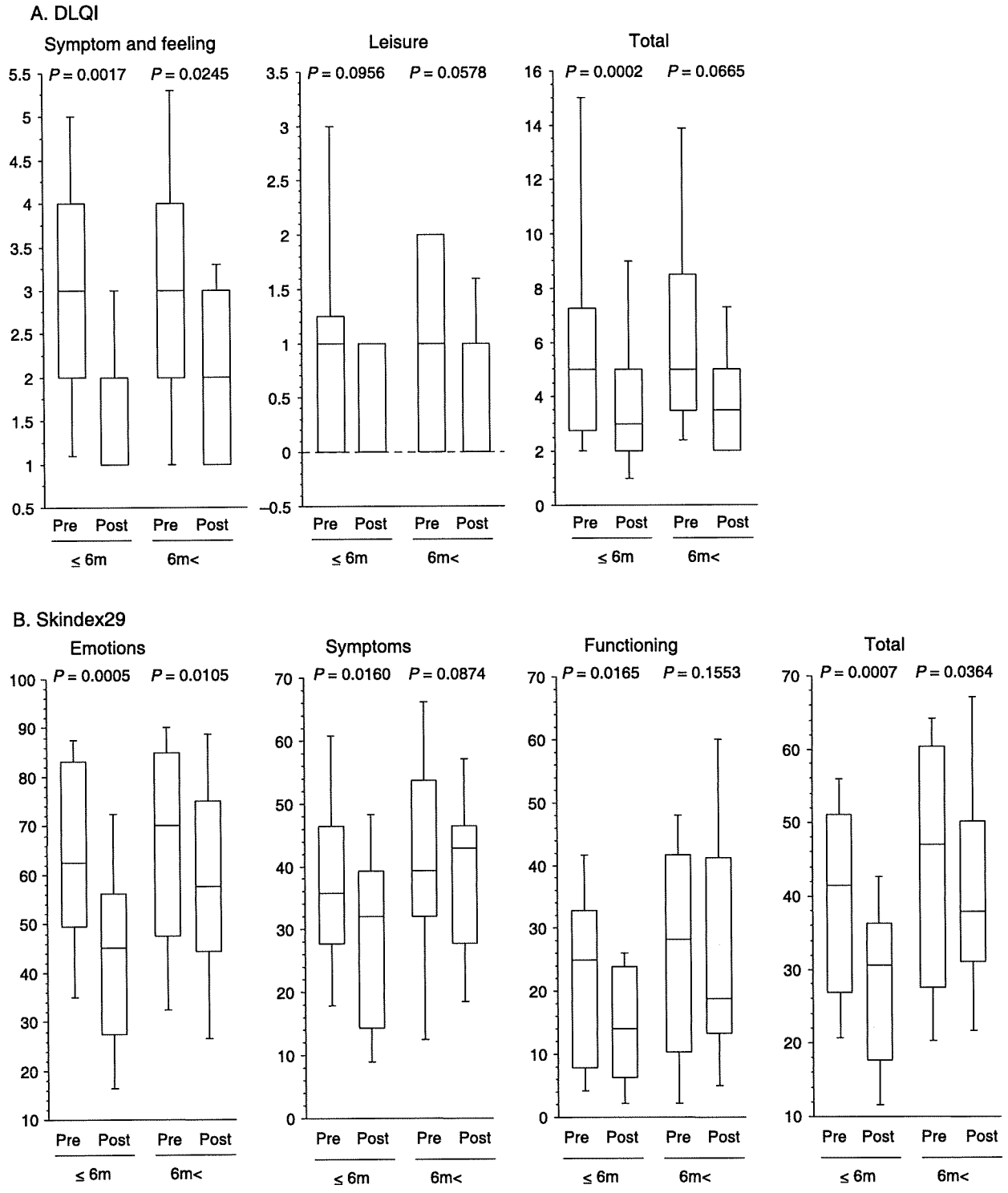

Figure 5. Disease duration dependency of quality of life scores pre- and post-treatment: ≤6 months, $n = 18–21$; >7 months, $n = 16–21$.

severity classification. There has been a report demonstrating that satisfaction of Japanese dermatologists for acne treatment before adapalene is low.[22] The QOL was improved in the patient group whose symptoms were alleviated, confirming the validity of the study and the close influence of the symptoms on the patients' QOL. Thus, our objective judgment was not estranged from the subjective symptoms of the acne patients. The improvements in the "emotion" of DLQI-J and the "symptom and feeling" of Skindex-29-J were particularly remarkable. On the basis of these results, we verified that a large burden developed in the mind of the patient when the facial symptoms were severe. In addition, it is noted that QOL was more markedly improved when the lesions were milder, the patients were older and the disease duration was shorter. The main reason for the absence of QOL improvement in the severe patients may stem from the short observation period, because the erythematous hue remained even after the inflammatory acne was healed.

There are two reasons why we chose RXM as treatment. Initially, RXM is approved as a therapy of acne by the Japanese government, whereas the vastly worldwide-recommended therapies (topical retinoids, benzoyl peroxide and isotretinoin)[1] are not yet approved in Japan. Another reason is perhaps more important. In addition to the strong antimicrobial activity to *Propionibacterium acnes*, RXM has various pharmacological functions and exerts a therapeutic effect on inflammatory acne. It is suggested that RXM alleviates acne by these pluripotential properties[9] and leads to improvement of QOL in patients with acne.

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