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ABSTRACT
Objective: To assess the association of CD40/CD40 ligand (CD40L) interactions with the development of skin fibrosis and autoimmunity in tight-skin (TSK/) mouse, which is a mouse model for human systemic sclerosis.

Methods: Newly born TSK/+ mice were treated with murine anti-CD40L monoclonal antibody (100 μg intraperitoneally weekly). Hypodermal thickness of 8-week-old female mice (defined as the thickness of a subcutaneous loose connective tissue layer beneath the panniculus carnosus) was measured under a light microscope. All skin sections were taken from the para-midline, upper back region. Serum anti-topoisomerase I autoantibody levels, serum immunoglobulin levels and plasma soluble CD40L levels were determined by enzyme-linked immunosorbent assay. For analysis of lymphocyte surface molecules, single cell suspensions of lymphocytes were stained by monoclonal antibodies. Proliferation of TSK/+ B cells and fibroblasts to anti-CD40 antibodies was assessed by the uptake of [3H]-labelled thymidine and bromodeoxyuridine, respectively.

Results: The blockade of CD40/CD40L interactions by anti-CD40L monoclonal antibody significantly reduced cutaneous fibrosis (65%) and anti-topoisomerase I autoantibody in TSK/+ mice. Anti-CD40L monoclonal antibody also normalised B lymphocyte abnormal activation in TSK/+ mice, demonstrated by hyper-g-globulinaemia. Furthermore, augmented CD40/CD40L interactions in TSK/+ mice were suggested by upregulated expression of CD40L on CD4+ T cells, elevated plasma soluble CD40L levels. The hyperresponsiveness to CD40 stimulation was also observed in TSK/+ B cells and fibroblasts.

Conclusions: Cutaneous fibrosis and autoimmunity in TSK/+ mice are closely correlated with CD40/CD40L interactions.

Systemic sclerosis (SSc) is an autoimmune disease, characterised by connective tissue involvement, with excessive extracellular matrix protein deposition in the skin and other visceral organs. The tight-skin (TSK/) mouse is a genetic animal model for human SSc: autoimmune components and cutaneous fibrosis observed in TSK/+ mice resemble those in human SSc.1–4 Although a tandem duplication within the fibrillin-1 gene in TSK/+ mice has been suggested to cause the SSc-like phenotype,5 immune components also contribute to skin fibrosis. Adoptive transfer of both T and B cells from TSK/+ mice induces cutaneous collagen deposition in wild-type mice.6 Deficiency in transforming growth factor-β, interleukin (IL)-4, IL-6 receptor, Stat-6, or CD19 as well as the administration of anti-IL-4 antibody, IL-12 or CpG oligodeoxynucleotides prevent or reduce skin fibrosis in TSK/+ mice.6,7 Thus, immune components can be a therapeutic approach in TSK/+ mice as well as patients with SSc.

Activated T cells express CD40 ligand (CD40L), which triggers various immune responses, including B cell survival, immunoglobulin class switching, and cytokine production8 through CD40/CD40L interaction. Circulating soluble CD40L (sCD40L) also has biological activities such as cell binding CD40L.9 CD40 is expressed on the surface of immune cells, including B cells, dendritic cells and monocytes/macrophages,10 as well as other cell types, such as endothelial cells11 and fibroblasts.12 A therapeutic approach targeting CD40L has been addressed using anti-CD40L monoclonal antibody (mAb). Administration of anti-CD40L mAb, which blocks the biological activity of CD40L/sCD40L, inhibits several animal autoimmune models, such as collagen-induced arthritis and lupus.13 14 Moreover, clinical studies using humanised anti-CD40L mAb have shown improvement in patients with autoimmune disorders, including lupus and immune thrombocytopenic purpura.15

In this study, we examined the effect of anti-CD40L mAb against the development of cutaneous fibrosis and autoimmunity in TSK/+ mice.

MATERIALS AND METHODS
Mice and in vivo treatment
TSK/+ mice with a C57BL/6 genetic background were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). To verify the TSK/+ genotype, polymerase chain reaction amplification of a partially duplicated fibrillin-1 gene was carried out using genomic DNA from each mouse as described.16 All studies were approved by the Committee on Animal Experimentation of Kanazawa University Graduate School of Medical Science. One-week-old TSK/+ mice were given either intraperitoneal 100 μg of murine anti-CD40L mAb (MR-1; BD PharMingen, San Diego, CA, USA) every week or the same amount of control mAb.

Assessment of skin fibrosis
The morphological characteristics of skin sections from 8-week-old female TSK/+ mice treated with anti-CD40L mAb were compared with those with
control antibody under a light microscope. All skin sections were taken from the para-midline, upper back region as full-thickness sections. Tissues were fixed in 10% formaldehyde solution for 24 h, embedded in paraffin, and stained with haematoxylin and eosin. Dermal and hypodermal thickness was measured for multiple transverse perpendicular sections using an ocular micrometer. Ten random measurements were taken per section. To determine hydroxyproline content in the skin, 6 mm punch biopsies were treated with chloroform:methanol (2:1 vol:vol) and were analysed as described.21

Flow cytometric analysis
Single cell suspensions of lymphocytes from blood were used. Leucocytes (0.5×10⁶ to 1×10⁶) were stained at 4°C using predetermined optimal concentrations of antibodies for 20 min. Cells with the forward and side light scatter properties of lymphocytes were analysed on a FACScan flow cytometer (BD Biosciences, San Diego, CA, USA).

Enzyme-linked immunosorbent assay for detecting plasma sCD40L levels
Specific enzyme-linked immunosorbent assay (ELISA) kits were used for measuring plasma sCD40L levels (Bender Medsystems, Vienna, Austria). Each sample was tested in duplicate.

In vitro cutaneous fibroblast proliferation
Trypsinised, third-passage fibroblasts (2×10⁶ cells/well in 0.2 ml) were cultured in triplicate in 96-well plates with Dulbecco minimal Eagle’s medium containing 10% fetal calf serum with indicated concentration (see fig 4) of anti-CD40 antibody (1C10; R&D Systems, Minneapolis, Minnesota, USA). Cellular proliferation was quantified by the addition of 10 mM bromodeoxyuridine (Roche Diagnostics, Mannheim, Germany) 20 min. Cells with the forward and side light scatter properties of lymphocytes were analysed on a FACScan flow cytometer (BD Biosciences, San Diego, CA, USA).

Enzyme-linked immunosorbent assay for detecting plasma IgM and IgG anti-topo I antibody in TSK/+ mice
By contrast, anti-CD40L mAb significantly reduced the levels of IgM and IgG anti-topo I antibody in TSK/+ mice compared with TSK/+ mice with the control antibody (0.38 (SD 0.05)) was similar to those with control antibody injection (0.41 (0.04)). This regimen was designed against skin sclerosis: the hypodermal thickness of 8-week-old TSK/+ mice treated after they were 4 weeks old (0.38 (SD 0.05)) was similar to those with control antibody injection (0.41 (0.04)). This regimen was designed around the observation that the difficulty to pinch up the back skin clearly appears at 4 weeks of age, although skin sclerosis in TSK/+ mice is recognisable by 1 week after birth and develops fully by ~2 months of age.12 Thus, blockade of CD40/CD40L interactions attenuated the development of skin fibrosis in TSK/+ mice.

RESULTS
Blockade of CD40/CD40L interactions by anti-CD40L monoclonal antibody attenuated the development of skin fibrosis in TSK/+ mice
One-week-old TSK/+ mice were given anti-CD40L mAb every week for seven weeks. Skin fibrosis in TSK/+ mice treated with anti-CD40L mAb was assessed by histopathology. The hypodermal thickness in TSK/+ mice was increased by ninefold compared with the wild-type littermates (p<0.0001, fig 1A,B). The TSK/+ mice treated with anti-CD40L mAb showed a markedly reduced hypodermal tissue, which was significantly 65% thinner than that found in TSK/+ mice (p<0.001) but remained significantly thicker than that of wild-type littermates (p<0.05). Anti-CD40L mAb administration in wild-type mice did not influence hypodermal thickness (data not shown). Cutaneous fibrosis was also assessed by quantifying the hydroxyproline content from skin samples (fig 1C). Hydroxyproline content in TSK/+ mice was increased by 24% relative to wild-type littermates (p<0.0001). The anti-CD40L mAb treatment reduced hydroxyproline content by 17% in TSK/+ mice compared with TSK/+ mice with the control antibody (p<0.001). Anti-CD40L mAb administration did not affect the development of pulmonary emphysema and cardiac hypertrophy in TSK/+ mice (data not shown).

Next, 4-week-old TSK/+ mice were treated with anti-CD40L mAb weekly until 8 weeks old. This regimen did not show any effect against skin sclerosis: the hypodermal thickness of 8-week-old TSK/+ mice that had been treated after they were 4 weeks old (0.38 (SD 0.05)) was similar to those with control antibody injection (0.41 (0.04)). This regimen was designed around the observation that the difficulty to pinch up the back skin clearly appears at 4 weeks of age, although skin sclerosis in TSK/+ mice is recognisable by 1 week after birth and develops fully by ~2 months of age.12 Thus, blockade of CD40/CD40L interactions attenuated the development of skin fibrosis in TSK/+ mice.

Blockade of CD40/CD40L interactions abrogated anti-topoisomerase I antibody production in TSK/+ mice
Next, we examined anti-topo I antibody production in TSK/+ mice treated with anti-CD40L mAb. Pooled sera from 8-week-old TSK/+ mice had mean IgM and IgG anti-topo I antibody levels that were 2.3- and 1.7-fold higher than those found in wild-type littermates (p<0.01 and p<0.005, respectively; fig 2). By contrast, anti-CD40L mAb significantly reduced the levels of IgM and IgG anti-topo I antibody in TSK/+ mice compared with TSK/+ mice with the control antibody treatment (p<0.05 and p<0.01, respectively; fig 2). Thus, anti-CD40L mAb normalised anti-topo I antibody production in TSK/+ mice.
expression levels on CD4+ T cells from TSK/+ mice. Because CD40L overexpression on CD4+ T cells indicates augmented CD40/CD40L interactions, as observed in several autoimmune diseases.24 Therefore, peripheral blood mononuclear cells from 4-week-old TSK/+ or wild-type littermates were assessed by two-colour staining with flow cytometric analysis (fig 3A,B). Although CD4+ T cells from wild-type littermates rarely expressed CD40L, CD4+ T cells from TSK/+ mice had a 7.5-fold increase in the percentage of CD40L+ cells in comparison with wild-type littermates (4.4 (1.3)% vs 0.53 (0.23)%, p<0.001, fig 3A). However, CD8+ T cells from TSK/+ or wild-type littermates rarely expressed CD40L on their surface (fig 3B). Thus, CD4+ T cells from TSK/+ mice frequently hyperexpressed CD40L. Next, plasma levels of sCD40L in TSK/+ mice were examined, as CD40L+ T cells may release sCD40L. TSK/+ mice had an 11-fold increase in sCD40L compared with wild-type mice at 4 weeks of age (p<0.0001), which was slightly decreased to a 7.7-fold increase at 8 weeks of age (p<0.001, fig 3C).

Proliferative responses to CD40 signalling was augmented in TSK/+ fibroblasts

To assess the association of fibroblast hyperplasia in TSK/+ mice with CD40/CD40L interactions, proliferative responses and the type I collagen α2 chain (COL1A2) mRNA levels of cultured fibroblasts were quantified. Proliferative responses and COL1A2 mRNA levels by anti-CD40 mAb stimulation (10 μg/ml) were significantly increased in TSK/+ fibroblasts compared with wild-type fibroblasts (118% increase, p<0.001 and 56% increase, p<0.05, respectively; fig 4A,B). Thus, hyperresponsiveness to CD40/CD40L signalling was observed in TSK/+ fibroblasts.

TSK/+ B cell hyperresponsiveness in association with CD40/CD40L interactions

As we have reported abnormalities of B cell responses in TSK/+ mice,11 25 the association of CD40/CD40L interactions with B cell hyperresponsiveness in TSK/+ mice was addressed. First, TSK/+ B cell proliferation to anti-IgM antibody, anti-CD40 mAb and LPS was assessed (fig 5A). Proliferation of B cells from

**Figure 1** Skin fibrosis in dorsal skin from wild-type littermates and tight-skin (TSK/+ ) mice treated with anti-CD40L mAb (αCD40L) or control antibody (CTL). (A) Representative histological sections stained with haematoxylin and eosin (×40). A triangle indicates the dermis, while double triangles show the subcutaneous loose connective tissue layer beneath the panniculus carnosus (arrow). Skin fibrosis was further assessed by quantitatively measuring hypodermal thickness (B) and skin hydroxyproline content (C). Horizontal bars represent mean hypodermal thickness (B) and mean hydroxyproline content (C). *p<0.001.

**Figure 2** Serum anti-topoisomerase (topo) I antibody levels. Horizontal bars represent mean OD levels. Values in parentheses represent the dilutions of pooled sera giving half-maximal OD values in enzyme-linked immunosorbent assays, which was determined by linear regression analysis to generate arbitrary units per millilitre. *p<0.05. **p<0.01. CTL, control; TSK, tight-skin mice.
8-week-old TSK/+ to anti-IgM antibody and anti-CD40 mAb was significantly increased compared with wild-type B cells (22% increase, p<0.05 and 53% increase, p<0.0001, respectively), while proliferation to LPS was normal. Thus, augmented proliferation in TSK/+ B cells was relatively dependent on the signals through CD40/CD40L.

The effect of CD40/CD40L blocking in vivo on B cell responsiveness was assessed by determining serum immunoglobulin levels in TSK/+ mice (fig 5B). Eight-week-old TSK/+ mice had significantly elevated IgM (p<0.005), IgG1 (p<0.01), IgG2a (p<0.003) and IgG2b (p<0.0005) levels compared with those of wild-type littermates, while IgG3 and IgA levels were not different from those of wild-type littermates. By contrast, 8-week-old TSK/+ mice treated with anti-CD40L mAb had significantly decreased IgG1 (p<0.0005), IgG2a (p<0.005), IgG2b (p<0.0001) and IgA (p<0.0001) levels compared with TSK/+ mice with control antibody treatment, while IgM levels were significantly higher than those of TSK/+ mice with control antibody (p<0.001). Thus, TSK/+ mice exhibited hyper-\(\gamma\)-globulinaemia that was associated with CD40/CD40L interactions.

**DISCUSSION**

In the current study, anti-CD40L mAb therapy showed remarkable effectiveness in TSK/+ mice even compared with our previous report of anti-CD20 mAb therapy, suggesting that CD40/CD40L interactions are critical for the development of skin fibrosis in TSK/+ mice. While dermal thickness is similar between TSK/+ mice and wild-type littermates, the hypodermal thickness is significantly increased in TSK/+ mice relative to wild-type littermates (fig 1A,B). Treatment by anti-CD40L mAb markedly reduced progression of hypodermal thickening in TSK/+ mice (fig 1A,B), although the treatment did not affect skin fibrosis in TSK/+ mice with established disease. As anti-CD40L mAb affected neither hypodermal thickness of wild-type mice nor dermal thickness of TSK/+ mice (fig 1A,B), the treatment did not induce cutaneous collagen deposition and autoantibody production in wild-type mice, while the infusion of purified T cells alone does not lead to the development of TSK syndrome.

Deficiency of CD19 as well as B cell depletion using anti-CD20 mAb resulted in markedly reduced skin fibrosis in TSK/+ mice. Furthermore, anti-CD40L mAb treatment normalised abnormal anti-topo I antibody production and hyper-\(\gamma\)-globulinaemia in TSK/+ mice (figs 2 and 5). Thus, anti-CD40L mAb therapy effectively attenuated the abnormal development of skin fibrosis and B cell activation in TSK/+ mice.

Although causal association between abnormal B cell activation and hypodermal hyperplasia in TSK/+ mice remains unknown in this study, some evidence has suggested the association. Adoptive transfer of both T and B cells from TSK/+ mice induces cutaneous collagen deposition and autoantibody production in wild-type mice, while the infusion of purified T cells alone does not lead to the development of TSK syndrome. Furthermore, the development of skin fibrosis correlated closely

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**Figure 3** Two-colour immunofluorescence of CD4+ (A) and CD8+ (B) T cells for CD40L expression. Representative staining is shown. Based on background staining the dot-plots were divided into four quadrants. The percentages of positive cells were indicated in the quadrants and right panels. (C) Plasma levels of sCD40L were determined by enzyme-linked immunosorbent assay. Statistical analysis is provided in the Results section. TSK, tight-skin mice.

8-week-old TSK/+ to anti-IgM antibody and anti-CD40 mAb was significantly increased compared with wild-type B cells (22% increase, p<0.05 and 53% increase, p<0.0001, respectively), while proliferation to LPS was normal. Thus, augmented proliferation in TSK/+ B cells was relatively dependent on the signals through CD40/CD40L.

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**Figure 4** (A) Fibroblast proliferative response to anti-CD40 monoclonal antibody (mAb). Fibroblasts were cultured with the indicated amounts of anti-CD40 mAb. Values represent the mean OD (SEM) of bromodeoxyuridine concentration. These results represent those obtained in four independent experiments. (B) mRNA levels of the type I collagen \(\alpha\)2 chain (COL1A2) in the cultured fibroblast. *p<0.05; **p<0.001. TSK, tight-skin mice.
with serum anti-topo I antibody levels in TSK/+ mice. The current study suggested that both augmented B cell and fibroblast response to CD40 signal influence the phenotype of TSK/+ mice.

As CD40 is expressed on various cell types, there are several potential mechanisms for the effectiveness of anti-CD40L mAb therapy in TSK/+ mice. First, ligation of CD40L to CD40 expressed by fibroblasts is likely to facilitate fibroblast proliferation. Proliferative response to CD40 signalling was significantly augmented in TSK/+ fibroblasts (fig 4A, B). The source of CD40L ligation remains unclear, as very little T cell infiltration in the cutaneous and subcutaneous tissue was observed in TSK/+ mice (fig 1A). TSK/+ mice had a marked increase in plasma sCD40L compared with wild-type littermates by 11-fold at 4 weeks of age and by 7.7-fold at 8 weeks, respectively (fig 3C). Therefore, increased sCD40L may lead to hyperactivation of fibroblasts in TSK/+ mice.

Consistent with the positive results in this study of anti-CD40L mAb in TSK/+ mice, critical roles of CD40/CD40L interactions have also been suggested in other animal autoimmune models. Moreover, clinical studies using humanised anti-CD40L mAb show improvement in patients with autoimmune disorders, including lupus, immune thrombocytopenic purpura and psoriasis. In SSc, blockade of CD40/CD40L interaction has inhibited anti-topo I antibody production in cultured T and B cells from patients with SSc with anti-topo I antibody. Serum and plasma levels of sCD40L, which activates CD40/CD40L interactions, are markedly elevated and correlated with the disease severity.

Figure 5 (A) B cell proliferation in response to anti-IgM antibody, anti-CD40 monoclonal antibody (mAb), or lipopolysaccharide (LPS). Values represent the mean cpm (SEM) of labelled thymidine. These results represent those obtained in four independent experiments. *p<0.05; **p<0.001. (B) Serum immunoglobulin levels. Pooled sera from 4-week-old and 8-week-old tight-skin (TSK/+) mice were used to determine immunoglobulin levels by isotype-specific enzyme-linked immunosorbent assays. Statistical analysis is provided in the Results section. CTL, control.
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CD4+ T cells from patients with SSc is increased.24 33 34 Thus, CD40/CD40L interactions play important roles in the development of autoimmune disorders, including SSc. In conclusion, the present study suggests that CD40/CD40L interactions may be possible targets in the therapy of SSc, although no therapy has been proven effective in suppressing or improving skin sclerosis in controlled studies.

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