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THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 30, Issue of July 26, pp. 27227–27231, 2002
Printed in U.S.A.

Mice Deficient in Nervous System-specific Carbohydrate Epitope HNK-1 Exhibit Impaired Synaptic Plasticity and Spatial Learning*

Received for publication, May 14, 2002
Published, JBC Papers in Press, May 24, 2002, DOI 10.1074/jbc.C200296200

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The HNK-1 carbohydrate epitope, a sulfated glucuronic acid at the non-reducing terminus of glycans, is expressed characteristically on a series of cell adhesion molecules and is synthesized through a key enzyme, glucuronyltransferase (GlcAT-P). We generated mice with a targeted deletion of the GlcAT-P gene. The GlcAT-P −/− mice exhibited normal development of gross anatomical features, but the adult mutant mice exhibited reduced long term potentiation at the Schaffer collateral-CA1 synapses and a defect in spatial memory formation. This is the first evidence that the loss of a single non-reducing terminal carbohydrate residue attenuates brain higher functions.

Glycosylation is a major post-translational protein modification, especially for cell surface proteins, which play important roles in a variety of cellular functions including recognition and adhesion. In the last decade, a number of glycosyltransferase genes and related genes have been cloned. Targeted deletion of these genes revealed the roles of cell surface glycans in the modulation of cellular interactions, particularly in the immune system (1, 2). We have been interested in the roles of a neural-specific carbohydrate, the HNK-1 carbohydrate, which is expressed on glycoproteins as well as on glycolipids and is postulated to be associated with cell adhesion, migration, and neurite outgrowth (3–5). The epitope is a sulfated trisaccharide, HSO_3-3GlcAβ1–3Galβ1–4GlcNAc (6, 7), and the inner structure, Galβ1–4GlcNAc, is commonly found on various glycoproteins and glycolipids. To elucidate the roles of the HNK-1 carbohydrate more clearly, we cloned two different glucuronyltransferases (GlcAT-P and GlcAT-S)[1] (8–11), which are key enzymes in the biosynthesis of the HNK-1 carbohydrate epitope (12, 13). In this study, we generated mice with a targeted deletion of the GlcAT-P gene and demonstrated clearly that the HNK-1 carbohydrate is in fact required for higher functions of the brain.

EXPERIMENTAL PROCEDURES

Targeted Disruption of the GlcAT-P Gene—Cloning of the genomic clones of the 129/Sv mouse GlcAT-P gene was described previously (10). Construction of the targeting vector is schematically represented in Fig. 1A. The neomycin resistance gene cassette in vector pPGKneobpA (14) and diphtheria toxin A (DT-A) gene cassette in vector pMC1DT-A (15) were used as positive and a negative selection markers, respectively. The targeting vector was transfected into E14-1 embryonic stem (ES) cells (16) by electroporation. Two ES clones among 525 tested revealed the desired homologous recombination (13C-2 and 22D-5). To generate chimeric mice, both clones were aggregated with C57BL/6 × BDF1 8-cell-stage embryos, and the embryos were transferred into the uteri of pseudopregnant mice. Both clones gave rise to germline chimeras. Mice heterozygous for the mutation were obtained by cross-breeding of the chimeras with C57BL/6 mice. The heterozygotes were further backcrossed with C57BL/6 mice for more than eight generations, and the resulting heterozygous mutants were interbred to obtain wild-type and homozygous littermates. The genotypes of the mice were determined by PCR and Southern blot analysis of genomic DNA prepared from tail tissue using a 5′-external probe (Fig. 1*B*).

Histochemical Staining—Mice (6 weeks old) were deeply anesthetized by diethyl ether inhalation and then perfused with phosphate-buffered saline followed by 4% paraformaldehyde in phosphate-buffered saline. The mouse brains were postfixed overnight followed by dipping into a 30% sucrose solution. For histological analysis, the sagittal sections (10 μm thick) were prepared, and the sections were stained with 0.5% thionine. For immunofluorescence staining, sections were incubated with the HNK-1 antibody and then incubated with the anti-mouse IgM antibody conjugated with FITC. These sections were visualized and digitized with a Fluoview laser confocal microscope system (Olympus). For double fluorescence staining, coronal sections of 8-week-old GlcAT-P −/− brains were prepared and incubated with biotinylated *Wisteria floribunda* agglutinin (WFA) and the HNK-1 antibody in 5% fetal calf serum. Then reactivity was visualized with rhodamine-avidin

* This work was supported in part by Grants-in-aid for Scientific Research B-12470497 (to T. K.), A-12308043 (to T. M.), and C-13680688 (to S. O.) from the Japan Society for the Promotion of Sciences and Grants-in-aid for Scientific Research on Priority Areas A-12053251 (to T. M.) and A-12033204 (to S. O.), and Special Coordination Funds for Promoting Science and Technology (to T. M.) from the Ministry of Education, Culture, Sports and Technology. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: GlcAT, glucuronyltransferase; GlcA, glucuronic acid; CAM, cell adhesion molecule; NCAM, neural cell adhesion molecule; EPSP, excitatory postsynaptic potential; LTP, long term potentiation; WFA, *W. floribunda* agglutinin; DT-A, diphtheria toxin A; ES, embryonic stem; GABA, γ-aminobutyric acid; $GABA_A$, GABA, type A; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; D-APV, D-(−)-2-amino-5-phosphonovaleric acid.

and a fluorescein isothiocyanate-conjugated secondary antibody followed by examination by laser confocal microscopy.

Electrophysiology—To compare GlcAT-P −/− and +/+ mice, most experiments were performed in a blind fashion, and the results were essentially identical to those of non-blind experiments; so all the data were pooled. 11–20-week-old GlcAT-P −/− and +/+ mice were decapitated under deep halothane anesthesia. Hippocampal slices (400 μm thick) were prepared with a vibratome slicer and placed in a holding chamber for at least 1 h. A single slice was then transferred to the recording chamber and submerged beneath a continuously perfusing medium that had been saturated with 95% O_2, 5%CO_2. The medium comprised 119 mM NaCl, 2.5 mM KCl, 1.3 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1.0 mM NaH_2PO_4, 26.2 mM $NaHCO_3$, and 11 mM glucose. All perfusing solutions contained 100 μM picrotoxin to block $GABA_A$ receptor-mediated inhibitory synaptic responses. Field potential recordings were made with a glass electrode (3 M NaCl) placed in the stratum radiatum. An Axopatch-1D amplifier was used, and the signal was filtered at 1 kHz and digitized at 10 kHz. To evoke synaptic responses, a bipolar tungsten stimulating electrode was placed in the stratum radiatum, and Schaffer collateral/commissural fibers were stimulated at 0.1 Hz. The ratio of excitatory postsynaptic potential (EPSP)/fiber volley amplitude (input-output relationship) of basal synaptic responses was measured in the presence of a low concentration of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (1 μM) to partially block AMPA receptors. This enables more accurate measurement of input-output relationships since the amplitude of EPSPs is usually much greater than that of fiber volleys. Furthermore, the use of a low concentration of CNQX reduces the non-linear summation of field EPSPs, especially when strong stimulus strengths are used. All experiments were performed at 25 °C. The data are expressed as means ± S.E. Student's *t* test was used to determine whether or not there was a significant difference ($p < 0.05$) in the mean between two sets of data.

Behavioral Tests—Behavioral tests were conducted in a blind fashion during the light phase at approximately the same time each day. Data were calculated as means ± S.E. and analyzed by means of one-way analysis of variance and Student's *t* test; *p* values greater than 0.05 were taken as not significant. The Morris water maze test (17) has been used for analysis of spatial navigation and hippocampus-dependent memory formation of rodents. The apparatus consisted of a circular pool (120-cm diameter, 30-cm depth) filled with water at 30 °C. A transparent platform (10-cm diameter) was placed at a fixed location in one quadrant of the pool, 0.5 cm below the surface of the water. Using a video tracking system with computerized data (Target/2, Neuroscience), we analyzed the escape latency (platform search time), path length (the distance traveled to reach the platform), and swimming velocity for each trial. 12–15-week-old mice were trained with blocks of four trials per day for 4 days. The water-filled multiple T-maze was used as another test for evaluating the spatial learning of mice. The apparatus was similar to that described previously (18). The time taken to reach the goal, as the escape latency, was recorded. 17–20-week-old mice were trained through three trials per day for 3 days.

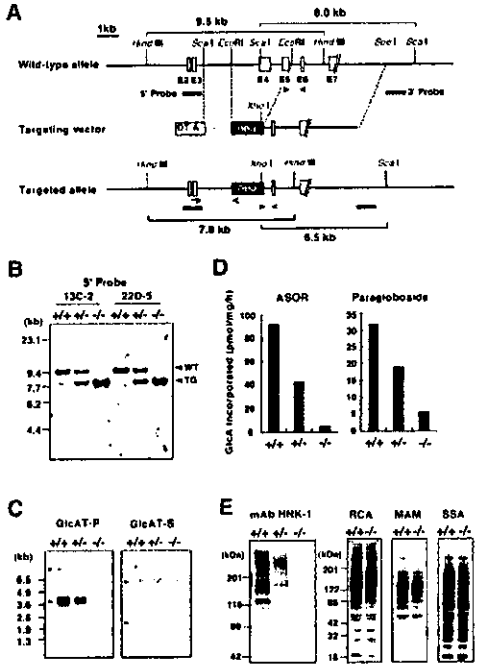

FIG. 1. **Generation and analysis of GlcAT-P −/− mice.** *A*, schematic diagram of the targeting construct. The primers used for the screening of ES cell clones and mice are indicated by *arrows* and *arrowheads*, respectively. The probes used for Southern blot analysis of ES cells and mice are also shown. *B*, Southern blot analysis. Genomic DNAs isolated from mouse tail tissue from GlcAT-P +/+, +/−, and −/− littermates were digested with the combination of *Sca*I and *Xho*I and then hybridized with the ^{32}P-labeled 5′ probe shown in *A*. Two independent lines of GlcAT-P −/− mice were analyzed. *C*, Northern blot analysis. The total RNAs were extracted from the whole brains of 10-week-old littermates and probed with GlcAT-P or GlcAT-S cDNA according to the procedure described previously (8). *Arrowheads* indicate the specific signals. *D*, glucuronyltransferase activity. The Nonidet P-40 extract was prepared from the whole brains of 10-week-old littermates as described previously (13) and was used as the enzyme source. The radioactivity of [^{14}C]GlcA incorporated into asialoorosomucoid (*ASOR*) and paragloboside was measured. *E*, Western and lectin blot analyses. The membrane proteins were prepared from whole brains of 10-week-old littermates. Protein bands were stained with the HNK-1 antibody (*left panel*) or various biotinylated lectins (*right panel*). mAb, monoclonal antibody; MAM, *M. amurensis* mitogen; SSA, *S. sieboldiana* agglutinin; RCA, *R. communis* agglutinin.

RESULTS

Generation of GlcAT-P-deficient Mice—Mice with targeted deletion of the GlcAT-P gene were generated using the strategy outlined in Fig. 1*A*. Most of the catalytic region of GlcAT-P, *i.e.* exons 4 and 5 (10), was replaced by a PGK-neo gene. The disrupted region is essential for the glucuronyltransferase activity (8). The homologous recombination was confirmed by Southern blot analysis using an external 5′ probe (Fig. 1*B*) and 3′ probe (data not shown). Two lines of GlcAT-P −/− mice were generated from two independent ES clones. They gave essentially the same results in all experiments described below. Genotyping of 207 progeny from F1 heterozygous intercrosses suggested that GlcAT-P −/− mice exhibit normal embryonic development. GlcAT-P −/− mice appeared to be normal, and there was no significant difference in body weight or brain size between GlcAT-P −/− and +/+ mice up to 20 weeks after birth. Northern blot analysis of total RNA from 10-week-old mouse brains revealed that the expression of GlcAT-P mRNA was completely abolished in GlcAT-P −/− mice (Fig. 1*C*). In GlcAT-P +/− mice, GlcAT-P mRNA was reduced by ~50%. The same blot probed with the cDNA of GlcAT-S, which is the second glucuronyltransferase involved in the biosynthesis of the HNK-1 carbohydrate (9), indicated that the expression level of GlcAT-S mRNA in GlcAT-P +/− and −/− mice remained as low as that in GlcAT-P +/+ mice (Fig. 1*C*).

GlcAT-P Is Responsible for Biosynthesis of the HNK-1 Carbohydrate on Both Glycoproteins and Glycolipids in Vivo—Since the HNK-1 carbohydrate is expressed on both glycoproteins and glycolipids, the effect of GlcAT-P gene disruption on the overall ability to synthesize the HNK-1 carbohydrate in the brain was examined. As shown in Fig. 1*D*, the glucuronyltransferase activity of GlcAT-P −/− mice toward the glycolipid acceptor (paragloboside) disappeared almost completely as did the activity toward the glycoprotein acceptor (asialoorosomucoid) in 10-week-old mice. The glucuronyltransferase activity in GlcAT-P +/− mice was reduced to about half of that in the GlcAT-P +/+ mice for both acceptor substrates. Thus, GlcAT-P is the most predominant glucuronyltransferase responsible for the biosynthesis of the HNK-1 carbohydrate, and the contribution of GlcAT-S to the HNK-1 carbohydrate biosynthesis is marginal at this age.

GlcAT-P-deficient Mice Lack the HNK-1 Carbohydrate in the Central Nervous System—As shown in Fig. 1*E*, the GlcAT-P −/− mice almost completely lost the HNK-1 carbohydrate as

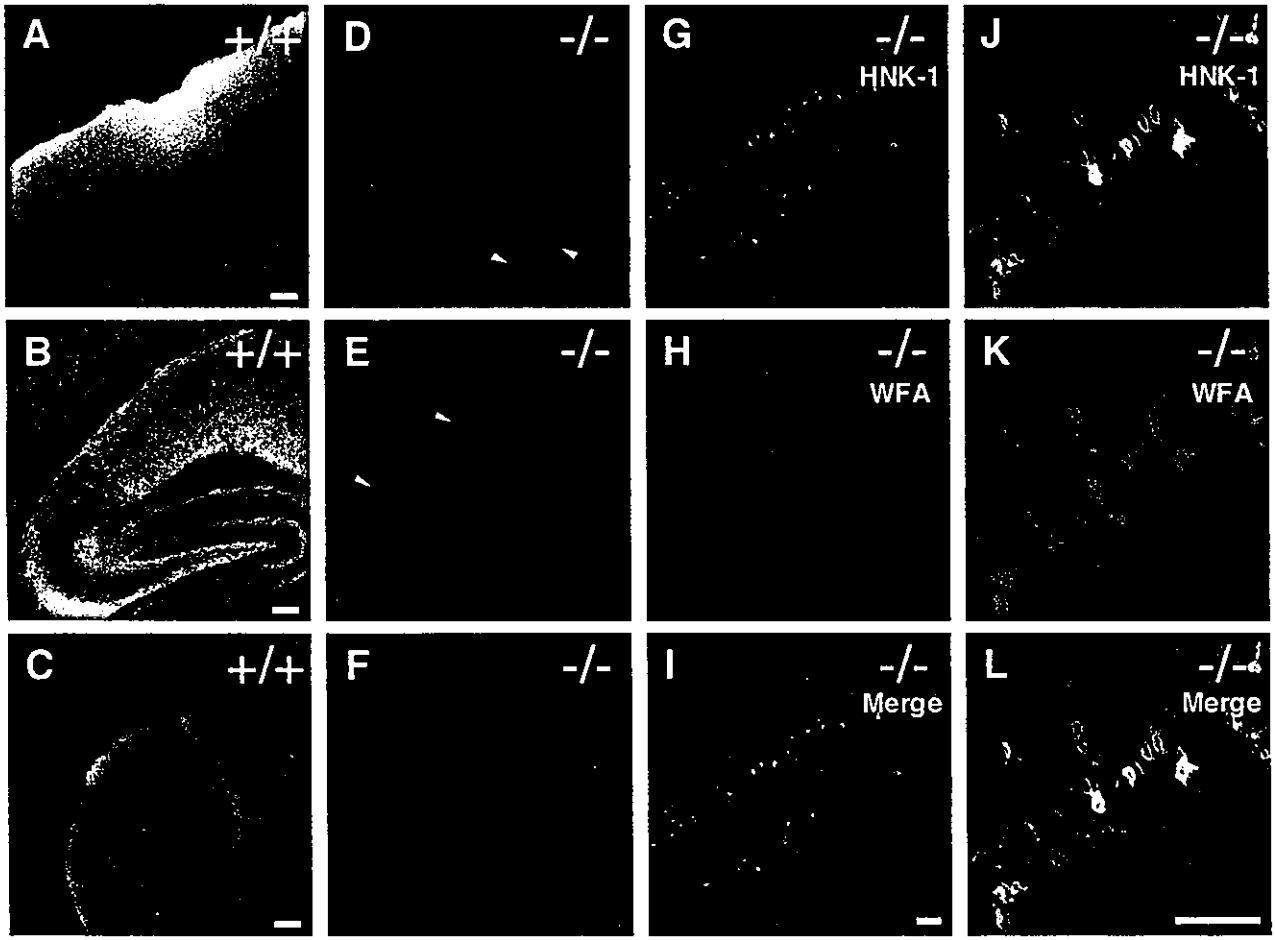

FIG. 2. *A–F*, immunohistochemical analysis of expression of the HNK-1 carbohydrate in mouse brain. Sagittal brain sections from 6-week-old wild-type (+/+, *A–C*) and GlcAT-P-deficient (−/−, *D–F*) mice were immunostained with the HNK-1 antibody. The HNK-1 carbohydrate was markedly diminished in the cerebral cortex (*A* and *D*), hippocampus (*B* and *E*), and cerebellum (*C* and *F*) of GlcAT-P −/− mice. A small amount of remaining HNK-1 carbohydrate was also detected in the cerebral cortex and hippocampus of GlcAT-P −/− mice (*arrowheads* in *D* and *E*). *G–L*, the remaining HNK-1 immunoreactivity in perineuronal nets of GlcAT-P (−/−) mice. Coronal sections of the cerebral cortex of adult GlcAT-P −/− mice were stained doubly with the HNK-1 antibody (*G* and *J*) and biotinylated WFA (*H* and *K*). Equivalent subpopulations of cortical neurons were stained with the HNK-1 antibody and WFA lectin (*I* and *L*). Images of magnified regions are also shown (*J–L*). *Scale bars*, 100 μm.

revealed on Western blot analysis. Several protein bands corresponding to over 100 kDa detected for GlcAT-P +/+ and +/− mice had disappeared in GlcAT-P −/− mice. The intensity of several protein bands in GlcAT-P +/− mice decreased to about half of that in GlcAT-P +/+ mice. Similarly immunohistochemical staining of 6-week-old mouse brains with the HNK-1 antibody revealed high levels of HNK-1 carbohydrate expression widely in the GlcAT-P +/+ mice brains including the cerebral cortex, hippocampus, and cerebellum (Fig. 2, *A–C*). In contrast, the immunoreactivity of the HNK-1 carbohydrate in the GlcAT-P −/− mice was reduced to almost negligible levels in all regions of the nervous system (Fig. 2, *D–F*). These findings clearly indicated that GlcAT-P is the principal enzyme responsible for the biosynthesis of the HNK-1 carbohydrate in the mature brain. There is at least the possibility that the lack of glucuronic acid at the terminus of glycans has an overall effect on the glycan structures in the brain since lectin blot analysis with the MAM (*Maackia amurensis* mitogen), SSA (*Sambucus sieboldiana* agglutinin), and RCA (*Ricinus communis* agglutinin) lectins, which specifically recognize the terminal structures of glycans, *i.e.* Siaα2–3Gal, Siaα2–6Gal, and β-Gal, respectively, exhibited almost indistinguishable profiles in wild-type and mutant mice (Fig. 1*E*). In GlcAT-P −/− mice, non-reducing terminal galactose is presumably substituted by sialic acid, resulting in a common terminal disaccharide, Sia-Gal.

Remaining HNK-1 Carbohydrate in the GlcAT-P −/− Mice—In GlcAT-P −/− mice, the HNK-1 carbohydrate disappeared almost completely as described above. However, further studies revealed that a trace of HNK-1 immunoreactivity remained on the surfaces of soma and proximal dendrites of a subset of neurons in some limited regions (Fig. 2, *D* and *E*, *arrowheads*). Some parts of interneurons were likely to be HNK-1-positive in the cerebral cortex, and such remaining immunoreactivity was not so significant up to 2 weeks after birth but seemed to increase gradually thereafter. These signal patterns and morphological features imply that the remaining HNK-1 carbohydrate in the GlcAT-P −/− mice corresponded to the perineuronal nets, which are known to comprise a lattice-like accumulation of the extracellular matrix on an unidentified subset of neurons (19). This was confirmed by double fluorescence staining of adult GlcAT-P −/− mice with the HNK-1 antibody and an *N*-acetylgalactosamine-binding lectin, WFA, a well known marker of perineuronal nets (19). In the cerebral cortex, both signals were detected in a restricted population of cortical neurons that were observed mainly in layers III–V (Fig. 2, *G–L*), although not all of the WFA-positive neurons were immunoreactive for the HNK-1 antibody (Fig. 2, *I* and *L*). These results revealed that the HNK-1 carbohydrate expressed in the perineuronal net structure was synthesized by an enzyme(s) other than GlcAT-P, presumably GlcAT-S.

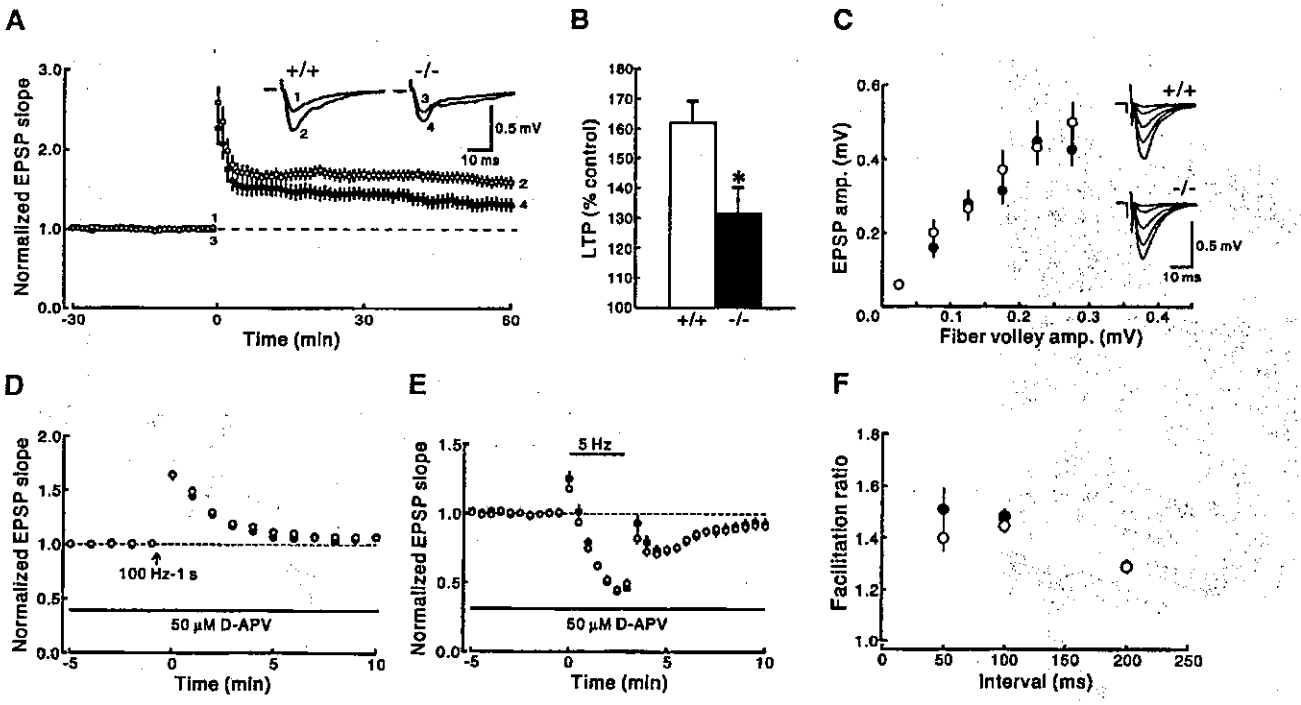

FIG. 3. *A–C*, LTP, but not basal synaptic transmission, is impaired in GlcAT-P −/− mice. *A*, the averaged time course of LTP in GlcAT-P +/+ (*open circles*, $n = 14$) and −/− (*closed circles*, $n = 14$) mice. Initial EPSP slopes were normalized in each experiment to the mean slope value during the control period (−30–0 min). A train of high frequency stimuli (100 Hz for 1s) was applied at time 0. Sample traces of field EPSPs (average of 10 consecutive responses) recorded at the times indicated by the *numbers* are shown in the *inset*. *B*, the summary of LTP calculated as the percent increase in the mean EPSP slope from 50 to 60 min after high frequency stimulation compared with the mean EPSP slope during the control period (−30–0 min). LTP in GlcAT-P −/− mice (*closed bar*) was significantly lower than that in GlcAT-P +/+ mice (*open bar*) ($p < 0.02$, t test). *C*, the input-output relationship of GlcAT-P −/− (*closed circles*) and +/+ (*open circles*) mice. 25 μM D-APV was present to block *N*-methyl-D-aspartate receptor-mediated synaptic responses. A low concentration of CNQX (1 μM) was also present. The data were first sorted by the range of fiber volley amplitudes, and then EPSP amplitudes were averaged within each range. Sample traces with various stimulus strengths are shown in the *inset*. *D–F*, the impairment of LTP in GlcAT-P −/− mice is not mediated by the changes in presynaptic release mechanisms. Analysis of the presynaptic short term plasticity in GlcAT-P +/+ (*open circles*) and −/− (*closed circles*) mice was performed in the presence of 50 μM D-APV. *D*, post-tetanic potentiation recorded in GlcAT-P +/+ ($n = 9$) and −/− ($n = 12$) mice. A train of high frequency stimuli (100 Hz for 1s) was delivered at time 0. *E*, paired-pulse facilitation in GlcAT-P +/+ ($n = 13$) and −/− ($n = 13$) mice. The *ordinate* indicates the ratio of the second field EPSP slope to the first field EPSP slope. With any interstimulus interval (50, 100, 200 ms), no significant difference was observed between the two genotypes. *F*, responses to prolonged low frequency stimulation (5 Hz for 3min) in GlcAT-P +/+ ($n = 12$) and −/− ($n = 13$) mice. *amp.*, amplitude.

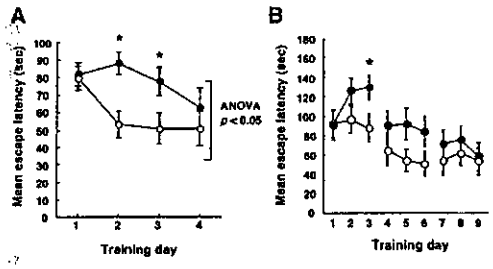

FIG. 4. **Impaired spatial learning in GlcAT-P −/− mice.** GlcAT-P +/+ ($n = 17$, *open circles*) and −/− ($n = 18$, *closed circles*) mice were tested with spatial learning tasks. *A*, mice were trained in a Morris water maze for 4 consecutive days. Learning performance is expressed as the mean escape latency of four trials per day. GlcAT-P −/− mice showed a significantly longer escape latency than GlcAT-P +/+ mice ($p < 0.05$, one-way analysis of variance (*ANOVA*)). *B*, learning performance in a water-filled multiple T-maze. Mice were given one session of three trials per day for 3 consecutive days. The mean escape latency on training day 2 is significantly different between the two genotypes ($p < 0.05$, t test).

Reduced LTP at the Schaffer Collateral-CA1 Synapses—The HNK-1 epitope is commonly expressed on a series of cell adhesion molecules (CAMs) such as the neural cell adhesion molecule (NCAM), L1, telencephalin, and tenascin-R (20). These CAMs are important constituents of the synaptic structure and play important and diverse roles in the regulation of synaptic plasticity. Aberrations of LTP in the hippocampal CA1 region occurred in mice deficient in NCAM and telencephalin (21, 22). Mice lacking extracellular matrix molecule tenascin-R were also shown to exhibit aberrant LTP in the CA1 region (23). With this background, we analyzed LTP in the CA1 region of GlcAT-P +/+ and −/− mice to examine the effect of HNK-1 carbohydrate deficiency on synaptic plasticity (Fig. 3). EPSPs were evoked by stimulating afferent fibers in the stratum radiatum of the CA1 region using the extracellular field potential recording technique. High frequency stimulation of afferent fibers (100 Hz for 1 s) gave rise to LTP of excitatory synaptic transmission in the GlcAT-P +/+ mice (161.7 ± 7.3% of baseline, $n = 14$), while the magnitude of LTP in the GlcAT-P −/− mice was significantly lower (131.9 ± 8.4% of baseline, $n = 14$; $p < 0.02$, t test) than that in the GlcAT-P +/+ mice (Fig. 3, *A* and *B*). In contrast, the initial potentiation after tetanus and the depolarization during tetanic stimulation were similar in magnitude to those in GlcAT-P +/+ mice (data not shown). The input-output relationship of AMPA receptor-mediated synaptic responses in the CA1 region was not significantly different between GlcAT-P +/+ and −/− mice (Fig. 3*C*), suggesting that basal synaptic transmission in GlcAT-P −/− mice is normal. We then examined three forms of presynaptic short term plasticity in the presence of D-(−)-2-amino-5-phosphonovaleric acid (D-APV), which blocks any *N*-methyl-D-aspartate receptor-dependent postsynaptic modification (Fig. 3, *D–F*). The magnitude of post-tetanic potentiation induced by tetanic stimulation (100 Hz for 1s) (Fig. 3*D*), the paired-pulse facilitation induced

by a pair of afferent fiber stimuli at 50-, 100-, or 200-ms intervals (Fig. 3*E*), and the synaptic responses to repetitive afferent fiber stimulation (5 Hz for 3 min) (Fig. 3*F*) were indistinguishable between GlcAT-P +/+ and −/− mice. These results indicated that the presynaptic function is intact in GlcAT-P −/− mice and suggested that presynaptic changes are not involved in the impairment of LTP in GlcAT-P −/− mice.

Impaired Performance in Spatial Learning Tasks in GlcAT-P-deficient Mice—In view of the reduced LTP, two types of spatial learning tests were carried out with 11–15-week-old GlcAT-P +/+ and −/− mice (Fig. 4). In the Morris water maze test, the time taken to reach the hidden platform (escape latency) was significantly longer for the GlcAT-P −/− mice than for the GlcAT-P +/+ mice during 4 days of training (Fig. 4*A*), although the swimming speed in the task was not significantly different between GlcAT-P +/+ and −/− mice (data not shown). In the water-filled multiple T-maze task, the GlcAT-P −/− mice showed increased escape latencies to the goal arm compared with the GlcAT-P +/+ mice, most significantly on day 2 during 3 days of training (Fig. 4*B*). These results suggest that the differences in performance of GlcAT-P −/− mice in the spatial learning tasks were related to impaired spatial learning, not to decreased motor activity.

DISCUSSION

The abnormality in higher brain functions of the GlcAT-P −/− mice, such as reduced LTP at the Schaffer collateral-CA1 synapses and defect in spatial memory formation, was similar to those in NCAM-deficient mice (21, 24), suggesting that the HNK-1 carbohydrate may function through modulation of the functions of CAMs. Besides NCAM, CAMs bearing the HNK-1 carbohydrate, such as telencephalin and tenascin-R, have also been shown to play important roles in the induction and expression of LTP (22, 23). The detailed molecular mechanisms by which the HNK-1 carbohydrate modulates the functions of CAMs are not clear at the moment. However, it should be noted that the HNK-1 carbohydrate on NCAM and L1 was shown to negatively regulate their homophilic binding activity.[2] The HNK-1 carbohydrate may weaken the homophilic interaction of CAMs expressed on the synapses and facilitate synaptic plasticity. It should be noted that only a subpopulation of CAMs express the HNK-1 carbohydrate and that the expression is independently regulated from the biosynthesis of CAMs, indicating that the HNK-1 carbohydrate is the characteristic functional component *in vivo* as a fine tuner that regulates synaptic plasticity or other brain functions. Alternatively the HNK-1 carbohydrate itself may be involved in LTP via interaction with binding proteins (receptors) on the cell surface or in the cell matrix. Several HNK-1 carbohydrate-binding proteins have been identified, such as laminin, selectins, SBP-1, and brevican (25–28). However, the association of these receptors or binding proteins with LTP has not been proved.

Recently Saghatelyan *et al.* (29) reported that a monoclonal antibody that recognizes the HNK-1 carbohydrate decreased perisomatic inhibitory postsynaptic currents and enhanced LTP in acute slices of the mouse hippocampus. This inhibition of perisomatic inhibitory postsynaptic currents by the antibody was also observed in NCAM-deficient mice but not in tenascin-R-deficient mice. The authors suggested that tenascin-R is a carrier molecule for the HNK-1 carbohydrate involved in the regulation of perisomatic inhibition of CA1 pyramidal neurons by GABAergic interneurons (29). In the present study, however, we detected reduced LTP in the presence of 100 μM picrotoxin that blocks the $GABA_A$ receptor-mediated inhibitory synaptic response, demonstrating that a mechanism other than the reduction of GABAergic inhibition of CA1 pyramidal neurons is involved in the attenuation of LTP in GlcAT-P −/− mice.

In the present study, we produced mice deficient in GlcAT-P, a glucuronyltransferase, which is involved in biosynthesis of the HNK-1 carbohydrate. The GlcAT-P −/− mice exhibited gross defects in functions of the nervous system such as LTP in the CA1 region and hippocampus-dependent spatial learning. This is the first study to demonstrate the involvement of a carbohydrate, notably of only a single non-reducing terminal carbohydrate residue, in higher ordered brain functions including learning and memory.

[2] K. Ohtsubo, S. Oka, A. Nakamura, Y. Mitsumoto, S. Yamamoto, K. Ono, J. Lütjohann, M. Schachner, U. Rutishauser, and T. Kawasaki, manuscript in preparation.

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IL-18 contributes to the spontaneous development of atopic dermatitis-like inflammatory skin lesion independently of IgE/stat6 under specific pathogen-free conditions

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Communicated by Charles A. Dinarello, University of Colorado Health Sciences Center, Denver, CO, June 5, 2002 (received for review January 30, 2002)

Atopic dermatitis (AD) is a pruritic inflammatory skin disease. Because IL-18 directly stimulates T cells and mast cells to release AD-associated molecules, Th2 cytokines, and histamine, we investigated the capacity of IL-18 to induce AD-like inflammatory skin disease by analyzing KIL-18Tg and KCASP1Tg, which skin-specifically overexpress IL-18 and caspase-1, respectively. They spontaneously developed relapsing dermatitis with mastocytosis and Th2 cytokine accumulation accompanied by systemic elevation of IgE and histamine. Stat6-deficient KCASP1Tg displayed undetectable levels of IgE but manifested the same degree of cutaneous changes, whereas IL-18-deficient KCASP1Tg evaded the dermatitis, suggesting that IL-18 causes the skin changes in the absence of IgE/stat6. KIL-18Tg and IL-1-deficient KCASP1Tg took longer to display the lesion than KCASP1Tg. Thus, AD-like inflammation is initiated by overrelease of IL-18 and accelerated by IL-1. Our present study might provide insight into understanding the pathogenesis of and establishing therapeutics for chronic inflammatory skin diseases including AD.

Atopic dermatitis (AD) is a common inflammatory skin disease (1, 2), characterized by pruritus, chronic relapsing course, genetic background, and occasional association with high serum levels of IgE. Although the mechanism underlying AD is still elusive, activated T cells, basophils, and mast cells seem to play a crucial role in induction of AD. Allergen-dependent cross-linkage of FcεR on basophils and mast cells activates them to produce Th2-related cytokines, such as IL-4, IL-13, and IL-5 and chemical mediators (3–8), suggesting the importance of antigen (Ag)/Ag-specific IgE in activation of basophils and mast cells (acquired type allergic response). However, we recently demonstrated the alternative, IgE-independent activation pathway (9, 10). IL-18 in the presence of IL-3 directly stimulates basophils and mast cells to produce these mediators in an IgE-independent manner *in vitro* (innate type allergic response) (9). IL-18 is a unique cytokine capable of strongly stimulating both IFN-γ and IL-4 production even in the absence of T cell antigen receptor engagement when it acts on freshly isolated T cells with IL-12 and IL-2, respectively (10–15). This innate style T cell activation is one of the outstanding properties of IL-18. Moreover, administration of IL-18 to normal BALB/c or C57BL/6 mice induces polyclonal IgE production in a $CD4^+$ T cell-, stat6-, and IL-4-dependent manner (10). IL-18, like IL-1β, is stored as biologically inactive precursor form (pro) in various cell types, including macrophages and keratinocytes, and becomes active after cleavage with caspase-1 or caspase-1-like enzyme (16–22). Previously, we showed that KCASP1Tg that overexpress caspase-1 in their keratinocytes spontaneously release biologically active IL-18, produce IgE partly, but obviously depending on the action of IL-18, and manifest chronic dermatitis under specific pathogen-free (SPF) conditions (14, 23). Thus, IL-18 might induce allergic disorders, particularly intrinsic atopic diseases characterized by the absence of elevation of proper Ag-specific IgE (2, 24).

Because caspase-1 also activates other cytokines such as IL-1β, it is important to address whether IL-18-transgenic mice that keratinocyte-specifically overexpress mature IL-18 alone, designated as KIL-18Tg, disclose phenotypes similar to those in KCASP1Tg (23). In addition, IL-18-transgenic mice may allow us to clarify whether IL-18, but not IgE, is responsible for inducing atopic phenotypes in the absence of specific allergen. Here, we showed that both KCASP1Tg and KIL-18Tg developed AD-like skin disease, which is characterized by the presence of high plasma levels of histamine, frequent skin-scratching, and mast cell accumulation in the lesion. However, KIL-18Tg required much longer latency to manifest the cutaneous lesions as compared with KCASP1Tg. IL-1-deficient KCASP1Tg required almost the same incubation time to develop the skin lesion as did KIL-18Tg, suggesting the potent disease-accelerating action of IL-1. In contrast, depletion of IL-18 almost completely abrogated pruritic dermatitis by diminution of mast cell accumulation in the skin as well as plasma histamine levels. In addition, stat6-deficient KCASP1Tg, although containing undetectable levels of IgE in their sera, developed pruritic dermatitis without any delay. Therefore, IL-18 can induce such inflammatory skin lesions without inducing allergen-specific IgE production. These results clearly demonstrate the importance of IL-18-dependent but IgE/stat6-independent atopic skin inflammation and may provide us with a classification of allergic responses into IgE/stat6-dependent acquired type and IL-18-dependent innate type.

Experimental Procedures

DNA Construct and Transgenic Mice. The cDNA-encoding region of the mouse mature IL-18 (25) was ligated into human keratin 14 (K14) promoter and rabbit β-globin intron, a kind gift from T. Tanaka of Kyoto University, by blunt-end ligation (Fig. 1*a*). The linear K14/IL-18 DNA fragment was injected into fertilized eggs of C57BL/6 mice as reported (23). A keratinocyte-specific mature IL-18-transgenic mice line (KIL-18Tg) was established. Of a total of 50 mice, two lines were transgenic for IL-18. Male

Abbreviations: AD, atopic dermatitis; Ag, antigen; SPF, specific pathogen-free; K14, keratin 14; WT, wild type; CD40L, CD40 ligand; PE, phycoerythrin.

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www.pnas.org/cgi/doi/10.1073/pnas.152337799

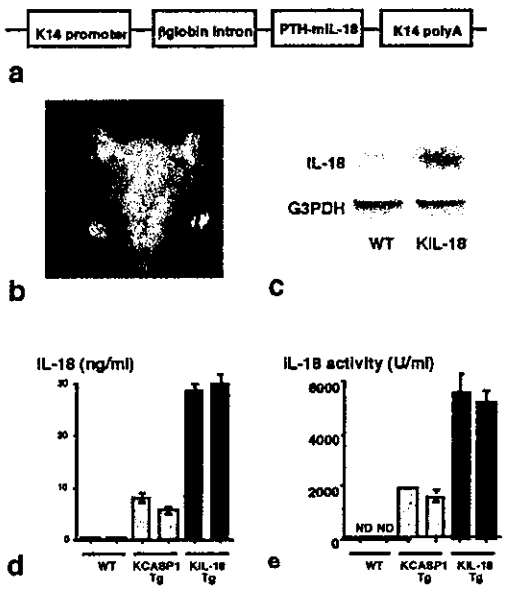

Fig. 1. Spontaneous development of chronic dermatitis in KIL-18Tg. (*a*) Schematic structure of the transgene for IL-18. The mouse mature IL-18 cDNA was ligated to the human keratin 14 promoter to drive its basal keratinocyte-specific expression. The transgene construct also contained the rabbit β-globin intron sequence and the K14 polyadenylation signal to aid in processing the transcript. (*b*) Cutaneous changes in KIL-18Tg. Skin alterations were observed at 24 weeks at the earliest. A result representative of three mice is shown. Similar results were obtained in three independent experiments. (*c*) Northern blotting analysis. Total RNA was extracted from the skin of KIL-18Tg (48 weeks old) or WT. Messenger RNA expressions for human preproPTH (IL-18) that was ligated to cDNA that encodes mature IL-18 and G3PDH were determined by Northern blotting analysis. (*d* and *e*) High serum levels of IL-18 in KIL-18Tg. Sera were sampled from KCASP1Tg, KIL-18Tg, or WT littermates at 36 weeks after birth. IL-18 concentration in each serum was determined by ELISA (*d*) or biological assay (*e*). Data are represented as mean ± SD of triplicates. Similar results were obtained in three independent experiments.

KIL-18Tg were mated with C57BL/6 wild-type (WT) females, and generated KIL-18Tg and WT offspring in a 1:1 ratio. All experiments were performed on mice heterozygous for the transgene compared with nontransgenic WT littermates.

Mice. Transgenic mice that keratinocyte-specifically overexpress human precursor caspase-1 gene containing the same human K14 promoter (female, 4–60 weeks old), designated as KCASP1Tg, were used for this study (23). IL-1α/β-deficient KCASP1Tg were generated by the cross of KCASP1Tg with IL-1α/β double-knockout mice with C57BL/6 background (26). Stat6-deficient KCASP1Tg or IL-18-deficient KCASP1Tg were generated by the cross of KCASP1Tg with stat6-deficient mice (27), and IL-18-deficient mice with C57BL/6 background (28), respectively.

Reagents. FITC-conjugated anti-CD3, phycoerythrin (PE)-conjugated anti-B220, FITC-conjugated anti-CD4, Cy-conjugated anti-CD8, FITC-conjugated anti-Mac-1, PE-conjugated anti-Gr-1, biotinylated anti-CD154 [CD40 ligand (L)], and PE-streptavidin were purchased from PharMingen. Culture medium generally used in this study was RPMI 1640 supplemented with 10% FCS, 100 units/ml of penicillin, 100 μg/ml of streptomycin, 50 μM 2-mercaptoethanol, and 2 mM L-glutamine.

Assay for IL-18 Activity. IL-18 activity in the serum of various mice was determined by using IL-18-responsive NK cell clone named LNK cells according to the method shown elsewhere (22, 29).

Northern Blotting Analysis. Total RNA was extracted from skin of KIL-18 and WT mice by using Isogen reagent (Nippon Gene, Toyama, Japan). Northern blotting analysis was performed by using ^{32}P-labeled cDNAs encoding murine IL-18 or glyceraldehyde-3-phosphate dehydrogense (G3PDH) according to the method described (23).

Flow Cytometry. Thymocytes or spleen cells from various mutant and WT mice were stained with various combinations of mAbs. Stained cells were analyzed by using a dual-laser FACScalibur (Becton Dickinson). Ten thousand cells were analyzed and data were processed with CELLQUEST (Becton Dickinson) (14, 22).

Histological Study. Skin specimens were sampled from various types of transgenic mice, fixed, and stained with hematoxylin and eosin. In some experiments skin specimens were stained with toluidine blue to identify mast cells by their positive metachromasia.

Cell Preparation. Splenic CD4$^+$ T cells were isolated by magnetic cell sorting after incubation with anti-CD4-binding magnetic beads (Miltenyi Biotec, Auburn, CA). Splenic CD4$^+$ T cells (1 × 106 per ml) were incubated in anti-CD3-bound 96-well plates for 48 h. Cytokine concentration in each supernatant was determined by ELISA.

ELISA for Cytokines and Ig. IL-4 and IFN-γ levels were measured by corresponding ELISA kits (Genzyme TECHNE). IL-18 concentration was determined by an ELISA kit from MBL (Nagoya, Japan). Plasma histamine levels were measured by RIA (SRL, Osaka). Serum levels of IgE, IgG1, and IgM were also measured by ELISA according to the methods described (14).

Frequency of Skin-Scratching. Mice were kept calm at least for 1 h, and then monitored by a video camera for 1 h. Frequency of scratching was counted at three distinct time points of 10-min duration as randomly selected.

Score for Skin Alterations. Skin alterations were observed with a week interval. Score was estimated by relative evaluation of skin changes to the maximum skin alterations of each line with individual genetic backgrounds at each time point.

Percent Levels of AD-Associated Parameter. Percent levels of serum IL-18 amounts, serum IgE and IgG1 concentration, plasma concentration of histamine, mast cell numbers in the skin specimens, and skin-scratching frequency observed in various transgenic mice compared with those in KCASP1Tg were calculated.

Results and Discussion

Transgenic Mice Overexpressing Mature IL-18 in Their Skin. Although KIL-18Tg were born normally and were healthy, they eventually developed skin diseases at about 6 months after birth under SPF conditions (Fig. 1*b*). In contrast, KCASP1Tg manifested skin disorders within 8 weeks (23). KIL-18Tg, like KCASP1Tg, frequently scratched their skin, particularly the skin lesion, which will be detailed later (see Fig. 5). Northern blotting analysis revealed the presence of mature IL-18 in the skin of KIL-18Tg (Fig. 1*c*) and 105 bp bigger endogenous IL-18 mRNA encoding proIL-18 in the skin of both WT and KIL-18Tg (data not shown). Like KCASP1Tg (23), KIL-18Tg contained the transgene selectively in the skin, but not in the liver, kidney, colon, lung, brain, or spleen (data not shown).

KIL-18Tg, having had high serum levels of IL-18 even at birth, persistently displayed much higher levels of IL-18 than did KCASP1Tg (Fig. 1*d*). Because their sera induced IFN-γ in LNK cells, a IL-18-responsive cell line (29), IL-18 in the serum of

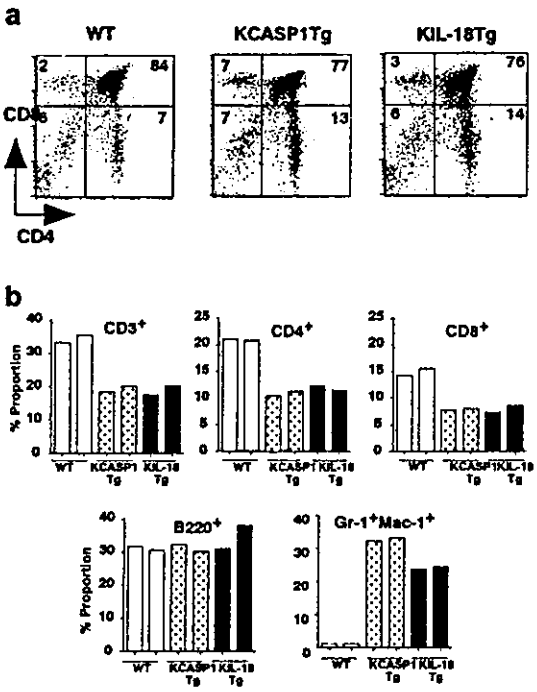

Fig. 2. Increase of splenic neutrophil proportion in both types of transgenic mice. Thymocytes were prepared from KCASP1Tg, KIL-18Tg, and WT littermates at 36 weeks after birth, and their expression of CD4 or CD8 was determined by flow cytometry (*a*). Spleen cells from various genotype mice were stained with PE-anti-B220, FITC-anti-CD3, FITC-anti-CD4 or PE-anti-CD8, or PE-anti-Gr-1 plus FITC-anti-Mac-1, and the percent proportion of each cell type was calculated (*b*). A representative result is shown. Similar results were obtained in three independent experiments.

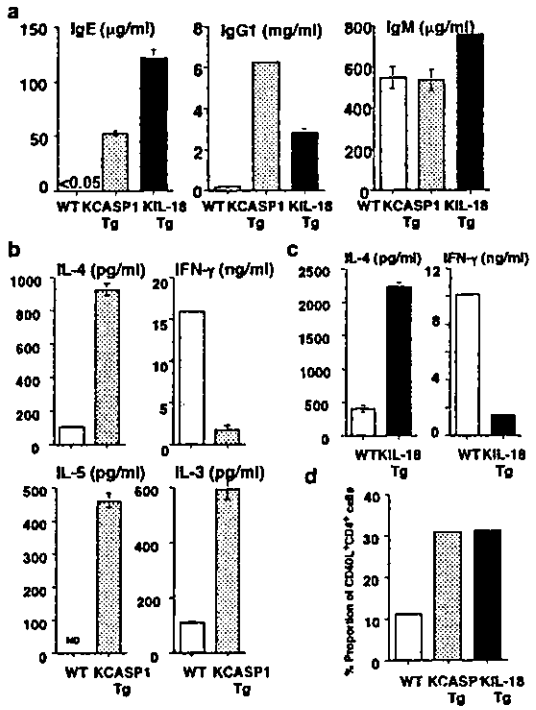

Fig. 3. Spontaneous deviation of splenic CD4+ T cells into Th2 cells. (*a*) High serum levels of IgE and IgG1 in both types of transgenic mice. Sera were sampled from the various types of mice (36 weeks old) and their serum levels of various types of Ig were measured by ELISA. Data are represented as mean ± SD of triplicate cultures. Similar results were obtained in three independent experiments. (*b* and *c*) Spontaneous development of Th2 cells in both types of transgenic mice. Splenic CD4+ T cells from KCASP1Tg or WT littermates at 12 weeks of age (*b*) or from KIL-18Tg or WT littermates at 36 weeks of age (*c*) were isolated by membrane attack complexes and were incubated with immobilized anti-CD3 for 48 h. Concentration of IL-4 and IFN-γ in each supernatant was determined by ELISA. Data are represented as mean ± SD of triplicate cultures. Similar results were obtained in three independent experiments. (*d*) Increase of CD40L-expressing CD4+ T cells in both types of transgenic mice. Spleen cells were isolated from KCASP1Tg, KIL-18Tg, or WT littermate at 36 weeks of age, and their CD40L expression gated on CD4+ cells were identified by flow cytometry. A representative result is shown. Similar results were obtained in three independent experiments. ND, not detected.

KIL-18Tg or KCASP1Tg is biologically active (Fig. 1*e*) (23). Both types of transgenic mice showed high levels of IL-18 in their sera (23). Downstream cytokines of IL-18, such as IL-4, IL-13, and IFN-γ, were not detected in either KCASP1Tg or KIL-18Tg serum by commercially available ELISA kits.

Neutrophil Accumulation and Th2 Deviation in the Spleen of KIL-18Tg and KCASP1Tg. Although both KIL-18Tg and KCASP1Tg showed almost intact T cell development in their thymus (Fig. 2*a*), their splenic lymphocytes showed relatively lower proportion of T cells as compared with WT (Fig. 2*b*). However, CD4+ T cells/CD8+ T cells ratio and proportion of B cells in their spleens seemed to be equal to those in WT (Fig. 2*b*). Proportion of neutrophils determined by Gr-1+ Mac-1+ cells was tremendously elevated in the spleen of KIL-18Tg or KCASP1Tg (Fig. 2*b*), which might be partly because of granulocyte/macrophage colony-stimulating factor, because T cells can produce granulocyte/macrophage colony-stimulating factor in response to IL-18 *in vitro* (30). Before the onset, neutrophil accumulation in the spleen of both types of transgenic mice was only slight, but was strikingly enhanced after the onset (data not shown), suggesting a possible contribution of neutrophils to the development of such cutaneous changes.

Next, we tested whether endogenously accumulated IL-18 in KIL-18Tg resulted in high-serum IgE/IgG1 and dominant Th2 response as did in KCASP1Tg (14). Consistently with previous reports (14, 15), both IgE and IgG1 levels were markedly elevated in KIL-18Tg and KCASP1Tg (Fig. 3*a*). We measured the capacity of freshly isolated splenic CD4+ T cells to produce cytokines in response to immobilized anti-CD3. CD4+ T cells from KCASP1Tg produced much more amounts of IL-3, IL-4, and IL-5 but smaller amounts of IFN-γ as compared with WT (Fig. 3*b*). CD4+ T cells from KIL-18Tg also produced greater amounts of IL-4 but less IFN-γ (Fig. 3*c*). We simultaneously examined whether their CD4+ T cells express CD40L, a critical molecule required for IgE production by B cells (31, 32). A 3-fold increase in the proportion of CD4+ T cells expressing CD40L was observed in both types of transgenic mice (Fig. 3*d*). These results indicate that IL-18 induces IgE/IgG1 response by causing CD4+ T cells to develop into Th2 cells and to express CD40L under SPF conditions.

Chronic Dermatitis in KIL-18Tg and KCASP1Tg. We next investigated the histology of AD-like skin lesions. KCASP1Tg showed remarkable skin alternations at 8 weeks, which started as severe erosive dermatitis, followed by reepithelization and lichenoid changes (Fig. 4; ref. 23). Finally, they developed prominent acanthosis and papillomatosis with intercellular edema and parakeratotic scale-crust in their epidermis (Fig. 4). In contrast,

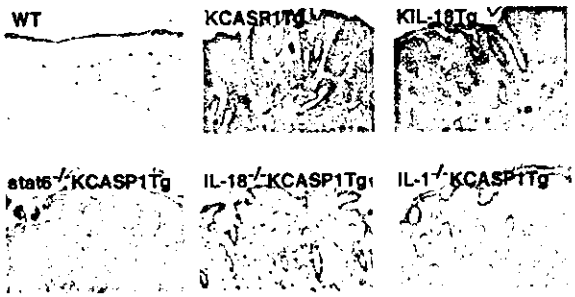

Fig. 4. AD-like inflammatory skin disease in KCASP1Tg and KIL-18Tg. Skins were sampled from WT (20 weeks old), KCASP1Tg (24 weeks old), KIL-18Tg (36 weeks old), stat6-deficient KCASP1Tg (24 weeks old), IL-18-deficient KCASP1Tg (24 weeks old), or IL-1 α/β-deficient KCASP1Tg (24 weeks old). The skin specimens were stained with hematoxylin and eosin. [Original magnification, ×100 (KCASP1Tg, stat6-deficient KCASP1Tg, IL-18-deficient KCASP1Tg, IL-1-deficient KCASP1Tg); ×400 (WT, KIL-18Tg).] Data are representative of three specimens sampled from two to five various mutant mice. The similar results were obtained in two independent experiments.

KIL-18Tg showed later onset of skin lesions, which started as focal skin alterations around their eyes but gradually extended to their face, head, and trunk (Figs. 1*b* and 5*a*). Skin alterations of KIL-18Tg slightly differed from those of KCASP1Tg and showed the presence of marked lichenification without scarring. Their epidermis is obviously acanthotic, although their dermis is severely infiltrated with lymphocytes and polymorphonuclear cells, such as neutrophils (Fig. 4; data not shown).

Mast cell number is remarkably and preferentially increased in the dermal infiltrates (Table 1, Fig. 5*b*) but not in the spleen, lungs, peripheral blood, or intestine (data not shown) of KCASP1Tg or KIL-18Tg, suggesting the contribution of local production of IL-18 to this mast cell accumulation. Because mast cells play a critical role in induction of skin-itching, we counted frequency of skin-scratching. After development of dermatitis, KIL-18Tg and KCASP1Tg much more frequently scratched their skin including the erosive lesion than did WT (Table 1, Fig. 5*b*). However, before onset of skin changes, both types of transgenic mice showed almost comparable frequencies of skin-scratching as did WT (data not shown). We showed previously that IL-18 stimulates $CD4^+$ T cells to produce IL-3, which together with IL-18 activates basophils and mast cells to release histamine *in vitro* (9, 30). Therefore, we measured plasma histamine levels. Histamine levels were obviously elevated in both types of transgenic mice (Table 1, Fig. 5*b*), suggesting IL-18-dependent activation of mast cells in the dermal infiltrates of both types of transgenic mice. Histamine concentration in KCASP1Tg is much higher than that in KIL-18Tg (Table 1, Fig. 5*b*), possibly reflecting the fact that mast cell numbers in KCASP1Tg were larger than those in KIL-18Tg (Table 1, Fig. 5*b*). These results suggested that their skin lesions might be intimately associated with itching and that IL-18 may cause the inflammatory cutaneous disease by activation of cutaneous mast cells, and possibly of lymphocytes and neutrophils.

IgE/stat6-Independent but IL-18-Dependent Pruritic Dermatitis. It is well established that allergen-specific IgE plays a critical role in hay fever or other allergic disorders (33). However, involvement of allergen-specific IgE in AD is not well defined, and involvement of Th1 cytokines has been suggested (34). KCASP1Tg and KIL-18Tg spontaneously develop inflammatory skin lesion with high concentration of IgE even without obvious immunization or infection, prompting us to examine the possibility that polyclonal IgE in these transgenic mice is not involved in the development

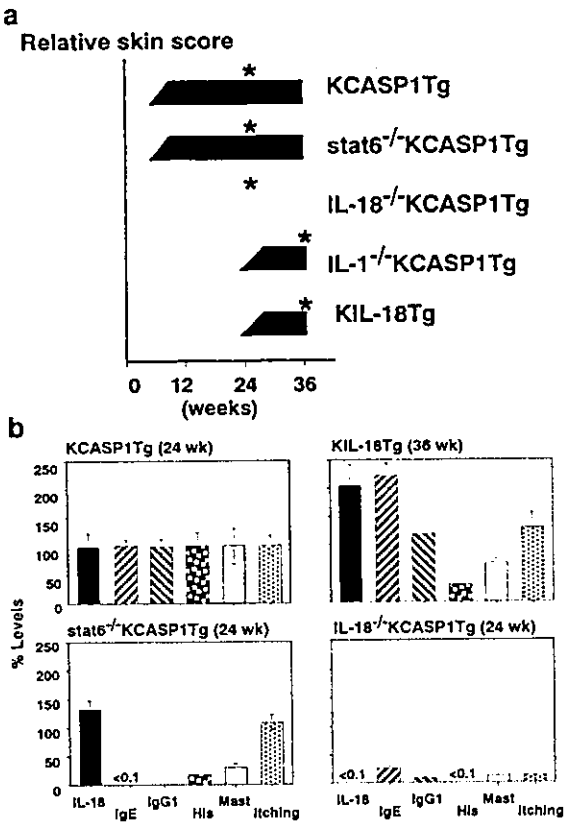

Fig. 5. IL-18-dependent, but stat6-independent pruritic dermatitis. (*a*) IL-18 is an initiating and IL-1, promoting factor for AD-like dermatitis. KCASP1Tg ($n = 5$), stat6-deficient KCASP1Tg ($n = 2$), IL-18-deficient KCASP1Tg ($n = 2$), or IL-1α/β-deficient KCASP1Tg ($n = 2$) and KIL-18Tg ($n = 5$) were kept under SPF conditions. Their skin alterations were scored at a week interval. A representative result of two to five mice in each experimental group is shown. Asterisks indicate the time points at which the indicated experiments (*b*; Table 1) were performed. The similar results were obtained in two independent experiments. ND, not detected. (*b*) Elimination of AD-associated parameters in IL-18-deficient KCASP1Tg. Percent levels of individual parameters in each experimental group compared with those in the KCASP1Tg group as shown in Table 1 were calculated. Data are represented as percent mean ± percent SD of each experimental group.

of these skin alterations. To address this possibility and to take advantage of prompt development of the inflammatory skin alterations, we constructed KCASP1Tg without IgE production by depletion of stat6 gene. As reported, stat6-deficient KCASP1Tg containing high serum levels of IL-18 showed no detectable levels of IgE and very little IgG1 (Table 1, Fig. 5*b*; ref. 14). Furthermore, these mice showed significant reduction in the number of accumulated mast cells and plasma level of histamine as compared with KCASP1Tg (Table 1, Fig. 5*b*). Defects in Th2 cell development, IL-4 production, and IL-4 signaling in stat6-deficient KCASP1Tg may result in such reduction in mast cell accumulation and plasma histamine levels, because IL-4 is an important mast cell growth factor (35, 36). Nevertheless, the cutaneous changes in stat6-deficient KCASP1Tg started almost at the same time as did those in KCASP1Tg (Fig. 5*a*). These results suggest that high levels of IL-18 are principally responsible for inducing skin alterations associated with itching. To prove this possibility, we crossed KCASP1Tg with IL-18-

Table 1. Characteristics of pruritic chronic dermatitis in KCASP1Tg

	IL-18, ng/ml	IgE, μg/ml	IgG1, μg/ml	Histamine, nM	Mast cell no., per 5 fields	Skin-scratching, per 10 min
WT	0.12 ± 0.05	ND	78 ± 51	ND	3.5 ± 1.3	11 ± 7
KCASP1Tg	9.5 ± 1.7	33.2 ± 4.1	6,178 ± 1,325	1,272 ± 257	56.3 ± 11.3	385 ± 48
KIL-18Tg	19.3 ± 3.6	73.5 ± 16.3	6,812 ± 2,133	360 ± 66	38.3 ± 4.3	500 ± 92
Stat6$^{-/-}$KCASP1Tg	12.5 ± 1.88	ND	2 ± 0.3	150 ± 37	16.9 ± 3.3	416 ± 84
IL-18$^{-/-}$KCASP1Tg	ND	8.6 ± 2.6	1,347 ± 266	ND	4.7 ± 2.3	23 ± 5

Serum, plasma, and skin specimens were sampled from five WT (20 weeks old), five KCASP1Tg (24 weeks old), five KIL-18Tg (36 weeks old), two stat6-deficient (stat6$^{-/-}$) KCASP1Tg (24 weeks old), or two IL-18-deficient (IL-18$^{-/-}$) KCASP1Tg (24 weeks old). IL-18, IgG1, and IgE serum levels were measured by ELISA. Plasma histamine levels were determined by RIA. Mast cell numbers were counted in the distinct five fields of skin specimens from various transgenic mice after being stained with toluidine blue at high magnification (×400). Various transgenic mice were kept under calm conditions and were recorded by a video camera for 60 min. Skin-itching was evaluated by skin-scratching frequency counted from three scenes (10 min) selected at random. Data are shown as mean ± SD (of triplicate culture) of two to five samples in each experimental group. ND, not detected.

deficient mice. Resultant IL-18-deficient KCASP1Tg showed neither frequent skin-scratching nor manifestation of dermatitis even at 6 months, although had low, but significant levels of IgE and IgG1 in their sera (Fig. 5). These results allowed us to conclude that IL-18 is principally responsible for inducing skin lesions in KCASP1Tg mice independently of elevation of IgE/IgG1.

Because IL-18-deficient KCASP1Tg showed striking diminution in the number of cutaneous mast cells and the levels of serum histamine (Table 1, Fig. 5*b*), IL-18, but not IgE, might account for the development of the inflammatory dermatitis by activation of mast cells and T cells. IL-18 acts on T cells and mast cells to produce IL-3 and IL-4 (9, 14, 30), which in combination might play an important role in mast cell accumulation. A significant relationship exists between reduction in mast cell number and histamine level ($P < 0.05$), although no obvious correlation exists between histamine levels or mast cell numbers and manifestation of itching. These results may indicate the presence of the threshold of histamine level that is required for induction of skin-itching. We suspect that KIL-18Tg and stat6-deficient KCASP1Tg have higher amounts of histamine than this threshold. Indeed, they displayed still much higher levels of mast cell numbers and histamine levels as compared with WT and also developed pruritic skin alterations (Figs. 4 and 5*b*). However, this finding does not deny the possibility that other factor(s), such as leukotrienes (37), might be involved in the development of skin-itching. Alternatively, IL-18-driven IL-4/IL-13 and/or some other factors might activate mast cells and/or other inflammatory cells to induce itching independently of stat6. We need further study to clarify the role of stat6-independent action of IL-4/IL-13 in this skin disease.

IL-1, as an Accelerator for AD. KIL-18Tg took much longer to develop their cutaneous alterations than did KCASP1Tg. Although KIL-18Tg displayed higher levels of serum IL-18 than did KCASP1Tg (Fig. 1*d*), KIL-18Tg started to manifest skin changes much later than KCASP1Tg (Fig. 5*a*), which led us to propose the possibility that IL-1β, another product of keratinocytes overexpressing caspase-1 (23), might be involved in the reduction of the incubation time. To investigate this possibility, we crossed KCASP1Tg with IL-1α/β double knockout mice (26). IL-1α/β-deficient KCASP1Tg, like KIL-18Tg, showed late onset of the skin alterations (Fig. 5*a*), suggesting that IL-1 might play a promoting role in the development of these atopic changes. The histopathological changes of IL-1α/β-deficient KCASP1Tg skin lesions are similar to those changes of KIL-18Tg but milder than those changes of KCASP1Tg (Fig. 4). Therefore, IL-1 overrelease seems to play a role in accelerating the skin alterations initiated by abnormally accumulated IL-18.

In this study we demonstrate that high accumulation of IL-18 is primarily responsible for inducing allergen/allergen-specific IgE-independent AD-like inflammatory skin disease in these transgenic mice. They frequently scratch their skin including the lesion and display high plasma levels of histamine, suggesting pruritic cutaneous changes (Fig. 5*b*). Like AD patients, their skin changes start to appear in their face and then expand to their trunks and extremities. All of the KCASP1Tg and KIL-18Tg early or lately suffer from the skin disease. They have high serum levels of IgE (Figs. 3 and 5*b*). Thus, the skin disease in both types of transgenic mice seems to fulfill the criteria of AD, although few eosinophils are found in the skin lesion (Fig. 4). Both types of transgenic mice are at least mouse models for inflammatory skin disease. To date, it is believed that allergen-specific IgE is essential to induce atopic phenotypes (4, 5, 9). However, our transgenic mice spontaneously develop AD-like inflammatory dermatitis without encountering with specific allergen (Figs. 1*b*, 4, and 5), suggesting that their polyclonal IgE and IgG1 play a minor role in activation of mast cells to produce the inflammatory changes in their skin. In fact, stat6-deficient KCASP1Tg that had very low levels of IgE and IgG1 in their sera still developed pruritic dermatitis similar to that in KCASP1Tg (Figs. 4 and 5). These results strongly suggest that neither IgE nor IgG1 is involved in the development of the cutaneous pathological changes in KCASP1Tg, although Ag-specific IgG1 as well as IgE are profoundly involved in the occurrence of anaphylaxis (38, 39). In human cases, a subgroup of AD patients exists who have low levels of IgE (2, 24). The depletion of the IL-18 gene in KCASP1Tg resulted in elimination of pathological cutaneous alterations (Fig. 4). These results strongly indicated that overrelease of IL-18 in the skin gives rise to the inflammatory cutaneous changes independently of IgE or stat6-mediated signaling. Thus, measurement of serum IL-18 levels may be important in the cases of AD. However, it apparently is more informative to measure serum levels of both IL-18 and IL-12 in patients with AD to determine their therapeutics whether against IgE or IL-18, because IL-18 shows IFN-γ-inducing activity in the presence of IL-12 (10).

Transgene-encoding human caspase-1 or active form murine IL-18 was selectively expressed in keratinocytes of KCASP1Tg and KIL-18Tg, respectively, under control of keratin 14 (ref. 23 and data not shown). Although the transgene is locally expressed in the skin but not in other tissues including spleen (23; data not shown), splenic CD4$^+$ T cells of these mutant mice spontaneously developed into Th2 cells under SPF conditions (Fig. 3 *b* and *c*). This development may be partly attributable to systemic elevation of IL-18 (Fig. 1 *d* and *e*), which has potential to induce Th2 cell development upon occasional exposure to intrinsic Ag, such as bacterial flora (10, 14). Alternatively, cutaneous dendritic cells might become Th2 cell-driving antigen-presenting cells in the circumstances of high IL-18 concentration and might migrate into peripheral immune organs including the spleen to participate in Th2 cell development. Further study is required.

After development of the cutaneous alterations, both KCASP1Tg and KIL-18Tg increased neutrophil numbers in their spleens (Fig. 2*b*). Comparable levels of neutrophil accumulation were observed in the skin lesions of stat6-deficient and normal KCASP1Tg (data not shown). Indeed, IL-18 has capacity to induce proliferation of neutrophils by induction of granulocyte/macrophage colony-stimulating factor (30), to activate neutrophils directly (40), and possibly to recruit neutrophils by induction of neutrophil chemotactic factor, such as IL-8 in human (41). Activated neutrophils might participate in the development of inflammatory skin changes in these mutant mice by releasing various potent effector molecules (42).

Induction and/or activation of AD are strongly influenced by bacterial infection (1, 2). Some microbe products induce IL-18 secretion by activation of Toll-like receptors, recently identified signaling receptors that specifically recognize microbe-derived molecules, leading to IL-18-dependent AD-like skin lesions (21). In fact, macrophages secrete IL-18 upon infection with various microbes including bacteria, virus, and protozoa (2, 43, 44). We are now investigating the microbe products that induce IL-18 release from keratinocytes.

We thank Dr. H. Tahara at the Institute of Medical Science (University of Tokyo) for providing us with cDNA of murine mature IL-18, Drs. S. Ootake and H. Tsumura (Mie University) for helpful technical assistance, and Dr. E. V. Fuchs (University of Chicago) for providing us with K14 promoter DNA. This work was supported in part by grants, grants-in-aid, and Hitec Research Center grant from the Ministry of Education, Science and Culture, Japan, and a grant-in-aid from the Mie Medical Research Foundation.

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Immunity, Vol. 17, 375–387, September, 2002, Copyright ©2002 by Cell Press

Antigen-Specific T Cell Sensitization Is Impaired in IL-17-Deficient Mice, Causing Suppression of Allergic Cellular and Humoral Responses

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Summary

Interleukin-17 (IL-17) is a proinflammatory cytokine produced by T cells. The involvement of IL-17 in human diseases has been suspected because of its detection in sera from asthmatic patients and synovial fluids from arthritic patients. In this study, we generated IL-17-deficient mice and investigated the role of IL-17 in various disease models. We found that contact, delayed-type, and airway hypersensitivity responses, as well as T-dependent antibody production, were significantly reduced in the mutant mice, while IL-17 deficiency of donor T cells did not affect acute graft-versus-host reaction. The results suggest that impaired responses were caused by the defects of allergen-specific T cell activation. Our findings indicate that IL-17 plays an important role in activating T cells in allergen-specific T cell-mediated immune responses.

Introduction

IL-17 is a T cell-derived proinflammatory cytokine originally named cytotoxic T lymphocyte-associated serine esterase-8 (CTLA-8) (Rouvier et al., 1993). Murine IL-17 is a 21 kDa glycoprotein, consisting of 147 amino acids, which has a 63% amino acid homology with human IL-17 (155 amino acids) (Yao et al., 1995b). It has no obvious homology with other cytokines but has a 57% homology with the predicted amino acid sequence of the open reading frame 13 (ORF13) of *Herpesvirus saimiri* (HVS) (also called vIL-17) (Rouvier et al., 1993).

Recently, human IL-17 has been found to be included in a novel cytokine family consisting of IL-17B, IL-17C, IL-17D, IL-17E, and IL-17F in addition to IL-17 and vIL-17; their identity with IL-17 is 16%–50% (Aggarwal and Gurney, 2002). A receptor for IL-17 (IL-17R) has been identified and found to share no homology with any other cytokine receptor family. Its mRNA expression shows a ubiquitous tissue distribution (Yao et al., 1995a). Although IL-17R has no obvious motifs in the intracellular domain, its signal transduction is shown to be mediated by tumor necrosis factor-associated factor 6 (TRAF6) but not by TRAF2 (Schwandner et al., 2000).

IL-17 is produced by TCRα/β$^+$CD4$^-$CD8$^-$ thymocytes, as well as activated CD4$^+$ and CD4$^+$CD45RO$^+$ memory T cells (Yao et al., 1995b). Although activated CD8$^+$ and CD8$^+$CD45RO$^+$ memory T cells are also known to produce IL-17 in humans (Shin et al., 1999), its expression is restricted to CD4$^+$ T cells in mice (Infante-Duarte et al., 2000). The expression in Th1 and Th2 cells seems to be different depending upon the conditions. While Aarvak et al. reported that IL-17 is produced by Th1/Th0 cells but not by Th2 cells, Albanesi et al. reported that both Th1 and Th2 cell clones from human skin-derived nickel-specific T cells produced IL-17 (Aarvak et al., 1999; Albanesi et al., 2000). On the other hand, IL-17 is produced by T cells expressing TNF-α but not by Th1 or Th2 cells in mice (Infante-Duarte et al., 2000).

IL-17 has pleiotropic activities including induction of TNF-α, IL-1β, IL-6, IL-8, G-CSF, and MCP-1 on various types of cells (Fossiez et al., 1998; Jovanovic et al., 1998), upregulation of ICAM-1 and HLA-DR expression on kerationocytes (Albanesi et al., 1999), induction of *iNOS* and cyclooxygenase-2 (*cox-2*) on chondrocytes (Shalom-Barak et al., 1998), induction of cox-2-dependent PGE2-mediated osteoclast differentiation factor (ODF) expression on osteoblasts (Kotake et al., 1999), and promotion of SCF- and G-CSF-mediated granulopolesis (Schwarzenberger et al., 2000). IL-17 acts on T cells as a costimulatory factor (Yao et al., 1995a), enhances allorejection via promotion of dendritic cell (DC) maturation (Antonysamy et al., 1999), and promotes tumor rejection by activation of NK cells (Hirahara et al., 2000). IL-17 has also been detected in the sera as well as the diseased organs and tissues of various patients, suggesting its involvement in the development of various human diseases such as rheumatoid and Lyme arthritis and osteoarthritis (Attur et al., 1997; Aarvak et al., 1999; Infante-Duarte et al., 2000), multiple sclerosis (Matusevicius et al., 1999), systemic lupus erythematosus (Wong et al., 2000), allograft rejection (Antonysamy et al., 1999), and asthma (Wong et al., 2001). Furthermore, IL-17 has been shown to be involved in the host defense mechanism against *Klebsiella pneumoniae* infection by using IL-17R$^{-/-}$ mice (Ye et al., 2001). Neither the exact pathophysiologic role of IL-17 nor its mechanisms of action in the immune system, however, have been completely elucidated.

In this study, we generated IL-17-deficient (IL-17$^{-/-}$) mice in order to elucidate the roles of IL-17 in various inflammatory diseases and immune responses. Using this model, we have shown that IL-17 is involved in diseases such as contact hypersensitivity (CHS), delayed-type hypersensitivity (DTH), and airway hypersen-

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