Bisecting GlcNAc structure is implicated in suppression of stroma-dependent haemopoiesis in transgenic mice expressing *N*-acetylglucosaminyltransferase III

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Several sugar structures have been reported to be necessary for haemopoiesis. We analysed the haematological phenotypes of transgenic mice expressing β -1,4 *N*-acetylglucosaminyltransferase III (GnT-III), which forms bisecting *N*-acetylglucosamine on asparagine-linked oligosaccharides. In the transgenic mice, the GnT-III activity was elevated in bone marrow, spleen and peripheral blood and in isolated mononuclear cells from these tissues, whereas no activity was found in these tissues of wild-type mice. Stromal cells after long-term cultures of transgenic-derived bone marrow and spleen cells also showed elevated GnT-III activity, compared with an undetectable activity in wild-type stromal cells. As judged by HPLC analysis, lectin blotting and lectin cytotoxicity assay, bisecting GlcNAc residues were increased on both blood cells and stromal cells from bone

marrow and spleen in transgenic mice. The transgenic mice displayed spleen atrophy, hypocellular bone marrow and pancytopenia. Bone marrow cells and spleen cells from transgenic mice produced fewer haemopoietic colonies. After lethal irradiation followed by bone marrow transplantation, transgenic recipient mice showed pancytopenia compared with wild-type recipient mice. Bone marrow cells from transgenic donors gave haematological reconstitution at the same level as wild-type donor cells. In addition, non-adherent cell production was decreased in long-term bone marrow cell cultures of transgenic mice. Collectively these results indicate that the stroma-supported haemopoiesis is compromised in transgenic mice expressing GnT-III, providing the first demonstration that the N-glycans have some significant roles in stroma-dependent haemopoiesis.

INTRODUCTION

Haemopoietic stem cell – stromal cell interactions are necessary for the growth and differentiation of haemopoietic cells and the distribution of blood cells into haemopoietic tissues [1]. Lectin-like molecules on the surface of blood cells and their complementary ligands (i.e. specific sugar chain structures) participate in several important roles of the interaction with stromal cells in haemopoietic tissues. In the homing process, lymphocytes adhere to high endothelial venules, which partly involves the interaction of carbohydrates on the endothelial cells with LEC-CAM, a surface lectin-like molecule on the lymphocytes [2]. In inflamed tissues, LEC-CAMs mediate the rolling of leucocytes on the cytokine-activated endothelial cells, an initial response for leucocytes to concentrate in the inflammation [3]. With regard to the adhesion to bone marrow stromal cells, mannose and galactose are responsible for haemopoietic cell – stromal cell interaction and the homing of haemopoietic stem cells to the bone marrow [4]. Granulocytic cell adhesion to haemonectin, one of the extracellular matrix proteins in the bone marrow, is mediated by mannose and galactose [5]. In contrast, mannose and fucose block the attachment of lymphocytes to endothelial cells [6].

In the biosynthesis of asparagine-linked sugar chains (N-glycans), β -1,4 *N*-acetylglucosaminyltransferase III (GnT-III;

EC 2.4.1.144) catalyses the transfer of GlcNAc to the core mannose at the β -1,4 position to form bisecting GlcNAc [7], as shown in Scheme 1. Our previous investigation showed that the metastatic potential of the B16 murine melanoma cells is decreased by the introduction of a GnT-III gene, indicating the anti-metastatic effect of GnT-III [8]. Moreover, expression of GnT-III is found to suppress the expression of hepatitis B virus in human hepatoma cell line HB611, indicating the anti-viral effect of bisecting GlcNAc [9]. Recently we reported that glycosylation by GnT-III enhances E-cadherin-mediated cell–cell binding to suppress metastasis of B16 melanoma cells [10]. These findings have provided evidence that bisecting GlcNAc structures are able to modify several biological events.

In haematological tissues, GnT-III activity is undetectable in the mononuclear cells isolated from peripheral blood and bone marrow of healthy individuals [11]. In mice, GnT-III is almost undetectable in haemopoietic tissues such as spleen and bone marrow [12]. In haematological malignancies, elevated GnT-III activity is observed in only two diseases: chronic myelogenous leukaemia in blast crisis and multiple myeloma [11]. These findings indicate that the expression of the GnT-III gene is strictly regulated to function at a limited stage of haemopoiesis. As mentioned above, several oligosaccharides seem to be involved in the interaction between blood cells and stromal cells, but it has

Abbreviations used: GnT, β -1,4 *N*-acetylglucosaminyltransferase; E-PHA, erythroagglutinating phytohaemagglutinin; L-PHA, leucoagglutinating phytohaemagglutinin; Con A, concanavalin A; FCS, fetal calf serum; CFU-GM, colony-forming units – granulocyte and macrophage; BFU-E, burst-forming units – erythroid; CFU-mix, multilineage colony-forming units; 2-PA, 2-aminopyridine.

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Scheme 1 Reactions catalysed by GnT-I and GnT-III

not been clarified whether haemopoiesis is affected by a specific sugar chain structure. In this study we established transgenic mice expressing GnT-III to elucidate the effect of bisecting GlcNAc structures on haemopoiesis.

MATERIALS AND METHODS

Reagents

Erythroagglutinating phytohaemagglutinin (E-PHA) lectin, leucoagglutinating phytohaemagglutinin (L-PHA) lectin and concanavalin A (Con A) lectin were purchased from EY Laboratories Inc. (San Metro, CA, U.S.A.). Recombinant human erythropoietin was supplied by Chugai Pharmaceutical Co. Ltd. (Tokyo, Japan). Recombinant murine interleukin 3, stem cell factor and recombinant human interleukin 6 were obtained from Kirin Company (Tokyo, Japan).

GnT-III expression vector

The expression vector pCAGGS contains a human actin promoter fused to a fragment of the human β -globin gene and polyadenylation signals (see Figure 1A). A plasmid containing a full-length GnT-III cDNA [13] was constructed as described [8] and termed Act-III.

Establishment of transgenic mice

The BDF1 mice used in this investigation were purchased from SLC Co. (Shizuoka Japan) and maintained under specific-pathogen-free conditions. After the microinjection of *SalI*-linearized Act-III into the pronuclei of BDF1-derived zygotes, the zygotes were introduced into the ampullae of a pseudo-pregnant BDF1 mouse, which gave birth to litters of 10–14 mice 21 days later. Newborn mice were genotyped by Southern blotting of *EcoRI*-digested genomic DNA isolated from tail tissues with a DNA fragment containing the coding region of the GnT-III gene as a probe. Transgene-positive F_1 mice were backcrossed with wild-type BDF1 mice to generate stable lines. Subsequently, transgene-positive mice were identified by PCR with genomic DNA from mice as templates and confirmed by Southern blotting of PCR products with the same probe as above. The PCR conditions were as follows: 94 °C for 3 min and then 30 cycles of 94 °C for 1 min, 63 °C for 2 min, and 72 °C for 2 min. The primers used were 5'-AAAGAATTCTAGCGGCGCAGGCGGATGGC-3' (anti-sense) and 5'-AAACATATGATCTCCTT-CCTGCACTTC-3' (sense) specific for rat GnT-III [13].

Cell preparations

Mice were killed by cervical dislocation after ether anaesthesia. For preparation of bone marrow cell suspensions, a single femur

was flushed with a syringe with a 27-gauge needle. For preparation of spleen cell suspensions, the spleen was resected from each mouse and teased with scissors. Peripheral blood was obtained by cardiac puncture. Mononuclear cells were isolated by Ficoll-Hypaque density centrifugation with Lymphoprep (Nycomed, Oslo, Norway).

Long-term cultured bone marrow and spleen stromal cells

Mice were killed and bone marrow cells and spleen cells were prepared as described above. After being washed once, bone marrow cells or spleen cells (10^7) were suspended in 10 ml of Fischer's medium supplemented with 20% (v/v) heat-inactivated fetal calf serum (FCS; Gibco BRL) and 0.1 μ M hydrocortisone (Sigma). Cells were gently pipetted to suspend them and incubated in 25 cm² culture flask (Corning) at 33 °C under air/CO₂ (19:1). The cultures were fed twice a week by the removal of half of the growth medium (5 μ l) and replenishment with the same amount of fresh medium. At 2 weeks after the incubation, confluent adherent layers of stromal cells were observed to spread on the bottom of the flask; they were photographed under a phase-contrast microscope. These were used as bone marrow stromal cells and spleen stromal cells for the assay for GnT-III activity and non-adherent cell production.

Determination of GnT-III activity

GnT-III activities were assayed with a pyridylaminated biantennary sugar chain as a substrate [13,14]. Briefly, samples were washed three times with PBS, suspended in PBS and sonicated for 10 min at 4 °C. The crude enzyme preparations were incubated in triplicate with the pyridylaminated biantennary sugar chain at 37 °C for 2 h. The reaction buffer consisted of 125 mM 2-(*N*-morpholino)ethanesulphonate, pH 6.25, containing 0.77 mM 2-aminopyridine (2-PA)-treated substrate, 20 mM UDP-GlcNAc, 10 mM MnCl₂, 200 mM GlcNAc and 0.5% (v/v) Triton-X. Samples were boiled for 3 min to stop the reaction and centrifuged at 15000 *g* for 5 min to remove debris. To separate bisecting GlcNAc from the unreacted substrate, samples were analysed on an HPLC system with a Shimadzu SCL-6A on a TSK gel ODS-80TM column (4.6 mm $\times$ 150 mm, TOSOH Corp., Japan) with an elution buffer of 20 mM ammonium acetate, pH 4.0, containing 0.25% (v/v) butan-1-ol for GnT-III at a flow rate of 1.2 ml/min at 55 °C. The 2-PA derivatives were detected with a fluorescence spectrophotometer with excitation and emission wavelengths of 320 and 400 nm respectively. The amount of bisecting GlcNAc was determined from the fluorescence intensity, with 399 pmol of standard pyridylaminated biantennary oligosaccharides. The specific activity of the enzyme was obtained by averaging the results of three independent experiments and was expressed as nmol of GlcNAc transferred/h per mg of protein. The protein concentration was

determined with a bicinchoninic acid kit (Pierce), with BSA as the standard.

HPLC analysis for N-glycans

Cell surface N-glycans were prepared and analysed by HPLC as described [15], with some modifications. In brief, isolated mononuclear cells (10^6) from peripheral blood were lysed for 30 min at 4 °C in 10 mM Tris/HCl, pH 7.0, containing 1 % (v/v) Nonidet P40. Insoluble materials and nuclei were removed by centrifugation at 14000 g for 20 min at 4 °C. The protein contents of the supernatants were determined with the bicinchoninic acid kit, with BSA as a standard. Then lipids were removed from 300 μ l of the supernatants (10 μ g of protein) by chloroform/methanol extraction, as described [16]. The precipitates were freeze-dried and dissolved in 50 mM Tris/HCl (pH 8.0)/50 mM 2-mercaptoethanol/2 % (w/v) SDS. Next, the samples were first incubated for 8 h with N-glycanase (0.5 unit; Genzyme) followed by a 16 h incubation with glycosidase mixture 1 comprising sialidase (*Arthrobacter*), β -galactosidase (*Aspergillus*) and α -fucosidases (*Streptomyces* and bovine kidney) (Takara Shuzo Co., Shiga, Japan). The released N-glycans were collected by phenol/chloroform extraction, as described [17]. After being desalted on a PD-10 column (Pharmacia), N-glycans were labelled by using 2-PA with HydraClub (Honen Corp., Tokyo, Japan), as described [15]. These pyridylaminated sugar chains were separated from the excess 2-PA by gel filtration of a Toyopearl HW-40F column (TOSOH Corp., Tokyo, Japan) equilibrated with 20 mM ammonium acetate, pH 6.0, evaporated and then dissolved in 20 mM ammonium acetate, pH 5.0. The 2-PA sugar derivatives were subjected to the same HPLC system and measured on a fluorescence spectrophotometer as above.

Lectin blotting

Spleen tissues from mice were homogenized in PBS, pH 7.4, containing 1 μ g/ml aprotinin and 0.5 μ g/ml leupeptin. Isolated mononuclear cells from spleen or long-term culture spleen stromal cells were washed twice with PBS. Then cell surface glycoproteins of the samples were solubilized for 20 min at 4 °C in an equal volume of lysis buffer containing 140 mM NaCl, 10 mM Tris/HCl, pH 7.5, 10 % (v/v) glycerol, 1 % (v/v) Nonidet P40, 1 mM PMSF, 1 μ g/ml aprotinin and 0.5 μ g/ml leupeptin. Homogenates or sera containing 10 μ g of protein were subjected to SDS/PAGE and then transferred to nitrocellulose membranes, as described [11]. The non-specific binding sites of the membranes had been blocked by 3 % (w/v) BSA in PBS. For the detection of bisecting GlcNAc residues with E-PHA, the membranes were desialylated by treatment with 0.2 units/ml sialidase (*Arthrobacter*, Nacalai Tesque, Kyoto, Japan) in PBS at 37 °C for 60 min [18]. The samples were then incubated with biotinylated E-PHA (2 μ g/ml) to evaluate the additional bisecting GlcNAc on the N-oligosaccharide [19]. Desialylation was confirmed by the abolished binding of *Salvia selanea* lectin, a marker for sialic acid residues. The signals were revealed with an enhanced chemiluminescence kit (Amersham) and exposed to X-ray films, in accordance with the manufacturer's instructions.

Lectin cytotoxicity assays

Alteration of surface oligosaccharides was evaluated by sensitivity to lectin-induced cytotoxicity, as described previously [20], with some modifications. In brief, cells from spleen or bone marrow were mixed with a haemolysing solution [150 mM

NH₄Cl/10 mM KHCO₃/1 mM EDTA (pH 7.3)] for 5 min at room temperature. After three washings with PBS, cells (2×10^4 per sample) were suspended in 500 μ l of RPMI 1640 medium containing 10 % (v/v) FCS, then incubated in triplicate for 60 min at 37 °C with various concentrations of E-PHA, L-PHA or Con A. Then the percentage of viable cells was determined by Trypan Blue exclusion through the inspection of more than 250 cells under a light microscope.

Blood cell counts and histology

A tail vein of each mouse was cut to collect several drops of peripheral blood into a heparinized capillary tube (Terumo, Tokyo, Japan). Leucocytes, erythrocytes and platelets were counted on an automatic cell counter (Sysmex F800; TOA Medical Electronics, Kobe, Japan) after appropriate dilution. Differential leucocytes were distinguished as neutrophils, lymphocytes and monocytes by microscopic inspection of blood smears after May Grunwald–Giemsa staining.

For histological examinations, mice were killed 8 weeks after birth by cervical dislocation after ether anaesthesia. Immediately, several organs were resected, weighed and fixed in 10 % (v/v) formaldehyde-containing PBS at 4 °C for 1 week. Then the fixed tissues were embedded in paraffin, sectioned at 8 μ m thickness and stained with haematoxylin–eosin by the standard procedure.

Haemopoietic colony assays

A total of 2×10^4 bone marrow or spleen cells were plated in 1 ml aliquots of 1 % (w/v) methylcellulose in α -MEM supplemented with 30 % (v/v) FCS, 2 mM glutamine, 0.1 % BSA (Fraction V; Sigma), 9 units of recombinant human erythropoietin, 30 ng of recombinant murine interleukin 3, 30 ng of recombinant murine stem cell factor, 300 ng of recombinant human interleukin 6, and antibiotics. Duplicate cultures were incubated at 37 °C under air/CO₂ (19:1) for 12 days. Colonies were counted on an inverted microscope and scored as multilineage colony-forming units (CFU–mix), burst-forming units – erythroid (BFU–E) or colony-forming units – granulocyte and macrophage (CFU–GM), confirmed by May Grunwald–Giemsa staining.

Bone marrow transplantation

Bone marrow cells were prepared from a single femur, as described above. Flushing and washing procedures were performed in RPMI 1640 medium supplemented with 2 % (v/v) heat-inactivated FCS and antibiotics. After filtration through a nylon mesh to remove the tissue debris, cells were resuspended at 10^5 cells/100 μ l and used for transplantation within 4 h after killing of the donor mice. Mice were irradiated at a dose of 10 Gy on a linear accelerator producing 12 MeV electrons. After 6 h, suspensions of bone marrow cells were injected intravenously into lethally irradiated mice via a lateral tail vein. Irradiated mice were maintained in sterilized cages under laminar airflow and fed with sterilized water containing antibiotics. At 1, 2, 3 and 4 weeks after transplantation, blood cells in peripheral blood were counted, as described above.

Determination of non-adherent cell production

In maintaining long-term bone marrow culture cells, half of the culture medium was changed twice a week as follows: the culture medium (approx. 10 μ l) was collected into a 15 ml tube. After centrifugation at 1200 g for 10 min, the supernatant was aspirated into a final volume of 5 ml, and fresh Fischer's solution (5 ml)

was added into the tube. Cells were resuspended well and returned to the same culture flask. The loss of non-adherent cells was minimal in this passage.

Non-adherent cell production was evaluated in triplicate, as described previously [21]. Briefly, in exchanging half of the culture medium for fresh medium, a small volume of the medium (approx. 50 ml) was used to determine the total number of non-adherent cells for the quantification of the production of non-adherent cells by the stromal cells. Then adherent stromal cells in the flask were detached by EDTA/trypsin treatment to determine the number of stromal cells. The number of non-adherent cells produced in the flask was corrected with the number of adherent stromal cells.

RESULTS

Generation of transgenic mice expressing GnT-III activity in bone marrow, spleen and peripheral blood cells

Transgenic mice that overexpressed GnT-III were created by injecting a rat GnT-III gene under the control of an actin promoter into fertilized mouse eggs, which were transferred into

Table 1 GnT-III activity in peripheral blood, spleen and bone marrow of wild-type and transgenic mice

GnT-III activity (nmol/h per mg of protein) was examined in peripheral blood (PB), spleen (SP), bone marrow (BM), the isolated mononuclear cells (MNC) from PB, SP and BM, and stromal cells from long-term cultures of spleen cells and bone marrow cells. Values are means $\pm$ S.D. for eight to ten mice in each group.

Tissue	GnT-III activity (nmol/h per mg of protein)			
	Wild-type	III-1	III-2	III-3
Peripheral blood (PB)	0.23 $\pm$ 0.06	4.71 $\pm$ 0.28	5.43 $\pm$ 0.17	6.74 $\pm$ 0.23
Spleen (SP)	0.18 $\pm$ 0.05	5.73 $\pm$ 0.11	7.84 $\pm$ 0.34	6.72 $\pm$ 0.34
Bone marrow (BM)	0.21 $\pm$ 0.06	7.53 $\pm$ 0.21	6.78 $\pm$ 0.22	7.45 $\pm$ 0.29
MNC from PB	0.13 $\pm$ 0.04	6.56 $\pm$ 0.39	6.43 $\pm$ 0.25	5.89 $\pm$ 0.15
MNC from SP	0.08 $\pm$ 0.03	5.33 $\pm$ 0.28	5.76 $\pm$ 0.27	6.35 $\pm$ 0.33
MNC from BM	0.11 $\pm$ 0.05	4.83 $\pm$ 0.27	4.99 $\pm$ 0.18	5.27 $\pm$ 0.36
Stroma cells from SP	0.29 $\pm$ 0.07	6.23 $\pm$ 0.23	5.39 $\pm$ 0.22	6.68 $\pm$ 0.24
Stroma cells from BM	0.23 $\pm$ 0.07	7.24 $\pm$ 0.31	6.54 $\pm$ 0.35	5.73 $\pm$ 0.41

Figure 1 Construction and generation of GnT-III transgenic mice

(A) Schematic representation of the GnT-III expression vector. The shaded box and black box indicate an actin promoter and poly(A) signal respectively. Under an actin promoter, transcription is initiated at the human β -globin intron and terminated at the poly(A) signals, resulting in a transcript of rat GnT-III fused with human β -globin intron. Translation of the transcript is initiated at ATG and terminated at TAG, resulting in the rat GnT-III protein. Two arrows indicate a pair of primers used for PCR. E, *EcoRI*; S, *SalI*. (B) Detection of the GnT-III transgene by PCR-Southern blot analysis. At 3 weeks after birth, genomic DNA was extracted from mouse tail pieces and amplified by PCR with a pair of primers. Then the PCR products were size-fractionated on formaldehyde/1% (w/v) agarose, blotted to nitrocellulose membranes and hybridized with ^{32} P-labelled probes overnight. After washing, the blots were autoradiographed at -80°C overnight. In the present blot, the signals of the transgene were detected as 700 bp bands in lanes 3, 6, 7, 8 and 9, indicating five transgene-carrying mice among the 17 mice examined.

four pseudopregnant F_1 mice. The structure of the expression vector containing rat GnT-III cDNA, Act-III, was as shown in Figure 1(A). After 20 days the four foster mice gave a total of 41 live F_1 litters; eight F_1 mice were found to contain Act-III, as confirmed by Southern blot analysis. Three F_1 mice were selected at random as founder mice, termed III-1, -2 and -3, and backcrossed with wild-type mice to generate stable lines. As shown in Figure 1(B), the founder mice transmitted the transgene to their offspring, as confirmed by PCR-Southern blot analysis, and were used as stable lines of transgene-carrying mice in this study.

As shown in Table 1, the GnT-III activity was below 0.3 nmol/h per mg in bone marrow, spleen and peripheral blood of wild-type mice. In all three lines of transgenic mice examined, the GnT-III activity was above 4.5 nmol/h per mg in the whole tissues of bone marrow, spleen and peripheral blood, and in the isolated mononuclear cells from these tissues. We also examined GnT-III activity in stromal cells after long-term cultures of bone marrow and spleen cells. Transgenic-derived stromal cells from bone marrow and spleen cells showed elevated GnT-III activity, in contrast with its lower level in wild-type-derived stromal cells (below 0.3 nmol/h per mg). These results indicate that the introduced GnT-III gene was expressed both in mononuclear blood cells and stromal cells in the bone marrow and spleen of transgenic mice in all three lines of transgenic mice.

Increased bisecting GlcNAc residues of surface glycoproteins on transgenic-derived haematological tissues

The alteration of bisecting GlcNAc composition on the isolated mononuclear cells was analysed through the detection of 2-PA-labelled N-glycans with bisecting GlcNAc by HPLC. The released N-glycans from spleen-derived mononuclear cells and stromal cells by N-glycanase were additionally digested with sialidase, β -galactosidase and α -fucosidase. These treatments removed neuraminic acid and galactose residues from the N-glycans terminal end and fucose residues from the N-glycans, resulting in the facilitated separation of N-glycans with bisecting GlcNAc from those without bisecting GlcNAc. As shown in Figure 2, the peak of N-glycans with bisecting GlcNAc was increased in spleen-derived mononuclear cells (Figure 2A) and long-term spleen culture cells (Figure 2B) from III-1, whereas the peak of N-glycans with bisecting GlcNAc was undetectable in these cells from wild-type mice. The similar alterations in HPLC profile

Figure 2 Detection of N-glycans with bisecting GlcNAc by HPLC

N-glycans were extracted from spleen-derived mononuclear cells (A) and long-term spleen culture stromal cells (B) through glycosidase digestion. The extracted N-glycans were labelled with 2-PA. Then the labelled glycans were separated by HPLC under the conditions for the separation of N-glycans with bisecting GlcNAc. The equivalent HPLC profile was reproducible in five independent mice in each group.

were also observed with III-2- and III-3-derived mononuclear cells and stromal cells from spleen (results not shown).

Alterations in surface oligosaccharide components in transgenic mice were also evaluated by lectin-induced cytotoxicity, as described [20]. Lectins are cytotoxic to animal cells and the cells that display a higher affinity for the lectin are susceptible to lectin-induced cytotoxicity at a lower concentration of lectin. Thus, in this assay, enhanced susceptibility to lectin-induced cytotoxicity indicates an increased binding of the lectin to the cells. The binding of E-PHA lectin to N-glycans is increased in the presence of bisecting GlcNAc in N-glycans [19] and was used as a positive marker. As shown in Figure 3, E-PHA-induced cytotoxicity was increased in III-1-derived mononuclear cells from spleen, bone marrow and peripheral blood cells in comparison with wild-type-derived bone marrow cells. L-PHA lectin recognizes triantennary or tetra-antennary structures of complex-type asparagine-linked sugar chains [22]. As the synthesis of complex-type triantennary or tetra-antennary structures was suppressed in the presence of bisecting GlcNAc, L-PHA was used as a negative marker for bisecting GlcNAc in this assay. L-PHA-induced cytotoxicity was decreased in transgenic-derived blood cells from bone marrow, spleen and peripheral blood cells, compared with that of wild-type-derived blood cells. Con A lectin has an affinity for the core mannose of biantennary structures of N-glycans [23]. As this affinity for the core mannose

Figure 3 Lectin-induced cytotoxicity in blood cells from wild-type and transgenic mice

Cells (2×10^4) of peripheral blood (○, ●), spleen (□, ■) and bone marrow (△, ▲) were haemolysed with NH_4Cl . After being washed with PBS, cells were incubated for 60 min at 37 °C with various concentrations of E-PHA, L-PHA or Con A (upper, middle and lower panels respectively). Then viable cells were determined by Trypan Blue exclusion. Values are means for eight wild-type mice (○, □, △) and ten III-1 (●, ■, ▲) were within 8% of the mean.

is suppressed by bisecting GlcNAc [23], Con A was also used as a negative marker. Transgenic-derived blood cells from bone marrow, spleen and peripheral blood were more resistant to Con A-induced cytotoxicity than those cells from wild-type mice. Similar alterations in lectin-induced cytotoxicity were also observed with III-2- and III-3-derived blood cells (results not shown).

We also employed Western blotting with E-PHA to examine whether cell surface glycoproteins are equally affected by the transgene. As shown in Figure 4, the affinity for E-PHA was equally increased in the surface glycoproteins in the whole spleen, isolated mononuclear cells from spleen and long-term culture stromal cells from spleen in transgenic mice. A high affinity for E-PHA was also found in whole bone marrow cells and long-term bone marrow culture stromal cells in transgenic mice (results not shown). Collectively, these results of HPLC, lectin cytotoxicity assay and lectin blotting indicate that GnT-III

Figure 4 E-PHA affinity for surface glycoprotein from wild-type and transgenic mice

Surface proteins were solubilized from homogenized spleen (left panel), isolated mononuclear cells from spleen (middle panel) and spleen-derived long-term-cultured stromal cells (right panel) of the following mice: lane 1, wild-type; lane 2, III-1; lane 3, III-2; lane 4, III-3. Solubilized proteins were subjected to SDS/PAGE (12% gel) under reducing conditions, followed by lectin blot analysis with biotinylated E-PHA. Before E-PHA probing, membranes were desialylated by treatment with sialidase. The positions of size markers are indicated at the left. The results were reproducible for five independent mice in each group.

gene transfection resulted in an increase in bisecting GlcNAc residues in cell surface glycoproteins in spleen, bone marrow and peripheral blood.

Pancytopenia, spleen atrophy, hypoplastic bone marrow and decreased haemopoietic colony formation in transgenic mice

Peripheral blood haematology of wild-type mice and three lines of transgenic mice are listed in Table 2. The numbers of leucocytes, erythrocytes and platelets were significantly decreased in all three lines of transgenic mice. In the leucocytes of the transgenic mice, neutrophils and lymphocytes were decreased significantly, whereas the numbers of monocytes and eosinophils of transgenic mice were not significantly different from those of wild-type mice. No significant abnormality was found in cell morphology in transgenic-derived peripheral blood cells. Macroscopically, spleen was atrophic in transgenic mice. As listed in Table 3, no significant differences were found in body weight and weight of organs between wild-type and transgenic mice, except the reduced weight of spleen due to the atrophy of transgenic spleen. Histologically, the spleen of III-1 mice showed a decrease in follicle numbers and a decrease in follicle diameter, as shown in Figure 5. The bone marrow in the femur of III-1 mice was hypoplastic compared with that in wild-type mice. These morphological and histological findings in III-1 were also observed in III-2 and III-3 mice (results not shown).

Table 2 Peripheral blood haematology

Values are means $\pm$ S.D. for eight to ten mice in each group. Statistical analysis was performed with Student's *t* test. * *P* < 0.05; ** *P* < 0.02; *** *P* < 0.01 compared with wild-type.

Blood cell type	Wild-type	III-1	III-2	III-3
Leucocytes (/ μ l)	5900 $\pm$ 340	2200 $\pm$ 240**	2800 $\pm$ 280**	2700 $\pm$ 220**
Neutrophils (/ μ l)	1300 $\pm$ 140	800 $\pm$ 80*	920 $\pm$ 80*	770 $\pm$ 70*
Lymphocytes (/ μ l)	4300 $\pm$ 370	1100 $\pm$ 140**	1000 $\pm$ 120**	1100 $\pm$ 100**
Monocytes (/ μ l)	230 $\pm$ 130	200 $\pm$ 100	220 $\pm$ 110	190 $\pm$ 110
Eosinophils (/ μ l)	80 $\pm$ 20	70 $\pm$ 30	80 $\pm$ 40	70 $\pm$ 30
10 ⁻⁴ $\times$ Erythrocytes (/ μ l)	921 $\pm$ 66	530 $\pm$ 49***	509 $\pm$ 43***	532 $\pm$ 48***
Haemoglobin (g/l)	158 $\pm$ 3	85 $\pm$ 5***	79 $\pm$ 4***	81 $\pm$ 6***
10 ⁻⁴ $\times$ Platelets (/ μ l)	91 $\pm$ 11	57 $\pm$ 7***	64 $\pm$ 8**	55 $\pm$ 7***

Table 3 Body weight and organ weight of wild-type and transgenic mice

Weights are means $\pm$ S.D. for six mice in each group. Statistical analysis was performed with Student's *t* test. ** *P* < 0.02 compared with wild-type.

	Weight (g)			
	Wild-type	III-1	III-2	III-3
Body	22.5 $\pm$ 1.9	23.5 $\pm$ 2.4	24.9 $\pm$ 2.3	23.1 $\pm$ 2.2
Brain	0.12 $\pm$ 0.02	0.13 $\pm$ 0.02	0.15 $\pm$ 0.03	0.13 $\pm$ 0.02
Heart	0.29 $\pm$ 0.04	0.31 $\pm$ 0.03	0.32 $\pm$ 0.03	0.26 $\pm$ 0.03
Liver	3.46 $\pm$ 0.31	3.31 $\pm$ 0.29	3.24 $\pm$ 0.29	3.51 $\pm$ 0.38
Spleen	0.35 $\pm$ 0.04	0.13 $\pm$ 0.02**	0.16 $\pm$ 0.03**	0.12 $\pm$ 0.02**
Kidney	0.21 $\pm$ 0.03	0.19 $\pm$ 0.03	0.20 $\pm$ 0.02	0.20 $\pm$ 0.03

We evaluated the numbers of haemopoietic precursors and erythroid precursors in the bone marrow and the spleen of transgenic mice and wild-type mice by the determination of the number of CFU-mix, CFU-GM and BFU-E, respectively. Compared with wild-type mice, numbers of CFU-mix, CFU-GM and BFU-E were decreased significantly both in bone marrow and spleen in all transgenic mice lines examined, as shown in Table 4.

Pancytopenia in transgenic mice after bone marrow transplantation

Leucocytes, erythrocytes and platelets in the peripheral blood were counted weekly after bone marrow transplantation. As shown in Figure 6, the recovery of leucocytes, erythrocytes and platelets was suppressed in III-1 recipients compared with wild-type recipients. Compared with wild-type donor cells, haemopoietic recovery by III-1-derived donor cells was significantly delayed for 3 weeks after bone marrow transplantation, but no significant differences were observed in blood cell counts between wild-type donor and III-1 donor at 4 weeks after transplantation. At 4 weeks after bone marrow transplantation, the leucocyte, erythrocyte and platelet levels of III-1 mice were 36 %, 55 % and 60 % of those of wild-type mice respectively. Similar results of delayed haemopoietic reconstitution and pancytopenia were also observed after bone marrow transplantation with III-2 and III-3 as recipients (results not shown).

Figure 5 Histology of transgenic and wild-type mice

Sections are shown of spleen from III-1 mice (A) and wild-type mice (B), and bone marrow from III-1 (C) and wild-type mice (D). Scale bars, 50 μm in (A) and (B); 5 μm in (C) and (D). Equivalent histology was observed in eight mice in each group.

Table 4 Colony formation by bone marrow cells and spleen cells from wild-type and transgenic mice

Bone marrow and spleen cells (2×10^4) from wild-type and transgenic mice were seeded in duplicate into 1.0% (w/v) methylcellulose containing Iscove's modified Eagle's medium supplemented with interleukins 3 and 6, stem cell factor, erythropoietin, BSA and FCS, and incubated for 12 days. Colonies were distinguished as CFU-mix, CFU-GM and BFU-E. Numbers of colonies are represented per 10^6 nucleated cells. Values are means $\pm$ S.D. for eight to ten mice in each group. Statistical analysis was performed with Student's *t* test. * $P < 0.05$; ** $P < 0.02$; *** $P < 0.01$ compared with the wild type.

	Colonies per 10^6 nucleated cells					
	Bone marrow			Spleen		
	CFU-mix	CFU-GM	BFU-E	CFU-mix	CFU-GM	BFU-E
Wild-type	140 $\pm$ 19	2720 $\pm$ 56	202 $\pm$ 23	52 $\pm$ 5	131 $\pm$ 21	54 $\pm$ 13
III-1	34 $\pm$ 5*	520 $\pm$ 23*	121 $\pm$ 12***	16 $\pm$ 3**	47 $\pm$ 13*	20 $\pm$ 11***
III-2	41 $\pm$ 6*	465 $\pm$ 30*	107 $\pm$ 15**	22 $\pm$ 4**	40 $\pm$ 12*	17 $\pm$ 6**
III-3	49 $\pm$ 7*	429 $\pm$ 31*	87 $\pm$ 21**	21 $\pm$ 4**	58 $\pm$ 13*	14 $\pm$ 5**

Dysfunction of transgenic-derived long-term-cultured bone marrow stromal cells expressing GnT-III

Stroma-dependent haemopoiesis was evaluated on the basis of non-adherent cell production on stromal cells. As shown in Figure 7, non-adherent cell production was decreased in the culture of all three lines of transgenic-derived stromal cells compared with the culture of native-derived stromal cells. During the incubation the number of non-adherent cells produced by transgenic-derived stromal cells was approx. 20–60% of that of non-adherent cells produced by wild-type-derived stromal cells. As shown in Figure 8, no significant differences in cell morphology or cell composition were found between long-term-cultured stroma cells between wild-type and transgenic mice, suggesting that the dysfunction of stroma-dependent haemopoiesis was not due to an alteration in cellular composition.

DISCUSSION

In this study we examined the haematological effect of GnT-III expression by establishing transgenic mice ectopically expressing GnT-III in haematological tissues. The transgenic mice had lower counts of leucocytes, erythrocytes and platelets in the peripheral blood and smaller numbers of haemopoietic colonies in bone marrow and spleen than those in wild-type mice. In transplantation with wild-type donor cells, haemopoietic reconstitution in the transgenic recipient was suppressed compared with that in the wild-type recipient mice. A similar suppression of haematological reconstitution in transgenic recipient mice was also found after transplantation with transgenic-derived donor cells. This indicates that the stromal cells in the transgenic recipient mice could not completely support the recovery of haemopoiesis. Compared with wild-type donor cells, haemo-

Figure 6 Haemopoietic reconstitution of wild-type and transgenic mice after bone marrow transplantation

At 1, 2, 3 and 4 weeks after bone marrow transplantation, heparinized peripheral blood was obtained and leucocytes (upper panel), erythrocytes (middle panel) and platelets (lower panel) were counted with an automatic cell counter. Bone marrow transplantation was performed as follows: ○, from wild-type to wild-type; □, from III-1 to wild-type; ●, from wild-type to III-1; ■, from III-1 to III-1. Values are means $\pm$ S.D. for eight independent wild-type or III-1 donors in each group. Statistical analysis was performed with Student's *t* test: a, $P < 0.02$; b, $P < 0.05$ compared with transplantation of wild-type donor into wild-type recipient; c, $P < 0.01$; d, $P < 0.02$; e, $P < 0.05$ compared with transplantation of wild-type donor into III-1 recipient.

poietic reconstitution was significantly delayed for 3 weeks after transplantation with transgenic-derived donor cells, but the delay was undetected at 4 weeks after the transplantation. This delay was due partly to the decrease in haemopoietic colonies in bone marrow cells in transgenic mice, although there remains a possibility that the expression of GnT-III in blood cells also caused inhibitory effects on the growth and differentiation of blood cells in a stroma-independent manner.

To obtain further evidence on the dysfunction of stromal cells in the transgenic mice, we examined non-adherent cell production

Figure 7 Non-adherent cell production by stromal cells established from wild-type and transgenic mice

At 2, 4, 6, 8, 10 and 12 weeks during incubation, non-adherent cells established from wild-type (○), III-1 (●), III-2 (■) and III-3 (▲) were collected by three washings to calculate the total number of non-adherent cells in the medium. Non-adherent cell production was represented as the cell number per 10^4 stromal cells. Values are means $\pm$ S.D. for eight mice. Statistical analysis was performed with Student's *t* test. a, $P < 0.05$; b, $P < 0.02$ compared with wild-type.

by transgenic-derived stromal cells and compared it with that of wild-type stromal cells. The production of non-adherent cells was decreased in transgenic-derived stromal cells. As haemopoietic cells interact with stromal cells to grow and differentiate into mature cells [1], the dysfunction of transgenic-derived stromal cells shown by these results led to the suppression of proliferation and differentiation of haemopoietic cells represented as CFU-mix, CFU-GM and BFU-E.

The results of the structural analysis based on HPLC, lectin cytotoxicity assay and lectin blot analysis indicated that, in transgenic-derived blood cells expressing high GnT-III, the bisecting GlcNAc structure was increased on the N-glycans, as confirmed by the increase in susceptibility to E-PHA-induced cytotoxicity and the decrease in L-PHA- and Con A-induced cytotoxicity. These alterations in lectin binding in the blood cells of transgenic mice were similar to that of K562 cells ectopically expressing GnT-III, as reported [24]. Several studies have shown that the attachment of haemopoietic cells to stromal cells is mediated by mannose and galactose residues on the stromal cells [4,5,21]. In GnT-III transgenic mice, galactose residues were presumed to be decreased because bisecting GlcNAc suppresses the elongation of complex-type sugar chains, and galactose residues are positioned at the termini of elongated complex-type sugar chains. In fact the number of elongated complex-type sugar chains decreased, as confirmed by the decreased L-PHA-induced cytotoxicity [22]. In contrast, the mannose residues were thought to be increased at the terminus of N-glycans in transgenic mice, because the inhibition of sugar chain elongation by bisecting GlcNAc results in an increase in the composition of core mannose with bisecting GlcNAc, two bisected mannose residues at the terminal end. However, the two bisected mannose residues are conformationally twisted in the presence of bisecting GlcNAc to cause an increase in sterically abnormal mannose residues, as determined by NMR [15,25]. This is also supported by the observation that the transgenic-derived blood cells were more resistant to Con A-induced cytotoxicity, because the binding of Con A to the terminal mannose residues is decreased in the presence of bisecting GlcNAc [23]. This bisected mannose core might not be as physiologically functional for the interaction

Figure 8 Phase-contrast photomicrographs of long-term-cultured bone marrow stromal cells from wild-type (a, c) and III-1 mice (b, d)

Haemopoietic colonies in wild-type-derived cultures, indicated as arrowheads in (a) and (c), were fewer in cultures established from III-1 mice. Scale bars, 20 μ m in (a) and (b); 2 μ m in (c) and (d).

with blood cells as the mannose residues on native stromal cells. Thus the increase in bisecting GlcNAc structures could lead to a decrease in galactose residues and increase in sterically abnormal and haematologically dysfunctional mannose residues and result in the inhibition of mannose- and galactose-mediated interaction of haemopoietic cells with stromal cells.

In the synthesis of N-glycans, β -1,2 *N*-acetylglucosaminyl-transferase I (GnT-I; EC 2.4.1.101) is essential for the synthesis of N-glycans (Figure 1) [26,27]. Fetal haemopoiesis in GnT-I-deficient mice was observed to be normal because there were no differences in the frequency of colony-forming cells and the nature of the colonies, and because of the normal proliferation and differentiation of haemopoietic cells in response to multiple cytokines [28,29]. These findings indicated that complex-type N-glycans were not essential, at least for fetal haemopoiesis in the liver. At present it is impossible to examine whether complex-type N-glycans are essential for haemopoiesis in the bone marrow and spleen in adult mice, because targeting the GnT-I gene is lethal at the embryo stage in mice. Our study revealed that haemopoietic colonies were fewer in the bone marrow and spleen of the transgenic mice expressing GnT-III, indicating that some parts of haemopoiesis were dependent on N-glycans in adult mice.

In summary, the present study of transgenic mice expressing GnT-III reveals that the stroma-dependent haemopoiesis is compromised through the introduction and the expression of the GnT-III gene. This indicates that the expression of GnT-III should be strictly regulated in haematological tissues, especially in stromal cells in bone marrow and spleen, because its expression in haematological tissues results in haematological disorders

such as pancytopenia, hypocellular bone marrow and spleen atrophy. Our study shows that glycosylation by N-glycans has some significant roles in haemopoiesis through post-translational modification.

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