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Rat chronic graft versus host disease (GvHD) analyses with no chronic GvHD system findings

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Ανάλυση της χρονίας αντίδρασης του μοσχεύματος κατά του ξενιστή (GvHD) ποντικού με ευρήματα μη χρονίας GvHD

Περίληψη στο τέλος του άρθρου

OBJECTIVE **Clinical and laboratory signs of chronic graft versus host disease (GvHD) were analyzed in an attempt to establish a bone marrow (BM) transplant system with no chronic GvHD.** METHOD **Using DA, Lewis and F_1 (DA×Lewis) hybrid strain rats, DA BM transplantation combined with a piece of DA spleen graft onto an F_1 host spleen were performed in F_1 hosts pre-injected with the CD2 antibody (Ab). To evaluate immune tolerance, extracellular signal regulated kinase 1 (ERK1) and ZAP70 kinase were blocked transiently at the time of BM transplantation.** RESULTS **Although acute rejection and GvHD were avoided in all the F_1 hosts, the F_1 hosts receiving either ERK1 Ab or ZAP70 kinase Ab began to show chronic GvHD 100 days post-transplant, except for the rats that received neither ERK1 Ab nor ZAP70 kinase Ab. Rebound activation of donor T cell ERK1 or ZAP70 kinase gradually reinforced chronic GvHD, confirmed not only by clinical findings, but also by flow cytometer and immunochemical electron microscope using ZAP70 kinase Ab. Judging from Western blot (WB) results of thrombospondin 1 (TSP 1) and Bcl-2, it was concluded that the BM and spleen of this chronic GvHD were affected more by autophagy than apoptosis. Large Bcl-2 complexes suggested Bcl-2 binding to Beclin (ATG7). Large quantities of TSP 1 were present in the spleen and BM, with hyperplasia of megakaryocytes.** CONCLUSIONS **An anergy, no chronic GvHD, was induced by a piece of spleen graft onto the host spleen, but not by transient ERK1 or ZAP70 kinase blockage, in which only partial adaptive tolerance was seen.**

Key words

Autophagy
Bcl-2
Bone marrow transplantation
CD2
Chronic graft versus host disease
Rat
Serine/threonine kinase
Spleen graft
Thrombospodin 1
Tolerance

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In order to improve the survival of grafted bone marrow (BM) cells having one haplotype identical to major histocompatibility complex (MHC) antigens, it was considered useful to evaluate the clinical pathology of rat chronic graft versus host disease (GvHD) and to investigate the effects of 2 signal transduction proteins, of serine/threonine kinane, extracellular signal regulated kinase 1 (ERK1) and ZAP70 kinase, on rat graft survival. ERK1-deficient mice have been reported to exhibit Th1 cell polarization with enhanced interleukin 12p70 (IL-12p70) and a prototypic Th1-mediated autoimmune disease of the central nervous system (CNS).[1] IL-27 (a novel member of IL-6/IL-12 family), IL-15, which is closely related to IL-2, and IL-4, which increases the release of Th2 cytokines combined with mast cell enhancement, activate intracellular ERK1/2 expression.[2–4] On the other hand, ZAP70 kinase associates with the ζ subunit of T cell receptors (TCR). On TCR stimulation, both ZAP70 kinase and Syk are recruited to phosphorylated CD3 and ζ subunits. Selective T cell-deficient (STD) patients with severe combined immunodeficiency (SCID) carried a mutation of ZAP70 kinase.[5] The STD patients with a ZAP70 kinase deficit function failed to produce IL-2 and showed a markedly reduced response to TCR stimulation. Human SCID due to a ZAP70 kinase defect was refractory to TCR-mediated immune activation in the absence of $CD3^+CD8^+$ cells, but with an abundance of $CD3^+CD4^+$ cells in the peripheral blood.[6] Recently, the signaling blockage of $CD3\varepsilon^{-/-}$ mouse T cells using anti-TCR and anti-CD4 antibodies was reported to result in adaptive tolerance, with suppression of calcium/NFAT and nuclear factor (NF)-κB pathways.[7] ZAP70 kinase activity was decreased markedly with only a modest decrease in ERK1/2 phosphorylation in the adaptive tolerant cells.

In the rat, BM transplantation of one identical MHC-haplotype, blockage of ERK1 or ZAP70 kinase was used, resulting in chronic GvHD with suppressed acute reactions.

However, partial adaptive tolerance was also recognized in several of the hosts with chronic GvHD. No GvHD with near to clonal anergy was found only in the hosts with a piece of spleen graft, but without exogenous kinase blockage.

MATERIAL AND METHOD

Experimental design

Parental DA females and LEW/SsN male rats were purchased from Japan SLC Co, Hamamatsu, Japan. DA, Lewis and F_1 (DA×Lewis) hybrid rats were bred at the Animal Center of Hamamatsu University School of Medicine. About 8 week-old F_1 rats were used as hosts. DA females and F_1 sibling males were used as donors. Experimental group (Exp) A, Exp B, Exp C and Exp D were composed of 4 F_1 males, 4 F_1 males, 4 F_1 females and 4 F_1 females, respectively. The anti-rat CD2 equivalent (Harlan Sera-lab Ltd, Sussex, England) was injected 5 times subcutaneously in amounts of 6 μL per male and 4 μL per female over the 5 days pre-transplantation in all 4 experimental groups.

Exp A males of average body weight (BW) of 218 g, Exp B males of 210 g BW, Exp C females of 145 g BW and Exp D females of 139 g BW were transplanted with a piece of 1/4 sized DA spleen. At the same time, separated DA BM at 1.8×10^6 cells per male was injected into the host spleen of Exp A together with an ERK1 antibody (Ab) (BD Biosciences, Tokyo, Japan) in amounts of 4 μL per male. Separated DA BM of $2.0–2.2\times10^6$ cells per rat was injected into the host spleens of Exp B and Exp C together with a ZAP70 kinase Ab (BD Biosciences, Tokyo, Japan) in amounts of 4 μL per rat. Exp D females were transplanted with DA BM of 2.3×10^6 cells per rat without Ab injection. Subsequently, one month post-transplantation, both DA BM of 2.5×10^6 cells and DA spleen of 3.1×10^6 per rat were subcutaneously injected into the abdomens of the Exp A males. In the same way, both DA BM of 2.5×10^6 cells and DA spleen of 3.6×10^6 cells per rat were injected into the Exp B males. One month post-transplantation, both a mixture of 3.6×10^6 BM cells and 5.5×10^6 spleen cells, and a mixture of 2.6×10^6 BM cells and 4.6×10^6 spleen cells both which had been separated from an F_1 sibling male were subcutaneously injected into the abdomens of Exp C and D females, respectively. Exp A, B, C and D rats were followed for 111 days, 115 days, 117 days and 123 days after the first transplantation.

Pathological findings

To confirm clinical signs of chronic GvHD, the size or weight of the thymus and spleen were checked at macroscopic examination. Tissue sections of BM, graft spleen, host spleen, lung and alopecia skin were prepared in all the F_1 hosts. For the electron microscopic examinations, alopecia circumscripta skin of an Exp A-1 rat and the spleen graft of an Exp B-1 rat that survived for 115 days were selected, and observed with a transmission electron microscope (JEM-1220, JEOL, Tokyo Japan). Host spleen lymphocytes of Exp A-4 and Exp B-1 were examined by electron microscopic immunochemistry using a pre-embedding colloidal gold marker system. Before fixing in 2% glutaraldehyde, separated host spleen cells were stained with anti-ZAP70 kinase Ab (BD Biosiences) at 4 °C for 20 min and then a monoclonal antibody (MoAb) sheep and mouse IgG_1 with gold colloidal particles-15 nm (EY Laboratories, Inc, San Mateo, CA, USA) at 40 °C for 15 min. The sectioned materials were observed with the transmission electron microscope (JEM-1220).

Flow cytometer (FCM) analyses

Fluorescent-labelled mesenteric lymph node (MLN), thymus, BM and spleen were measured by the FCM of an EPICSR XL-MCL System (Beckman Coulter, Fullerton, CA, USA). In Exp A, B, C and D, respectively, the thymus and spleen, the MLN, thymus and spleen, the MLN, BM and spleen, and the MLN, thymus, BM and spleen were studied in the FCM analyses. The positive cell % of fluorescein isothiocyanate (FITC) anti-rat CD25 monoclonal antibody (MoAb) (Cedar Lane Laboratories Limited, Ontario, Canada), that is, IL-2 receptor (IL-2R), was measured in the lymphocytes of the MLN and spleen. R-phycoerythrin (R-PE)-conjugated mouse anti-rat CD90 (thy-1)/mouse CD90.1 (Thy-1.1) MoAb (BD Biosciences, Tokyo, Japan) was applied for the Thy-1 positive cell % of the thymus, BM and spleen. Spleen natural killer (NK) cells were stained with FITC anti-rat NK cell MoAb (Cedar Lane Laboratories Limited, Ontario, Canada). Gamma/delta (γδ) T cell receptor (TCR) positive cells were stained with a mouse anti-rat TCR gamma/delta FITC (Serotec, Kidlington, Oxford, UK). For all the labeled antibody methods, separated cells were incubated at 40 °C for 30 min and then immediately fixed in 0.5% paraformaldehyde. The spleen cell preparations of CD25 and Thy-1 were doubly stained with FITC anti-CD25 MoAb and R-PE anti-Thy-1 MoAb.

Western blot (WB) analyses

Frozen host spleen and BM of Exp A and Exp B and frozen BM of Exp C and Exp D were lysed to apply a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel (PAG). The fractioned proteins using 1-dementinal mini-slab gel apparatus (Marysol Co, Ltd, Tokyo, Japan) were transferred to immobilon PVDF transfer membranes (Nihon Millipore Ltd, Yonezawa, Japan). The immune-blotted spleen membranes were stained with thrombospondin 1 (TSP 1) Ab (Santa Cruz Biotechnology, Inc, Santa Cruz, California, USA). The immune-blotted membranes of BM were stained with TSP 1 Ab and anti-Bcl-2 (Ab-1) mouse MoAb (Calbiochem, Darmstadt, Germany). The secondary antibody, biotin conjugated anti-rabbit IgG (Sigma, Saint Louis, Missouri, USA) was further conjugated with ExtrAvidin-Peroxidase (Sigma). For red chromo-reactions, 3-amino-9- ethylcarbazole (Sigma) was used as the substrate.

RESULTS

In this study, acute GvHD and graft rejection were avoided in all the experimental systems of Exp A, B, C and D. However, chronic GvHD became a problem in the

F_1 rats of Exp A injected with ERK1 Ab and the F_1 rats of Exp B and C injected with ZAP70 kinase Ab. Although there were individual variations in chronic GvHD severity, it was clear that ZAP70 kinase suppression of Exp B and C caused milder chronic GvHD than ERK1 suppression of Exp A. Exp D F_1 rats, which were not injected with any Ab to signal transmitting protein, had the best clinical findings without chronic GvHD. More than 40% volume of the graft spleen survived for 111–123 days post-spleen and BM transplantation in all the F_1 hosts, except for one Exp A-1 male, in which a rejected necrotic spleen graft was removed 1 month post-transplantation. Lymph node (LN) structure in the remaining graft spleens was disturbed or had disappeared, with extramedullary mild erythropoiesis at the time of sacrifice. Exp D females with good clinical signs maintained LN structure quite well in the remaining graft spleens.

Table 1 summarizes the clinical and laboratory findings of chronic GvHD in Exp A and Exp B rats. Host spleen pathology of chronic GvHD was characterized by germinal center (GC) activation of LN, decreased numbers of LN lymphocytes and increased extramedullary hematopoiesis (especially erythropoiesis) in the red pulp. One Exp A-3 male, which had mostly severe chronic GvHD, showed weakness with BW loss of 80 g and skin redness similar to acute GvHD signs 3.5 months after the first transplantation. This Exp A-3 rat showed severe hypoplastic BM, shown in figure 1. Mast cells and lymphocytes proliferated with decreased numbers of erythroid-myeloid immature cells and megakaryocytes. Figure 2 shows one of the characteristic skin findings of chronic GvHD in an Exp A-1 rat.

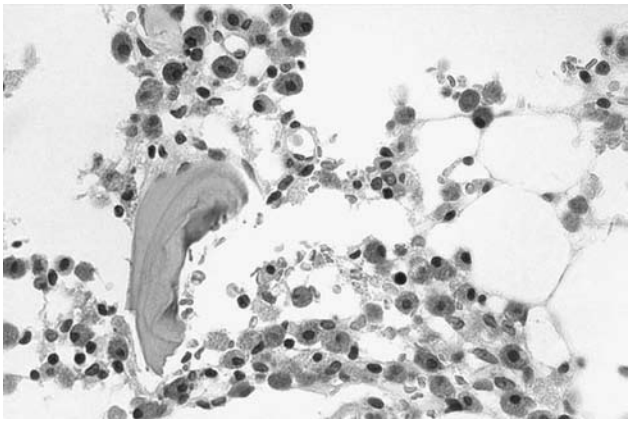

Figure 1. Bone marrow (BM) of an Exp A-3 male with the most severe chronic GvHD 111 days post-BM and spleen transplantation. This hypocellular BM showed massive proliferation of basophilic mast cells and lymphocyte proliferation. BM in chronic GvHD loses markedly erythroblastic, myeloblastic and megakaryocytic cell proliferation (Hematoxylin-eosin stain, ×400).

Alopecia circumscripta of the abdominal skin was caused by hyperkeratinization. Other characteristic signs of chronic GvHD were increased TSP 1 and Bcl-2 proteins, shown in figures 3a and 3b. Extramedullary megakaryocytes in the host spleens were increased to 1.1, 0.5 and 0.6 per oil field (×1,000) in the Exp A-3 and A-4, and Exp B-2 rats with moderate to severe chronic GvHD, respectively. All three rats had splenomegaly, and especially, in the Exp A-4 rat, which had a remarkably large spleen of 2.34 g. As shown in the WB of figure 3a, the host spleens in Exp A-3 and A-4 indicated high levels of TSP 1 (90–100 kDa), in which WB the large band detection of TSP 1 was difficult in control

Table 1. Chronic GvHD observed in the F_1 (DA×Lewis) males followed for 111–115 days after the first transplants of bone marrow (BM) and spleen (Sp).

Exp No (Sex)-Rat No		Thymus atrophy	Sp weight (g)	Sp weight SI[a]	TSP-1 in Sp[b] (Increase)	Bcl-2 in BM[b] (Increase)	Chronic GvHD
A (M)-	1[c]	(–)	0.87	2.6	(–)	(–)	Mild
–	2	(–)	0.86	2.4	(–)	(–)	Mild
–	3	(+)	1.10	4.0	(+)	(–)[d]	Severe
–	4	(m+)[e]	2.34	7.2	(+)	(+)	Moderate
B (M)-	1	(–)	0.89	2.5	(–)	(–)	Mild
–	2	(+)	1.05	3.1	(–)	(s+)[f]	Moderate
–	3	(+)	0.78	2.2	(–)	(–)	Mild
–	4	(–)	0.79	2.2	(+)	(–)	Mild
Controls (M)-	1,2,3,4	(–)	0.58±0.1	1.8±0.1	(–)	(–)	(–)

[a]Spleen (Sp) weight (mg)/body weight (g); [b] See figures 3a and 3b; [c] See figure 2; [d] See figure 1; [e] Moderately atrophic; [f] Slightly increased
TSP-1: Thrombospondin-1

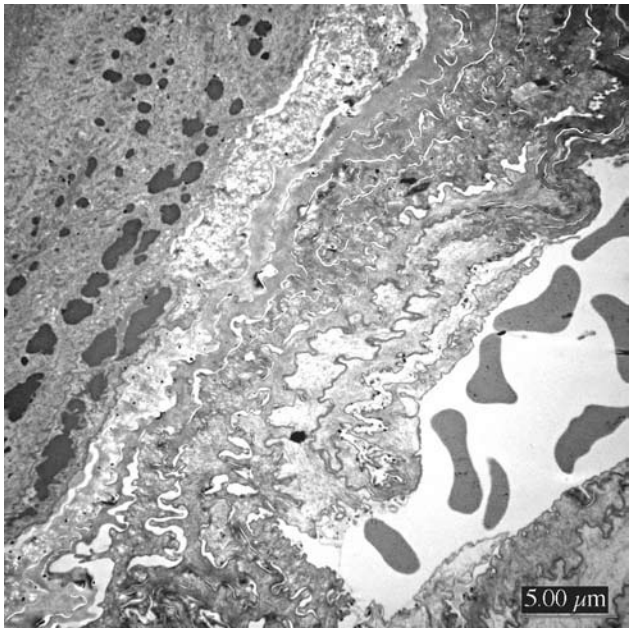

Figure 2. Electron micrograph of skin alopecia circumscripta found in an Exp A-1 male with mild chronic GvHD. This localized area of abdominal skin had markedly decreased hairs and hair follicles 111 days post-bone marrow (BM) and spleen transplantation. The hair follicle indicated the hair lost completely with hyperkeratinization and bleeding. The root sheath of this hair follicle has many large and amorphous kerato-hyaline granules of high density. Localized skin hyperkeratinization is recognized as a sign of chronic GvHD (Uranyl acetate-lead citrate double stain ×4,000).

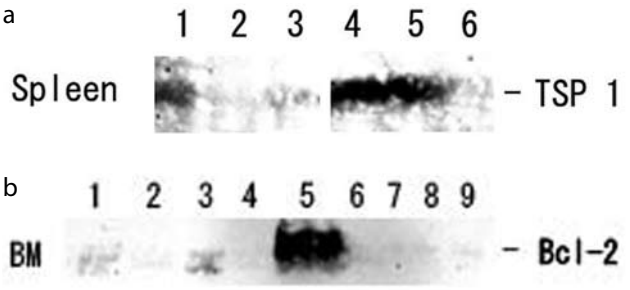

Figure 3. Western blot (WB) analyses of the spleen (fig. 3a) and bone marrow (BM) (fig. 3b) in Exp A and B males with chronic GvHD. (a) Spleen WB results of Exp A and B males stained with thrombospondin 1 antibody (TSP 1 Ab). This figure shows TSP 1 protein of 90–100 kDa. The rat spleens of the Exp A-3 rat (lane 5) and the Exp A-4 rat (lane 4) demonstrated large amounts of TSP 1, in which spleen megakaryocytes were increased with severe to moderate chronic GvHD. Only hyperplastic BM of Exp A-4, but not hypoplastic BM of Exp A-3, had elevated expression of TSP 1 (data not shown here). The Exp B-4 male of lane 1 with mild chronic GvHD shows moderately increased TSP 1. Two control male spleens showed only an about 45 kDa band of TSP 1, which was detected in all the rats (data not shown). Spleen samples in each lane are obtained from Exp rats as follows: Lane 1 of Exp B-4, lane 2 of Exp B-3, lane 3 of Exp B-2, lane 4 of Exp A-4, lane 5 of Exp A-3 and lane 6 of Exp A-2. (b) BM WB results of Exp A and B males stained with Bcl-2 Ab. This figure shows Bcl-2 complex proteins of 90–115 kDa. The Exp A-4 rat BM of lane 5 had markedly high levels of Bcl-2 complexes shown by a wide band. Bcl-2 complex proteins increased slightly in the Exp B-2 rat BM of lane 3. BM samples in each lane are obtained from Exp rats as follows: Lane 1 of Exp B-4, lane 2 of Exp B-3, lane 3 of Exp B-2, lane 4 of Exp B-1, lane 5 of Exp A-4, lane 6 of Exp A-3, lane 7 of Exp A-2, lane 8 of Exp A-1 and lane 9 of a control female.

males. The control males had an only about 45 kDa band fainty. The Exp B-2 rat with moderate chronic GvHD did not show a high level of TSP 1 in the spleen. The Exp A-4 rat with hyperplastic BM showed strongly increased TSP 1 in the BM, but the hypoplastic BM in the Exp A-3 rat (fig. 1) did not show TSP 1 clearly. One Exp B-4 rat with mild chronic GvHD demonstrated moderately increased TSP 1 also in the spleen. The Exp B-4 rat spleen had activated GC and plasma cell proliferation, but no increase in megakaryocytes. The Exp A-4 lungs showed perivascular and peri-bronchiole masses of plasma cells (activated B cells). The Exp B-4 lungs showed perivascular basophilic mast cell infiltration. It was likely that TSP 1 was mainly released from disturbed platelets. Bcl-2 in the BM is demonstrated in the WB shown in figure 3b. The hyperplastic BM of Exp A-4 expressed massive and large Bcl-2 complexes of 90–115 kDa. Exp B-2 BM with moderate chronic GvHD expressed slightly elevated large Bcl-2 complexes.

Exp C females re-immunized with BM and spleen cells obtained from a F_1 sibling male had milder chronic GvHD than those of Exp B males re-immunized with BM and spleen cells obtained from a DA female, even though both of the F_1 rats with the same ZAP70 kinase Ab were firstly transplanted with DA BM and the spleen in the same way. Increased BM Bcl-2 complexes and non-complex BM Bcl-2 of 24–26 kDa were detected in an Exp C-2 female and in the Exp C-3 and C-4 females, respectively, but not any BM Bcl-2 in Exp D rats on the same WB membrane as the Exp C rats. Abdominal alopecia circumscripta was found in the Exp C-1 and C-4 rats. The average spleen weights (spleen index) were 0.49 g (2.2), 0.44 g (2.1) and 0.38 (1.8) in the Exp C, Exp D and 6 control female rats, respectively. The Exp C females had mild chronic GvHD. Clinical chronic GvHD was avoided only in the Exp D females.

Figure 4 and table 2 show the results of FCM in the F_1 hosts. Figure 4 shows the Thy-1 positive cell % in the thymus of panel A and the BM of panel B. As shown in panel A-1 of the Exp A-3 rat with severe chronic GvHD and in panel A-3 of the Exp B-2 rat with moderate chronic GvHD, advanced chronic GvH reactions (GvHR) tended to increase thymus Thy-1 expression with atrophic changes. The Exp B-2 rat of panel A-3 with 21.6% of Thy-1-positive thymus cells had chronic GvHD more mildly than that of the Exp A-3 rat of panel A-1. Strong thymus Thy-1 expression protected the host from the severe development of chronic GvHD. As shown in panel B-1 of figure 4, the control female BM expression of Thy-1 was 37.0±7.8%. The Exp C rats of

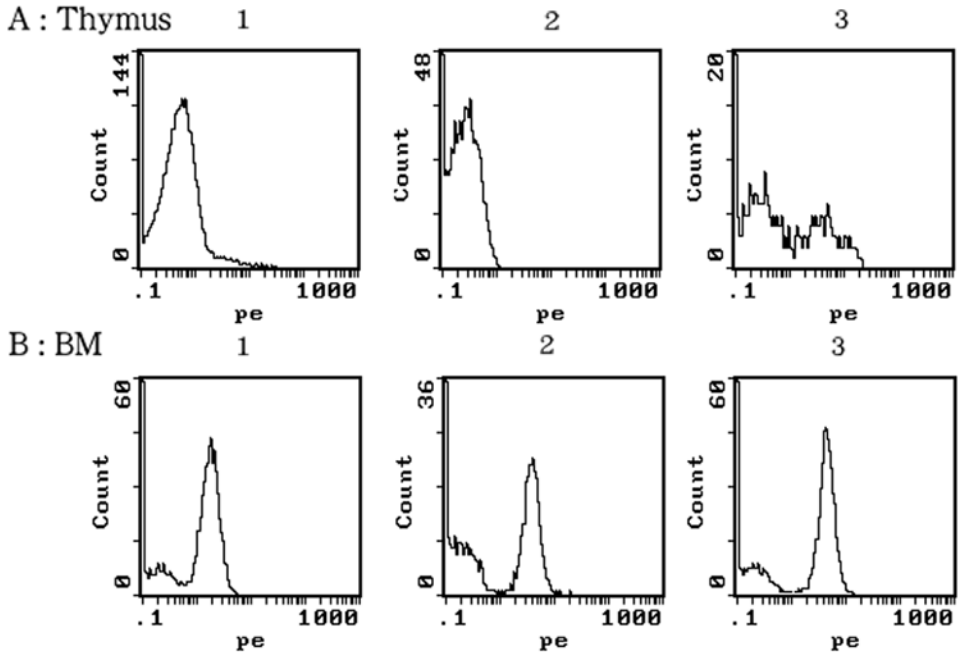

Figure 4. Flow cytometry (FCM) thymus results (panel A) in Exp A and B and FCM bone marrow (BM) results (panel B) in Exp C and D. Both thymus and BM cells were stained with the R-PE-conjugated mouse anti-rat Thy-1 (CD90) MoAb. Panel A-1 was obtained from the thymus of Exp A-3 with severe chronic GvHD. Panel A-2 was obtained from the thymus of Exp B-1 with mild chronic GvHD and panel A-3 from the thymus of Exp B-2 with moderate chronic GvHD. Panel A-3 of Exp B-2 with thymus atrophy has a Thy-1-positive peak of 21.6%, while in panel A-1 of Exp A-3 with thymus atrophy, Thy-1-positive cells account for 6.2%. Panel A-2 of Exp B-1 without thymus atrophy had 0.1% of Thy-1- positive cells. In panel B-1 of a control female, panel B-2 of Exp C-3 and panel B-3 of Exp D-4, Thy-1-positive BM cells are 43.6%, 21.6% and 39.7%, respectively. The Exp C-3 rat had mild chronic GvHD, but the Exp D-4 rat had not.

Table 2. Flow cytometry (FCM) results in the mesenteric lymph node (MLN), thymus, bone marrow (BM) and spleen of F_1 rats.

Exp	Rat	MLN	Thymus	BM	Spleen			
No	No	CD 25%	Thy-1%	Thy-1%	NK%	γδTCR%	CD25%	Thy-1%
A	-1,2	NT[a]	0.1	NT	2.1	1.0	0.9	2.2
	-3	NT	6.2[b]	NT	2.8	0.8	1.6	5.0
	-4	NT	4.9	NT	3.3	0.7	1.3	2.8
B	-1	15.8	0.04[b]	NT	1.0	0.7	0.7	2.1
	-2	11.1	21.6[b]	NT	1.4	0.6	0.5	0.5
	-3,4	2.7	0.4	NT	1.6	0.9	1.0	2.2
C	-1-4	3.3±0.1	NT	22.7±1.5[b]	3±0.4	NT	2±0.1	5±0.6
C (Graft)[c]	-1,2,3,4				3±0.4	NT	2±0.2	6±0.8
D	-1-4	3.0±0.1	1.8±1.4	36.5±3.6[b]	3±0.3	NT	1±0.2	6±0.2
CM	-1-4	3.6±0.5	0.1±0.1	NT	4±0.7	2±0.3	2±0.2	7±0.6
CF	-1-6	2.6±0.4	0.3±0.2	37.0±7.8[b]	4±0.2	NT	2±0.1	5±2.0

Exp C, C (Graft), D, CM and CF data are shown by M±SD. [a]Not tested; [b]See figure 4; [c]Measured lymphocytes were present in Exp C spleen grafts
CF: Control females

panel B-2 had 22.7±1.5% Thy-1-positive BM cells, while the Exp D rats of panel B-3 had 36.5±3.6% Thy-1-positive BM cells. Although the Exp C and D rats expressed the Thy-1 antigen more strongly than those of the control female, the Exp C rats had mild chronic GvHD, but not the Exp D rats. CD25 (IL-2R)-positive MLN cells of Exp B-1 and B-2 rats were increased to 11.1–15.8%, suggesting the activation of ZAP70 kinase (tab. 2). The positive cell % values of NK, γδ TCR, CD25 (IL-2R) and Thy-1 were within the normal ranges in the host spleens. The spleen grafts of the 4 Exp C rats showed a normal % of NK cells, CD25 (IL-2R) cells and Thy-1 cells. However, the spleen graft of Exp B-2 showed slightly elevated 4% level of γδ TCR-positive cells (data not shown in table 2). As the Exp B-2 rat showed 11.1% of CD25 (IL-2R)-positive MLN cells, AZP70 kinase activation might be brought at least in the MLN.

Figures 5a and 5b show lymphocytes prepared from the host spleens for immunochemical electron microscopy. Before fixing, the figure 5a T cells separated from the host spleen of the Exp A-4 rat with moderate chronic GvHD had been incubated with ZAP70 kinase Ab and then a MoAb

sheep anti-mouse IgG_1 coupled to gold colloidal particles of 15 nm. The activated T cell of figure 5a had only a small perinuclear area that reacted to gold particles, but the nucleolus had accumulations of gold particles in the rat injected with ERK1 Ab at BM transplantation. From the results of immunochemical electron microscopy, it was judged that the donor T cell activation had not occurred based on ZAP70 kinase activation in the Exp A-4 rat with moderate chronic GvHD and that the donor T cell activation of the Exp A-4 rat injected with ERK1 Ab had occurred based on ERK1 activation. On the other hand, the figure 5b T cell was separated from the host spleen of the Exp B-1 rat with mild chronic GvHD, in which ZAP70 kinase Ab had been injected at BM transplantation. Before fixing, this lymphocyte was also incubated with ZAP70 kinase Ab and then a MoAb sheep anti-mouse IgG_1 coupled to gold colloidal particles of 15 nm. As 15.8% of MLN lymphocytes expressed CD25 (IL-2R) in this Exp B-1 rat, ZAP70 kinase activation in the donor T lymphocyte was suspected before fixing. Several perinuclear areas and several parts of nuclear membrane of the donor T cell were involved in strong ribosomal activation based on ZAP70 kinase Ab reactions to activated ZAP70 kinase. Figure 6 shows a graft spleen of the Exp B-1 rat (fig. 5b) that survived for 115 days with a small erythroblastic island in the graft. Compared with nuclear development, the cytoplasm of the erythroblasts developed more poorly, which was a similar finding to that of erythroblasts in hemolytic disease. It was shown that the survival of extramedullary erythroblasts was shorter than that of normal erythroblasts in the Exp B-1 rat with ZAP70 kinase activation.

DISCUSSION

To protect against acute GvHD, an anti-rat CD2 MoAb which recognized thymocytes and peripheral T cells, but not B cells, was injected before transplantation of one haplotype identical BM. As observed earlier in subtotal-thymectomy rats,[8] in this study, acute GvHD was also avoided by the CD2 MoAb pre-injection in non-thymectomized rats. CD2 antigens were expressed on T cells and also on immunoglobulin superfamily molecules, a monocyte subset with high-affinity IgE receptor I (FcεRI), and NK cells. The injected CD2 MoAb might suppress not only simple CD2-positive

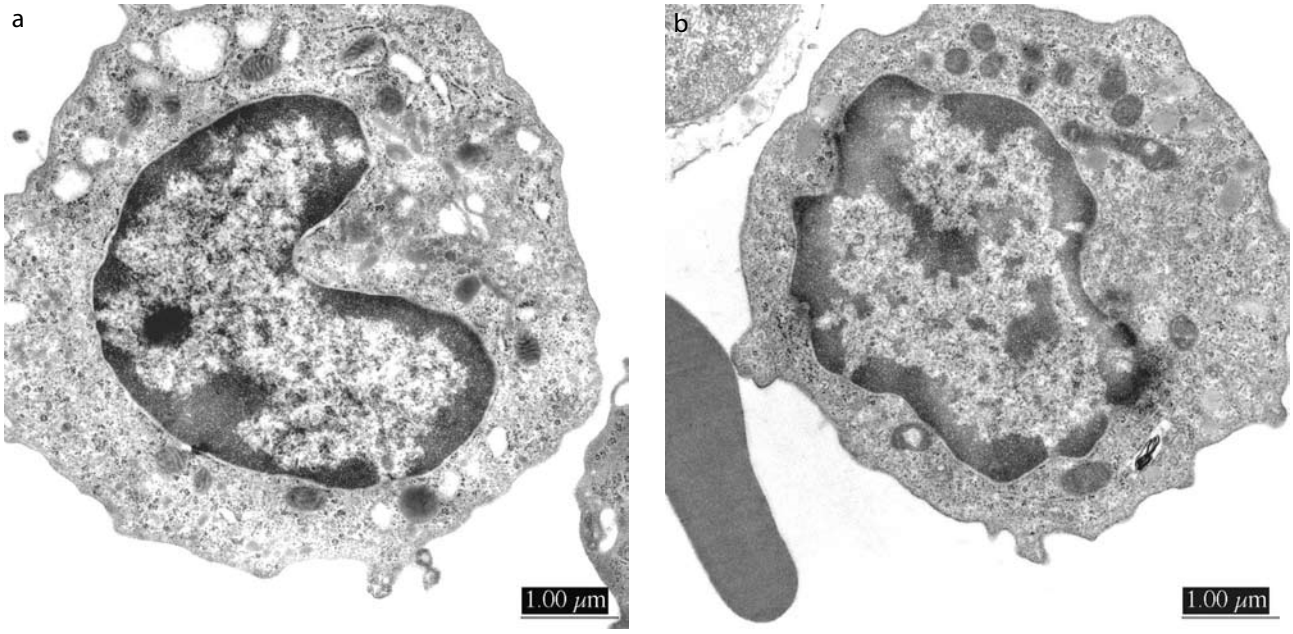

Figure 5. Activated lymphocytes of chronic GvHD host spleens examined by electron microscopy immunochemistry with 15 nm gold particle-labeled ZAP70 kinase antibody (Ab). (a) Activated lymphocyte separated from the host spleen of Exp A-4 with moderate chronic GvHD, in which ERK1 Ab had been injected at bone marrow (BM) transplantation. At the perinuclear cistern side, a small number of gold particles is observed. Massive accumulation of gold is present in the nucleolus. The perinuclear cistern became larger and surrounded by activated ribosomes. In the cytoplasm, many rough-surfaced endoplasmic reticula (RER) were activated, resulting in vacuolar formation. Small mitochondria of a high density matrix with long cristae are recognized. This activated T cell did not show ZAP70 kinase activation (Uranyl acetate-lead citrate double stain, ×20,000). (b) Lymphocyte separated from the host spleen of Exp B-1 with mild chronic GvHD. This lymphocyte has centrioles under the nucleus. At several areas of the perinuclear membrane, ribosomal particles of high density accumulate massively. In the cytoplasm, many small mitochondria of high density begin to degenerate. Small degenerated mitochondria of low density are also present. This T lymphocyte of Exp B-1, in which ZAP70 kinase Ab was injected at BM transplantation, must have developed ZAP70 kinase activation gradually (Uranyl acetate-lead citrate double stain, ×20,000).

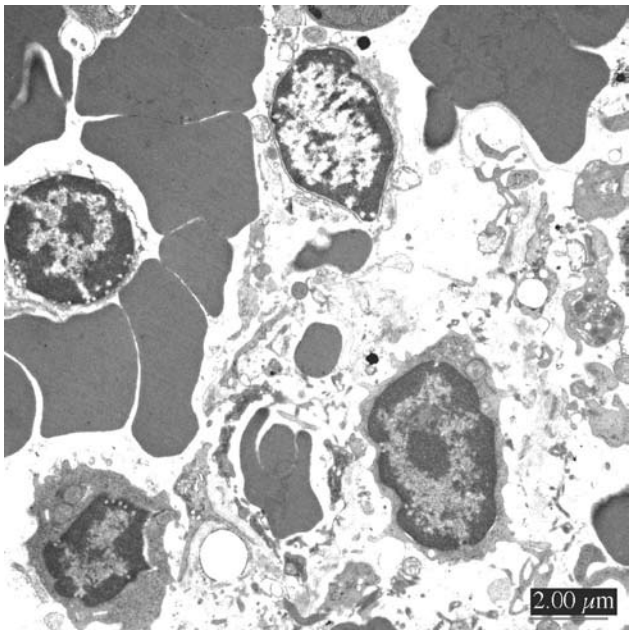

Figure 6. A small erythroblastic island found in the spleen graft of Exp B-1 with mild chronic GvHD, which is the same rat as shown in figure 5b. This DA spleen graft survived on the F_1 host spleen for 115 days post-transplantation. The two upper erythroblast nuclei were already denucleated from cytoplasm in earlier maturing stages. Under the two cells are a polychromatophilic erythroblast (right) and an orthochromatic erythroblast (left), respectively. The poly-chromatophilic erythroblast has a nucleus of condensed chromatin with a large nucleolus and marrow cytoplasm. The narrow cytoplasm has degenerated mitochondria and a rough-surfaced endoplasmic reticulum (RER). The orthochromatic erythroblast has an enlarged space between the nucleus and cytoplasm. The cytoplasm of the lower two cells has many villous projections. All four cells connected with loops of macrophages showed poorly developed cytoplasm. Nuclear membranes of the four cells had many pores (Uranyl acetate-lead citrate double stain, ×8,000).

T cell functions, but also more complicated immune reactions such as cell-mediated cytotoxicity, interferon-γ (IFN-γ) secretion, phosphinositol turnover, immediate type hypersensitivity, or invasive NK cells.[9,10]

A characteristic protective approach to prevent graft rejection, as shown earlier,[8] was BM transplantation into the host spleen, instead of venous injection. However, in this experiment, a piece of the donor spleen was transplanted onto the host spleen at the time of BM transplantation. The donor spleen grafts became a good marker of BM graft survival and GvHD in these experimental systems. Apart from one rat with necrotic spleen graft rejection, the other hosts had grafts that survived well, with more than 40% remaining. Chronic GvHR occurred not only in the host spleens, but also developed in the spleen grafts, which was shown by lymphocyte depletion and short survival of extramedullary erythroblasts. In the rats without GvHD, spleen grafts had the best pathological findings at the time of sacrifice.

T cell adaptive tolerance was examined in the rats injected with EFK1 Ab or ZAP70 kinase Ab. The signal transduction proteins of ERK1 and ZAP70 kinase were blocked by the Abs. If adaptive tolerance had been established in T cells injected with ERK1 Ab or ZAP70 kinase Ab, the T cells should show hyporesponsive immunity.[7] However, unfortunately, among the 4 rats of the ERK1 Ab experimental system, one rat rejected the spleen graft and all 4 rats showed mild to severe chronic GvHD. ERK1 inhibition in T cells gradually led to donor T cell activation in a state of ERK1 rebounded activation. In contrast to donor T cells, host plasma cells detected in the spleen and lungs might have suppressed ERK1/2 and produced IgG2b in chronic GvHD.[1] Host T cells that were rejected by donor T cells did not induce ERK1 activation. It has been suggested that IL-27 production, and then IL-15 production led to donor T cell activation combined with ERK1 activation (2–3). At an advanced stage of severe chronic GvHD, one rat of the ERK1 Ab experimental system showed marked proliferation of basophilic mast cells in the BM. As IL-4 enhances mast cell proliferation with increased Th2 cytokine (IL-3, IL-5 and IL-13) release in a state of ERK1/2 activation, IL-4 secretion has been shown indirectly in the severe chronic GvHD rat.[4] $ERK^1/_2$ activation in the donor T cells was suggested easily. Among the 8 rats of the ZAP70 kinase Ab systems, all showed mild to moderate chronic GvHD. Two of the 8 exhibiting ZAP70 kinase activation had activated CD25 (IL-2R)-positive MLN cells, but had normal CD25 (IL-2R)-positive spleen cells.[11] IL-2 gene transcription was initiated based on produced transcription factors in the rats of the ZAP70 kinase Ab system.[12] Subsequently, $CD4^+CD25^+T_{reg}$ cells of MLN, which would inhibit graft rejection, were controlled by IL-2 dominant mechanisms.[13] ZAP70 kinase rebound activation of spleen donor T cells induced $CD4^+CD25^+T_{reg}$ cells in the MLN of the rats. It was shown that in the rats injected with ZAP70 kinase Ab, partial adaptive tolerance had been established to a greater degree than the rat injected with ERK1 Ab.[7] In the hosts with no chronic GvHD, near to clonal anergy may have been induced by inhibition of the RAS/MAPK pathway.[7] Spleen grafts having the same MHC antigens as BM grafts may block the RAS/MAPK pathway of the donor T cells.

Supportive data for chronic GvHD was that the rats of the ERK1 or ZAP70 kinase Ab systems strongly expressed TSP 1 and Bcl-2 in the spleen and/or BM. As TSP 1 regulates cell growth, motility and apoptosis, it is reasonable to expect that the chronic GvHD rats will strongly express TSP 1. TSP 1 is released from platelet α granules in response to thrombin. TSP 1 inhibits angiogenesis by impairing endothelial cells.[14] Recently, TSP 1 expression was found in 17.5%

of non-Hodgkin's lymphoma, associated with aggressive histology and short overall survival.[15] In this study, TSP 1 was concluded to have been produced from the release of disturbed platelets, probably combined with impaired endothelial cells and lymphocyte distraction. On the other hand, the Bcl-2 protein of 24–26 kDa, which is located in the outer membranes of mitochondria and appeared on the endoplasmic reticulum (ER) after dissociation, functions as an inhibitor of apoptosis, necrosis and autophagy.[16] Bcl-2 protein suppresses apoptosis caused by the release of caspase-activating proteins. Bcl-2 and Bcl-X_1 suppress autophagy, which is initially induced for cell survival, but ends in cell death, in binding to Beclin (ATG7). As marked expression of Bcl-2 complexes shown in this chronic GvHD suggested Bcl-2 binding to Beclin (ATG7), autophagy changes were considered to be more predominant than apoptotic changes in the chronic GvHD of this study.

ΠΕΡΙΛΗΨΗ

Ανάλυση της χρονίας αντίδρασης του μοσχεύματος κατά του ξενιστή (GvHD) ποντικού με ευρήματα μη χρονίας GvHD

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ΣΚΟΠΟΣ Αναλύονται τα κλινικά και τα εργαστηριακά ευρήματα της χρονίας αντίδρασης του μοσχεύματος κατά του ξενιστή (GvHD), με σκοπό τη δημιουργία ενός συστήματος μεταμόσχευσης μυελού των οστών (ΜΜΟ) χωρίς εμφάνιση GvHD. **ΥΛΙΚΟ-ΜΕΘΟΔΟΣ** Χρησιμοποιήθηκαν DA, Lewis και F_1 (DA×Lewis) υβριδικά ποντίκια και έγινε ΜΜΟ σε συνδυασμό με σπληνικό μόσχευμα στο σπλήνα ενός F_1 ποντικιού που είχε προηγουμένως λάβει αντίσωμα CD2 (Ab). Για την αξιολόγηση της ανοχής, δεσμεύτηκαν παροδικά κατά τη μεταμόσχευση οι κινάσες ERK1 και ZAP70. **ΑΠΟΤΕΛΕΣΜΑΤΑ** Αν και η οξεία απόρριψη του μοσχεύματος και η GvHD δεν εμφανίστηκαν σε όλα τα F_1 ποντίκια, τα ποντίκια F_1 που πήραν αντίσωμα είτε κατά της ERK1 είτε και κατά της ZAP70 άρχισαν να εμφανίζουν χρονία GvHD 100 ημέρες μετά από τη μεταμόσχευση, ενώ δεν εμφανίστηκε αντίδραση GvHD στα ποντίκια που δεν πήραν αντίσωμα και για τις δύο παραπάνω κινάσες. Η παλίνδρομη ενεργοποίηση των Τ κυττάρων του δότη από τις ERK1 ή ZAP70 κινάσες ενίσχυσε σταδιακά τη χρονία GvHD, που επιβεβαιώθηκε όχι μόνο από τα κλινικά ευρήματα, αλλά επίσης και με την κυτταρομετρία ροής και την ανοσοχημεία με ηλεκτρονικό μικροσκόπιο, χρησιμοποιώντας αντίσωμα κατά της ZAP70 κινάσης. Εκτιμώντας τα αποτελέσματα της θρομβοσπονδίνης 1 (TSP 1) και της Bcl-2 με Western blot, συμπεραίνεται ότι η μεταμόσχευση μυελού και σπλήνα στη χρονία GvHD έχει ως αποτέλεσμα κυρίως αυτοφαγία παρά απόπτωση. Μεγάλα συμπλέγματα Bcl-2 υποθέτουν σύνδεση της Bcl-2 με Beclin (ATG7). Στο σπλήνα και το μυελό ευρίσκονται μεγάλα ποσά TSP 1 με υπερπλασία των μεγακαρυοκυττάρων. **ΣΥΜΠΕΡΑΣΜΑΤΑ** Ανεργία και μη εμφάνιση χρονίας GvHD προκλήθηκε από ένα σπληνικό μόσχευμα στο σπλήνα του ξενιστή, ενώ δεν εμφανίστηκε με παροδική δέσμευση των κινασών ERK1 ή ZAP70, που συνοδευόταν μόνο από μερικά προσαρμοσμένη ανοσιακή ανοχή.

Λέξεις ευρετηρίου: Ανοσιακή ανοχή, Bcl-2, CD2, Θρομβοσπονδίνη 1, Κινάση σερίνης/θρεονίνης, Μεταμόσχευση μυελού των οστών, Ποντίκια, Σπληνικό μόσχευμα, Χρονία αντίδραση μοσχεύματος κατά ξενιστή

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