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Interaction of merosin (laminin 2) with very late activation antigen-6 is necessary for the survival of CD4$^+$ CD8$^+$ immature thymocytes

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SUMMARY

The laminin α2-chain is a component of merosin, a member of the laminin family molecules, which is mainly expressed in the basement membranes of striated muscle. It is known that laminin α2 gene (*lama2*) null mutant mice (dy^{3k}/dy^{3k}) exhibit congenital muscular dystrophy (CMD). Because the laminin α2-chain is also expressed in the thymus, the role of merosin in the thymus was examined. In association with the onset of muscular dystrophy, CD4$^+$ CD8$^+$ double-positive (DP) thymocytes disappear by apoptotic cell death, while CD4$^+$ CD8$^-$ or CD4$^-$ CD8$^+$ thymocytes remain. In order to study the mechanisms leading to the selective death of DP cells in the absence of merosin, the role of the interaction between very late activation antigen-6 (VLA-6), a candidate merosin ligand in the thymus, and merosin was examined. The *in vitro* survival of thymocytes from normal mice was maintained by the addition of either anti-VLA-6 monoclonal antibodies (mAbs) or merosin. Furthermore, when the normal thymocytes were cultured on thymic epithelial cell lines, viable DP cell recoveries on wild-type epithelial cells were better than on cells from null mutant mice. The results suggest that DP cells are more sensitive to an uncharacterized apoptotic death signal, and that survival is supported by the interaction between VLA-6 and merosin.

INTRODUCTION

The thymus is the organ in which most T cells develop. T-cell precursors originate from fetal liver during the embryonic stage and from bone marrow in an adult. In the fetal thymus, prothymocytes enter the non-vascularized thymic rudiment by encapsulation. The thymus is composed of thymic lymphocytes (thymocytes) and non-lymphoid stroma. The thymic stroma consists largely of epithelial cells derived from the pharyngeal pouch during development, and monocytes and dendritic cells derived from bone marrow. Furthermore, fibroblasts and various extracellular matrix (ECM) molecules permeate the whole framework. Cellular interactions between stromal cells and thymocytes play crucial roles in T-lymphocyte development.[1–4] There are two types of cellular interaction between these cell types: direct cell-to-cell interactions through major histocompatibility complex (MHC)/T-cell receptor (TCR), intracellular adhesion molecule-1 (ICAM-1)/lymphocyte function-associated antigen-1 (LFA-1), and LFA-3/CD2, and bridging by ECM molecules. In the thymus, laminins, fibronectin and type IV collagen interact with thymocytes through their respective ligand.

Laminins are components of basal laminae throughout the body, and play essential roles in the organization of molecular networks of basal laminae, the interaction with cell-surface components and signal transduction into the cells. Laminin consists of three subunits, α-, β- and γ-chains (nomenclature for laminins by Burgeson *et al.*[5]). To date, five α-chains (α1–5) have been shown to assemble into at least 11 heterotrimers.[6] Merosin (laminin 2) is a 700-MW glycoprotein composed of α2- (300 MW), β1- (200 MW), and γ1- (200 MW) chains. Merosin is found in the basement membranes of striated muscle, Schwann cells, trophoblasts,[7,8] kidney, heart, skin,[9,10] central nervous system[11] and intestine.[12] Merosin expression has also been found in the thymus following analysis using the reverse transcription–polymerase chain reaction (RT–PCR),[13,14] and possible roles of the merosin–integrin interaction in thymocyte development have been suggested.

Merosin is a specific ligand for dystroglycan in the muscle, and the interaction produces an axis connecting the ECM and subsarcolemmal network.[15] The specific absence of merosin

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results in a congenital muscular dystrophy (CMD) and an autosomal recessive muscle disorder.[16] Researchers have identified two naturally occurring mouse models for this disease, dy/dy[17–20] and dy^{2j}/dy^{2j}.[21,22] Very recently we established laminin α2-chain null mutant mice (dy^{3k}/dy^{3k}) by targeted disruption of the *lama2* gene.[23] The homozygous mice are characterized by growth retardation and severe muscular dystrophy symptoms and succumb to undetermined causes by 5–6 weeks of age. In the degenerating muscles, considerable amounts of apoptotic cell death are detected.[23] We then examined the thymus of dy^{3k}/dy^{3k} mice to investigate the role of merosin in T-cell development. We describe here severe thymic atrophy in dy^{3k}/dy^{3k} mice, and report this atrophy to be associated with the selective apoptotic cell death of $CD4^+$ $CD8^+$ double-positive (DP) thymocytes. The possible role of merosin in the maintenance of DP cells in the thymus is discussed.

MATERIALS AND METHODS

Mice

Heterozygous *lama2* gene-targeted mice[23] were maintained in our animal facility by mating with normal BALB/c mice. Heterozygous mice were interbred to obtain homozygous mice. Specific pathogen-free BALB/c mice aged 5–6 weeks were purchased from Charles River Japan (Tokyo, Japan).

Genotyping of the *lama2* deficiency was performed by PCR on tail genomic DNA. The PCR primers for the wild-type (WT) *lama2* allele were: 5′-CCAGATTGCCTACGTAATTG-3′ and 5′-CCTCTCCATTTTCTAAAG-3′. The primer pairs for the mutant allele were: 5′-CTTGGGTGGAGAGGC-TATTC-3′ and 5′-AGGTGAGATGACAGGAGATC-3′, which are present in the *neo* gene. Mice showing WT *lama2*-negative and *neo*-positive PCR products were defined as *lama2* homodeficient (dy^{3k}/dy^{3k}). *Lama2* homodeficient mice are hereafter referred to as $mer^{-/-}$.

Establishment of thymic epithelial cell lines

Thymi of $mer^{-/-}$ or WT mice were obtained, and thymic epithelial cell (TEC) lines were established according to previously published methods.[24] TEC lines derived from $mer^{-/-}$, WT and normal BALB/c mice were termed S7HoE1, S7wtE1 and S1Bc, respectively. The cell lines were maintained in Dulbecco's modified Eagle's medium (Nissui, Tokyo, Japan) containing 10% fetal calf serum (FCS; Boehringer Mannheim, Castle Hill, Australia) with kanamycin (100 mg/l; Meiji Pharmaceutical Co., Tokyo, Japan).

Antibodies

Biotinylated anti-CD4 (GK1.5) and fluorescein isothiocyanate (FITC)-conjugated anti-CD8 (53-6.7) (from the American Type Culture Collection [ATCC], Rockville, MD) were prepared in our laboratory. Hamster monoclonal antibodies (mAbs) to mouse integrin α_6 (HMα6), α_2 (HMα2), and β_1 (HMβ_1-1),[25,26] and rat mAb to α_4 (CAS-9)[27] (a gift from Dr T. Kina, Kyoto University, Kyoto, Japan) were used. Phycoerythrin (PE)-conjugated anti-CD4 (RM4-5), biotinyl anti-TCRβ (H57) and biotinyl-anti-Vβ8 (F23.1) antibodies were obtained from PharMingen (San Diego, CA). PE-conjugated streptavidin, PE-Cy5-conjugated streptavidin and FITC-conjugated goat anti-hamster immunoglobulin G (IgG) were purchased from DAKO Japan (Tokyo, Japan), Cedarlane (Ontario, Canada), and Organon Teknika (West Chester, PA), respectively. mAb to mouse anti-human laminin α2-chain (2D9) was kindly provided by Dr H. Hori (Tokyo Medical and Dental University, Tokyo, Japan).[28] Horseradish peroxidase-conjugated goat anti-mouse IgG was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Other reagents

Human merosin, bovine gelatin, Sephadex G-10 and 7-amino actinomycin D (7-AAD) were purchased from Chemicon (Temecula, CA), Wako Pure Chemicals (Osaka, Japan), Pharmacia Biotech (Uppsala, Sweden) and Sigma Chemical Co. (St. Louis, MO), respectively.

Flow cytometry

Single-cell suspensions were prepared from thymi. Thymocytes (500 000) were stained with each antibody and flow cytometric profiles were analysed using an EPICS ELITE (Coulter Electronics, Hialeah, FL) or a FACSCalibur analyser and CELLQuest software (Becton-Dickinson Immunocytometry Systems, Mountain View, CA).

Thymocyte survival assay

Freshly isolated normal BALB/c thymocytes were passed through a Sephadex G-10 column to remove adherent cells, and cultured in RPMI-1640 medium, with or without purified human merosin or various mAbs, at 2×10^5 cells/400 μl/well in 48-well culture plates (Sumitomo Bakelite, Tokyo, Japan) at 37° in 5% CO_2. Eight hours later, the cells were harvested and stained with 7-AAD (used at 1 μg/ml). The 7-AAD-negative cells were then counted by using flow cytometry.

Co-culture of thymocytes on a TEC line

FCS was passed through a gelatin-conjugated affinity column to remove serum ECM molecules (such as fibronectin) and the eluate is hereafter referred to as FCS′. TECs were cultured in RPMI-1640 supplemented with 2·5% FCS′ (FCS′/RPMI) at 2×10^4 cells/ml/well in 24-well culture plates (Sumitomo Bakelite). Forty-eight hours later, monolayers of TECs were overlaid with normal thymocytes (that had been passed through a Sephadex G-10 column), in FCS′/RPMI, at 5×10^5 cells/ml/well. Forty-two hours later, the thymocytes were harvested and stained with biotinyl-anti-CD4 (GK1.5) plus PE-streptavidin, FITC-anti-CD8 (53-6.7) and 7-AAD, then analysed by flow cytometry.

DNA-fragmentation assay

Two million freshly isolated thymocytes were fixed in 70% ethanol overnight at 4°, washed with phosphate-buffered saline (PBS) and resuspended in 100 μl of phosphate-citrate buffer (pH 7·6) containing 192 mM Na_2HPO_4 and 4 mM citric acid, and incubated for 30 min at room temperature. The cells were incubated with RNase A (0·2 mg/ml; Sigma) at 37° for 30 min, then with propidium iodide (PI) (100 μg/ml; Sigma) for 30 min in the dark. The DNA content of the cells was analysed by flow cytometry.

RT–PCR

Total RNA was isolated from thymus, muscle, thymocytes or TEC lines using TRIzol (Gibco BRL, Grand Island, NY), and

treated with DNase I (Perkin-Elmer, Branchburg, NJ). RNA was transcribed to cDNA using an AMV Reverse Transcriptase First-Strand cDNA Synthesis Kit (Life Sciences, St. Petersburg, FL). cDNA was amplified by using Ampli*Taq* (Perkin Elmer) in buffer containing 1·5 mM $MgCl_2$. Primers for the laminin α2-chain were the same as used for genotyping. The primer pairs for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were: 5′-CCATCACCATCTTCCAGGAG-3′ and 5′-CCTGCTTCACCACCTTCTTG-3′.

Western blot analysis

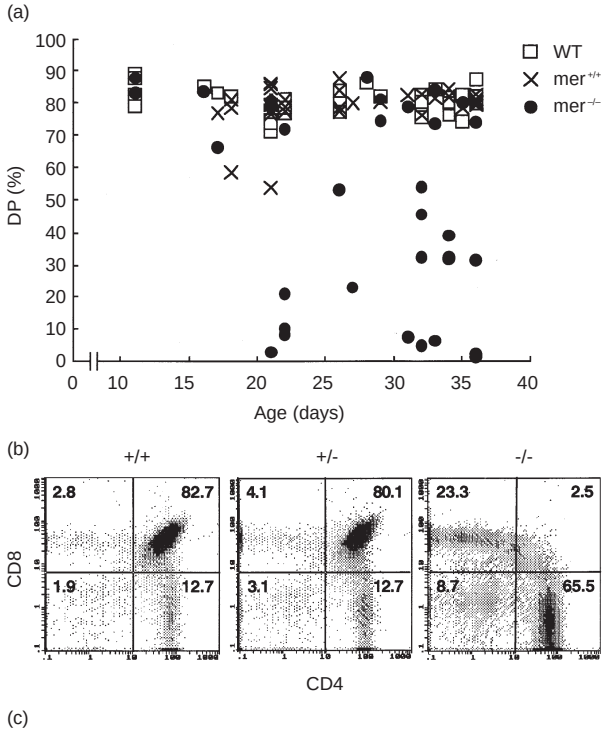
Thymus (10 mg, wet weight), spleen (10 mg, wet weight), and muscle (2 mg, wet weight) were homogenized in 40 μl of buffer containing 15% sodium dodecyl sulphate (SDS), 70 mM Tris-HCl (pH 6·7), 10 mM EDTA and 5% 2-ME, and boiled for 5 min. The supernatants were separated by electophoresis (5% SDS–PAGE) and electrotransferred to Protran Nitrocellulose filters (Schleicher & Schuell, Dassel, Germany). The membranes were reacted with 2D9, a mAb to human laminin α2, then with horseradish peroxidase-conjugated goat anti-mouse IgG. The enhanced chemiluminescence method (Luminescence Reagent Set, Wako, Japan) was applied for the visualization of laminin α2 bands.

RESULTS

Reduction of DP thymocytes in mer$^{-/-}$ mice

Mer$^{-/-}$ mice were indistinguishable from their heterozygous or WT littermates up to postnatal week 2–3, then showed growth retardation and overt dystrophic symptoms.[23] Because merosin is also expressed in the thymus, thymocyte development in mer$^{-/-}$ mice was examined. In association with the onset of dystrophic symptoms, the number of DP thymocytes decreased (Fig. 1a), but CD4$^+$ CD8$^-$ and CD4$^-$ CD8$^+$ single-positive (SP) cells were less affected (Fig. 1b). Total thymocyte number in WT, mer$^{+/-}$, or mer$^{-/-}$ mice during this experiment was 260, 108, or 3·0 ($\times 10^6$ cells/mouse), respectively. The numbers were different in each individual, and mer$^{-/-}$ mice had fewer cells at age 2–3 weeks after birth than WT or mer$^{+/-}$ mice, but there was no significant difference between WT and mer$^{+/-}$ mice in replicate experiments. The expression of SP cell TCR-β and Vβ8 were similar to the WT littermates (Fig. 1c), but the total thymocyte number was reduced, as indicated in the figure legend. Although the data are not shown in this report, splenic cell number in mer$^{-/-}$ mice also decreased to 10–33% of the level observed in WT littermates at age 4–5 weeks. However, the relative compositions of the B220$^+$, CD4$^+$ and CD8$^+$ fractions did not differ from WT littermates.

In order to study the mechanisms for the selective decrease in DP cell number in the absence of merosin, the thymi of mer$^{-/-}$ mice and their littermates were examined histologically. Haematoxylin and eosin staining of the thymus of a mer$^{-/-}$ mouse, compared with that of a WT mouse (Fig. 2a), showed a very thin outer cortex with thymocytes scattered in the inner cortex and medullary area (Fig. 2b). The cortico-medullary junction of the mer$^{-/-}$ thymus was obscure. In the thymus of a WT mouse, a few apoptotic thymocytes were seen (Fig. 2c). On the other hand, a large number of apoptotic cells were observed in the subcapsular and outer cortical areas of a mer$^{-/-}$ mouse (Fig. 2d). An electron micrograph (Fig. 2e) of the outer cortex of a WT mouse thymus (the same as Fig. 2c) showed apoptotic thymocytes phagocytosed by a macrophage and many viable immature thymocytes. Figure 2(f) (the same as Fig. 2d) showed apoptotic cells at various stages of degeneration, mainly in the vacuoles of the epithelial thymic nurse cell-like structure, as described previously,[29] and mature thymocytes were conspicuous instead of immature thymocytes, as seen in Fig. 2(e). A small number of apoptotic thymocyte-containing macrophages were also observed (data not shown). The subcapsular and outer cortex was rich in epithelial cells, as shown in a section from another mer$^{-/-}$ mouse thymus (Fig. 2g). These results indicate that the thymus in Fig. 2(g) shows a more progressive stage of DP cell depletion than that of Fig. 2(d). An electron micrograph (Fig. 2h) showed the subcapsular cortex of a thymus (the same as Fig. 2g) in which three epithelial cell nuclei and one apoptotic cell are seen. Many small vacuoles with



(c)

Mouse	DP (%)	CD4 SP		CD8 SP	
		H57 (%)	Vβ8 (%)	H57 (%)	Vβ8 (%)
+/+	74·8	99·0	26·3	96·2	22·2
-/-	6·6	99·3	20·2	99·2	27·7

Figure 1. Selective cell death in the thymus of mer$^{-/-}$ mice. (a) Thymocytes from wild-type (WT), mer$^{+/-}$ and mer$^{-/-}$ mice of various ages were stained with biotinyl-anti-CD4 (GK1.5) plus phycoerythrin (PE)-streptavidin and fluorescein isothiocyanate (FITC)-anti-CD8 (53-6.7), and the percentages of double-positive (DP) cells are shown. (b) Typical flow cytometric patterns of each thymus from the same littermates are shown (36-day-old mice). (c) Frequencies of T-cell receptor (TCR)-positive cells in single-positive (SP) thymocytes are shown (from 33-day-old mice). Cells were stained with PE-anti-CD4 (RM4-5), FITC-anti-CD8 (53-6.7), and biotinyl-anti-TCR plus PE-Cy5-streptavidin. Total cell numbers of WT and mer$^{-/-}$ mice were 310 and 9·5 ($\times 10^6$ cells/mouse), respectively.

small amounts of non-digestible electron-dense materials derived from apoptotic thymocytes, as described previously,[29] were observed within the epithelium.

DNA fragmentation of the thymocytes was then examined by PI staining. As shown in Fig. 3, a flow cytometric study revealed that apoptotic thymocytes in WT and mer$^{-/-}$ mice comprise 3·0% (Fig. 3a) and 28·3% (Fig. 3b), respectively, of total thymocytes. Although the data are not shown in this report, we then examined cell death by the TUNEL assay. In contrast to WT mice, in which some apoptotic cells appeared to be randomly scattered throughout the thymus, thymocyte apoptosis was observed intensively in limited regions in the mer$^{-/-}$ thymus. These data suggest that DP thymocytes in the mer$^{-/-}$ thymus are removed by apoptotic cell death.

Expression of laminin α2 and integrin α_6 in the thymus

Expression of the laminin α2-chain in the thymus was examined. Thymi from BALB/c mice of various ages were obtained and Western blotting studies were performed. Expression of the laminin α2-chain was observed in thymi from mice at various stages of development (from neonates to adults), as well as in adult mouse skeletal muscle (Fig. 4a, 4b). However, the Sephadex G-10 pass-through fraction of thymocytes, which is depleted in adherent thymic cells, showed no laminin α2-band (Fig. 4a, lane 2). These results suggest that merosin is expressed on the adherent fraction of thymic cells, namely thymic stromal cells. To confirm this, TEC lines from WT and mer$^{-/-}$ mice were examined, by using RT–PCR, for their expression of the mRNA for the laminin α2-chain. As shown in Fig. 4(c), neither purified thymocytes nor TECs from mer$^{-/-}$ mice showed laminin α2-chain bands, while TECs and skeletal muscle from WT mice did. Laminin α2 expression was also observed in the spleen of normal mice of various ages (Fig. 4d).

VLA-6 is known to be a ligand for merosin in the thymus.[13,14] Therefore, the expression of integrin α_6 on thymocytes was examined by using flow cytometry. As shown in Fig. 5, integrin α_6 expression on thymocytes was observed for mice at various stages of development (from neonates to adults). Thus, both laminin α2 and integrin α_6 are already expressed in neonatal mice.

Merosin and anti-VLA-6 antibodies rescue DP thymocytes from cell death

To study the mechanisms for the selective death of DP cells in the absence of merosin, the role of merosin or VLA-6 in the viability of DP cells was examined *in vitro*. Normal adult thymocytes were cultured in the presence or absence of merosin or various integrin mAbs, and cell survival was examined. As shown in Fig. 6, a reduced cell recovery was observed in the group receiving no proteins, or in the presence of control rat IgG. In contrast, the viability of adult BALB/c thymocytes was significantly enhanced in the presence of merosin, or mAb to integrins α_6 or β_1. The mAb to integrin β_1 produced better viability than the mAb to integrin α_6. Integrin α_4, but not integrin α_2, is expressed on thymocytes; however, mAbs to these integrins had no effect on the viability of DP cells.

To elucidate the role of merosin on the interaction between thymocytes and TECs, normal thymocytes were cultured on various TEC lines. S7HoE1, the TEC line from mer$^{-/-}$ mice, had almost no enhancing effect on thymocyte viability when compared with the absence of TECs (Fig. 7). On the other hand, S1Bc and S7wtE1, which are TEC lines from WT and normal BALB/c mice, respectively, produced better recovery of viable DP cells from culture. We attempted co-culture of thymocytes and S7wtE1 cells in the presence of an antilaminin

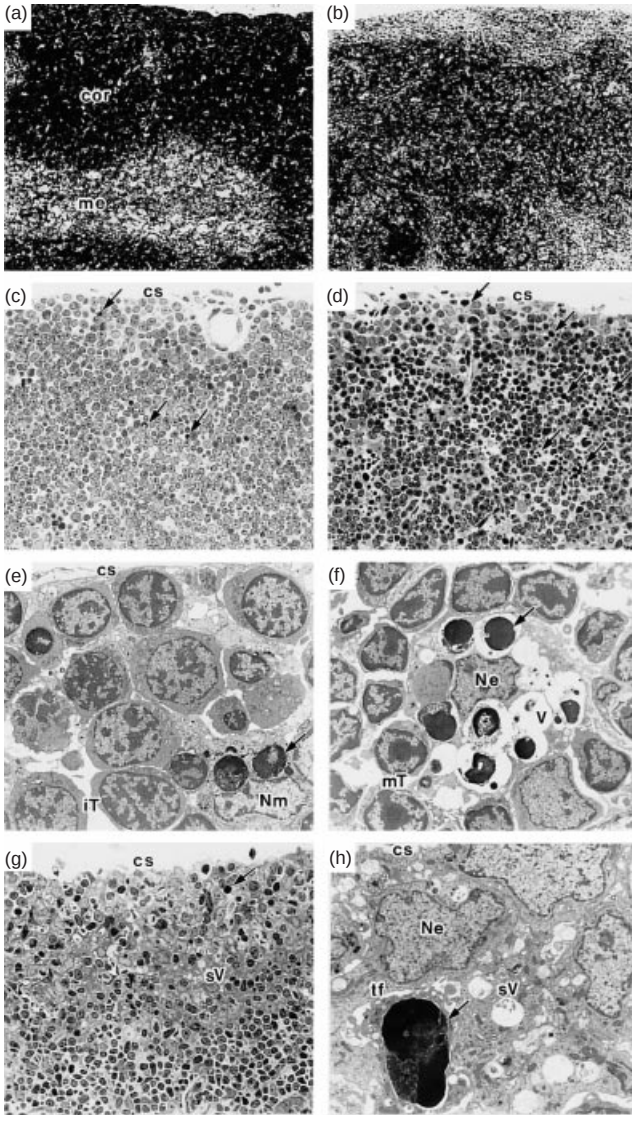


Figure 2. Histological studies of the thymus of mer$^{-/-}$ mice. The thymus of a wild-type (WT) (a) or mer$^{-/-}$ (b) mouse (22 days old) was obtained and a paraffin-embedded section of one lobe from each was stained with haematoxylin and eosin (×60). The frequencies of double-positive (DP) thymocytes were determined, using another lobe, to be 77·9% (a) and 21·1% (b). Epoxy resin-embedded thin sections of the thymi of a WT mouse (c) (×240) or mer$^{-/-}$ mice (22 days old) (d) and (g) (×240) were stained with 1% toluidine blue. Electron micrographs of (c), (d) and (g) are shown in (e) (×1900), (f) (×1900) and (h) (×2600), respectively. Ultrathin sections were stained with 1% uranyl acetate and lead citrate. →, apoptotic thymocyte; cor, cortex; cs, capsule; iT, immature thymocyte; me, medulla; mT, mature thymocyte; Ne, nucleus of thymic epithelial cell; Nm, nucleus of macrophage; sV, small vacuole; tf, tonofilament; V, vacuole.

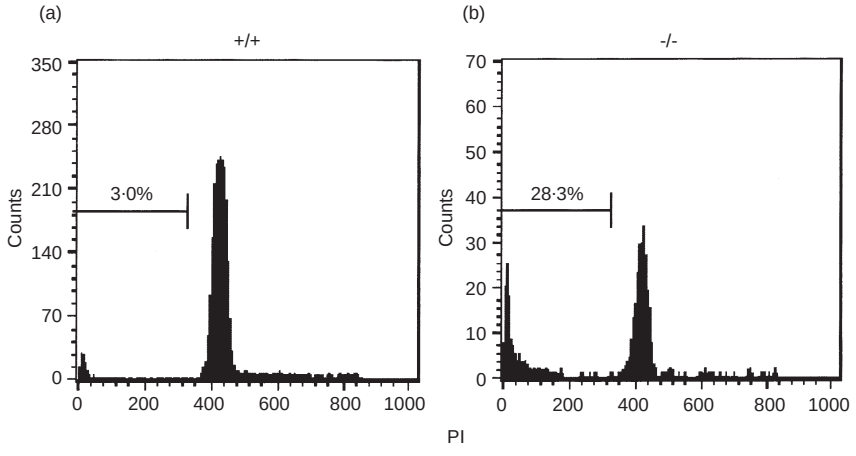


Figure 3. DNA fragmentation of double-positive (DP) thymocytes from mer$^{-/-}$ mice. Freshly isolated thymocytes from wild-type (WT) and mer$^{-/-}$ mice (34 days old) were fixed in 70% ethanol, stained with propidium iodide (PI) and analysed by using flow cytometry. The frequencies of DP cells in WT and mer$^{-/-}$ mice were 79·0% and 31·2%, respectively.

α2 antibody, but the thymocyte viability was inhibited only partially (data not shown). This result may be explained by the fact that the antibody used in the experiment did not sufficiently block the binding of merosin with thymocytes.

Taken collectively, these results suggested that the interaction of merosin from TEC with VLA-6 (or with some undefined molecule(s) in the β_1-integrin family) in DP thymocytes may play a crucial role in the survival of DP thymocytes.

DISCUSSION

Severe thymic atrophy has previously been observed in laminin α2-chain null mutant (*dy*^{3k}/*dy*^{3k}) mice in association with growth retardation and muscular dystrophy. Histological and cellular analyses revealed that the thymic atrophy was a result of selective apoptotic cell death of DP thymocytes. Thymic and splenic abnormalities associated with bodyweight loss in naturally occuring dystrophic mice (*dy*/*dy*) was first described in 1976.[30,31] The histological features of the *dy*/*dy* mice were similar to the results obtained in this study, although TCR-β-positive mature-type T cells were detected in the dystrophic thymus. Merosin is expressed in the spleen and the splenocyte number is also reduced in mer$^{-/-}$ mice, but the proportions of mature T and B lymphocytes are similar to those in normal mice (data not shown). This suggests that merosin has some functions in the maintenance of splenocytes, but the mechanisms underlying this issue are not yet known.

It will be of interest to discover why thymocyte death occurs at a certain age after birth in association with the onset of muscular dystrophy. We examined the developmental changes

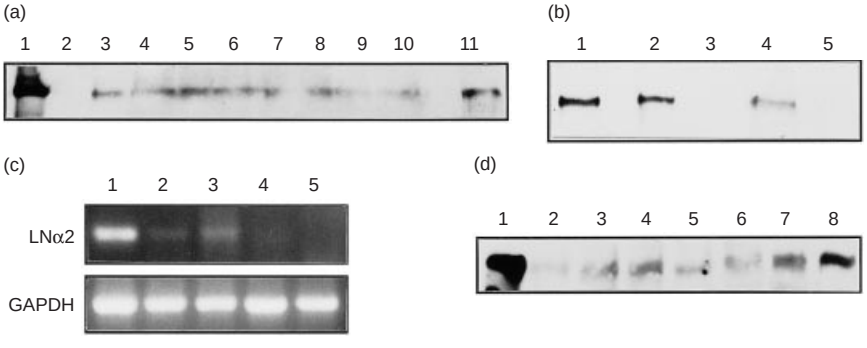


Figure 4. Expression of the laminin α2-chain in the thymus. (a) Western blotting pattern of laminin α2-chain expression in the normal BALB/c thymus. Human merosin (lane 1 as a positive control), the Sephadex G-10 pass-through fraction of thymocytes (lane 2), thymi (lane 3–10, from 54-, 35-, 25-, 21-, 18-, 12-, 7- and 0-day-old mice, respectively), or muscle (lane 11, 54-day-old mice). (b) Western blotting pattern of laminin α2-chain expression in muscle and thymus. Muscle of normal BALB/c (lane 1), mer$^{+/-}$ (lane 2), or mer$^{-/-}$ (lane 3), and thymus of mer$^{+/-}$ (lane 4), or mer$^{-/-}$ (lane 5) mice. (c) Reverse transcription–polymerase chain reaction (RT–PCR) analysis of laminin α2-chain expression in thymic epithelial cell (TEC) lines. RNA from the muscle of normal BALB/c mice (lane 1), TEC line S1Bc (from normal BALB/c mice) (lane 2), TEC line S7wtE1 (from WT mice) (lane 3), S7HoE1 (from mer$^{-/-}$ mice) (lane 4), or Sephadex G-10 pass-through normal thymocytes (lane 5). GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LN, lymph node. (d) Western blotting pattern of laminin α2-chain expression in normal BALB/c mouse spleen. Human merosin (lane 1 as a positive control), spleens (lanes 2–6 from 54-, 40-, 29-, 21- and 12-day-old mice, respectively), thymus (35-day-old mice) (lane 7), or muscle (54-day-old mice) (lane 8).

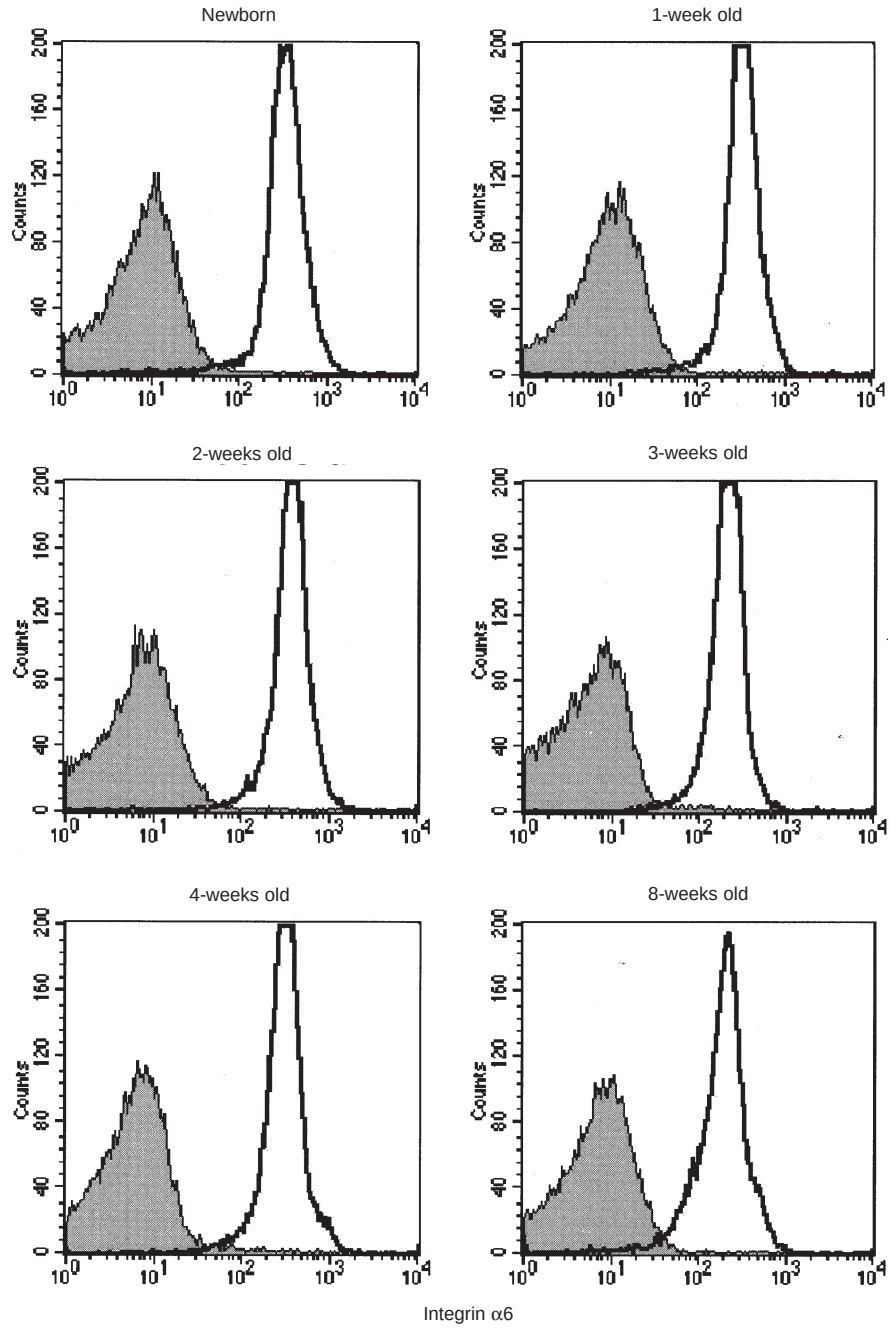


Figure 5. Expression of integrin α_6 on thymocytes from normal BALB/c mice. Thymocytes were isolated from mice at the ages indicated, stained with anti-integrin α_6 plus fluorescein isothiocyanate (FITC)-anti-hamster immunoglobulin G (IgG) and analysed by using flow cytometry. Shaded areas indicate controls without the first antibody.

in merosin and VLA-6 expression in normal thymus. Merosin expression in the thymus was observed from the neonatal to adult (day 54) stages. VLA-6 expression was also found from the neonatal period. These results suggest that the apoptotic death of DP thymocytes is not associated with the timing of the expression of either merosin or VLA-6 in the thymus. It is speculated that neonatal mutant muscles contain some unidentified trophic factors which prevent apoptotic cell death. Such a similar mechanism may be applicable to the thymus.

In a previous study we demonstrated that the degenerating muscle in the $mer^{-/-}$ mice contains a markedly higher number of TUNEL-positive nuclei compared to the muscles of their WT littermates.[23] As described in this work, the number of DP thymocytes was also decreased by apoptotic cell death in association with muscle cell death. It is well known that the injection of glucocorticoids or severe physical stress induces thymic atrophy associated with a selective loss of DP cells. Very recently, Vacchio *et al.* reported that a significant amount of glucocorticoids can be synthesized by TECs, and that locally produced glucocorticoids may be a key element in antigen-

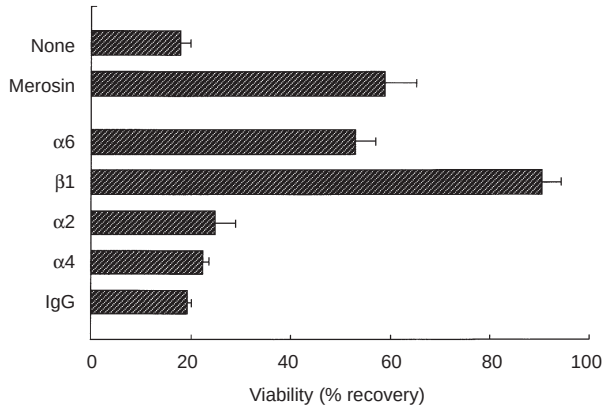


Figure 6. Increased viability of normal thymocytes cultured in the presence of merosin or anti-very late activation antigen-6 (VLA-6) monoclonal antibodies (mAbs). Normal BALB/c thymocytes (from 4-week-old mice) were prepared by passing the thymus cells through a Sephadex G-10 column to remove adherent cells. The cells were cultured for 8 hr in serum-free medium with various anti-integrin mAbs or human merosin (protein concentration 10 μg/ml/5×10^5 cells). Immunoglobulin G (IgG) indicates normal rat IgG. 7-amino actinomycin D (7-AAD)-negative cells were counted. The data shown are mean ± SD (of three independent cultures) and are representative of three separate experiments.

specific thymocyte selection.[32,33] We do not intend to exclude the role of glucocorticoids produced by either the adrenal gland or the thymus in the deletion of DP cells; however, questions arise as to whether the apoptotic death of DP thymocytes is a result of a simple secondary effect of muscular dystrophy and which role merosin plays in the thymus.

Experiments were therefore designed to determine the effect of merosin or VLA-6, a ligand of merosin in the thymus, on the survival of DP thymocytes *in vitro*. Because merosin is produced by TECs, normal thymocytes were depleted of TECs and then cultured in the presence or absence of exogenous proteins. Moreover, the addition of fibronectin supported the viability of the DP thymocytes (data not shown), and FCS was depleted of fibronectin by passing it through gelatin-conjugated gel. These experiments enabled us to determine the *in vitro* effects of merosin and integrins on the survival of DP thymocytes. As clearly shown in Figs 6 and 7, merosin, as well as the mAb to integrins α_6 or β_1, supports the viability of DP thymocytes. That anti-integrin β_1 produced better cell viability than anti-α_6 suggests that β_1 integrin(s) other than VLA-6, such as VLA-3,[14] may also participate in the interaction with merosin in the thymus. The mAbs to integrins α_2 or α_4 had no supportive effect. These effects were confirmed using TEC lines from WT or mer$^{-/-}$ mice. TECs from mer$^{-/-}$ had little effect on thymocyte viability, while TECs from WT or normal BALB/c mice enhanced viability. This result is in agreement with the earlier observation by Vachon *et al.*[34] that merosin promotes myotube stability by preventing apoptosis *in vitro*. Such a preventive mechanism may be operating through merosin and VLA-6 in the thymus. Recently, it has also been shown that the co-ligation of VLA-4 and TCR rescues human thymocytes from dexamethasone-induced apoptosis.[35] Agea *et al.* reported that costimulation of CD3/TCR with integrin protects against the apoptotic cell death of antigen-specific T lymphocytes.[36]

Here we hypothesize that a certain apoptotic death signal(s), including glucocorticoids, is delivered continuously to DP thymocytes and muscular cells, but is blocked by the interaction of merosin-integrin $\alpha_6\beta_1$ in the thymus, and merosin-dystroglycan or merosin-integrin $\alpha_7\beta_1$[37] in muscle. It is suggested that the interaction of ECM proteins and thymocytes influences the intrathymic migration and differentiation of T lymphocytes in the thymus.[1] There is increasing evidence that integrins transmit signals into cells and suppress anchorage-related apoptosis (anoikis).[38,39] Investigation of signalling pathways via ECM proteins will reveal the molecular mechanisms that prevent apoptotic cell death of lymphocytes and muscular cells.

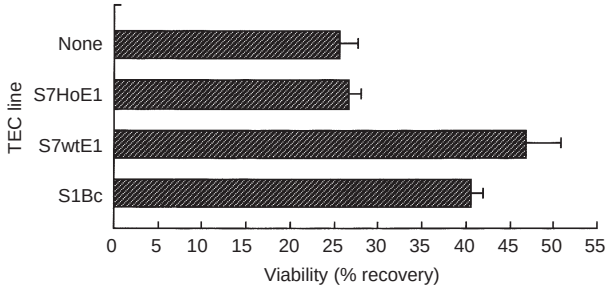


Figure 7. Increased viability of double-positive (DP) thymocytes cultured on laminin α2-chain-expressing thymic epithelial cell (TEC) lines. Sephadex G-10 pass-through normal BALB/c thymocytes (from 6-week-old mice) were cultured for 42 hr in the 2·5% FCS′ (see the Materials and methods for a description of FCS′)-containing medium, and stained with anti-CD4, anti-CD8 and 7-amino actinomycin D (7-AAD). 7-AAD-negative DP cells were counted by using flow cytometry. The frequencies of double-negative (DN), DP, CD4 single-positive (SP), or CD8 SP thymocytes at 0 hr were 1·8%, 84·5%, 9·3%, or 4·4%, respectively. The data shown are mean ± SD (of three independent cultures) and are representative of three separate experiments.

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