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Is There a Role for Cellular Prion Protein in Intrathymic T Cell Differentiation and Migration?

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Key Words
Thymocyte differentiation · Cellular prion protein · Thymic hypoplasia · Laminin · Cell migration

Abstract
The cellular prion protein (PrP^C) is expressed in the nervous and immune systems. Functionally, PrP^C has been suggested to participate in neuron survival, neuritogenesis and T lymphocyte activation. Moreover, PrP^C interaction with laminin influences neuronal adhesion and neurite extension. Nevertheless, so far the physiological role of PrP^C has not been completely elucidated, particularly in the immune system. The aim of the study was to evaluate the possible participation of PrP^C in intrathymic T cell development. We evaluated T cell differentiation markers in thymocytes and peripheral lymphocytes, as well as thymocyte death in PrP^C-null or PrP^C-overexpressing (Tga20) mice, compared to wild-type controls. In these same animals, we ascertained laminin-driven thymocyte migration. Compared to controls, only marginal differences were found in PrP^C-null animals. However, Tga20 mice exhibited a severe thymic hypoplasia, with 10–20% lymphocytes compared to wild-type counterparts. In particular, the frequency of CD4+CD8+ cells was largely reduced, and this was accompanied by a dramatic increase in the frequency of CD4–CD8– thymocytes, which could be as high as 60–65% of the whole-cell suspensions. Moreover, Tga20 mice exhibited an increase in thymocyte death, comprising the CD4+CD8+, as well as CD4+ and CD8+ single-positive cells. Additionally, laminin-driven migration was largely impaired in Tga20 mice, in which we also found a significant decrease in total T lymphocytes in the spleen and lymph nodes. Our results show that PrP^C overexpression alters intrathymic T cell development, a defect that likely has a negative impact in the formation of the T cell peripheral pool.

Introduction

The cellular prion protein (PrP^C) is a glycosylphosphatidylinositol-anchored glycoprotein concentrated in lipid raft microdomains, expressed on a wide range of tissues, including tissues in the nervous and immune systems [1]. Although it is well defined that the pathogenesis of prion diseases occurs through the direct interaction between the constitutively expressed isoform PrP^C and the scrapie

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protease-resistant isoform prion (PrP^{Sc}), with conversion of PrP^{C} to PrP^{Sc} [2], the precise physiological role of PrP^{C} remains unclear.

The ability of PrP^{C} to bind a variety of molecules has been used as a tool to understand PrP^{C} functions. The PrP^{C} binding to laminin was suggested to be involved in neuronal adhesion, as well as neurite extension and maintenance. In addition, other adhesion molecules like laminin receptor, heparan sulfate and neural cell adhesion molecule have been described as PrP^{C}-binding proteins. Moreover, PrP^{C} interactions with Bcl-2 family members and stress-inducible protein-1 have been suggested to protect neurons from apoptosis. Accordingly, PrP^{C} capacity to bind and chelate copper seems important in neuronal resistance to oxidative stress [for reviews, see 3, 4].

Although the nervous system is the main site for PrP^{C} expression, this molecule is also widely expressed in the immune system [1]. Importantly, PrP^{C} expression is increased upon T lymphocyte activation [5], PrP^{C} engagement induces the recruitment of signaling cascade molecules [6], and PrP^{C} is involved in MHC-peptide-driven T lymphocyte-dendritic cell interactions [7]. Additionally, it seems to modulate phagocytosis, likely being involved in inflammatory responses [8].

In situations of PrP^{C} overexpression, deviations of physiological functions were observed, including delivery of proapoptotic messages when PrP^{C}-mediated signaling exceeds a certain threshold [9, 10], as well as PrP^{C} overexpression in brain endothelial cells in a rat model of cerebral ischemia [11].

In the thymus, PrP^{C} is expressed by thymocytes [12], thymic epithelial cells (TEC) and dendritic cells [1]. More recently, it has been reported that PrP^{C} transgenic mice exhibit an intense thymic hypoplasia with depletion in the CD4+CD8+ thymocyte subpopulation, paralleled by accumulation of CD44–CD25+CD4–CD8– cells, possibly arrested in the DN3 stage of differentiation, differentially impacting on αβ and γδ T cell lineages [10].

The thymus is a primary lymphoid organ in which bone marrow-derived cell precursors undergo T lymphocyte differentiation. Such a process involves sequential expression of various proteins and rearrangements of T cell receptor (TCR) genes, as the developing thymocytes migrate in the thymic lobules. This migration allows thymocytes to encounter cortical and medullary nonlymphoid microenvironments through distinct cell-cell and cell-matrix interactions, which interfere with the differentiation process. Finally, positively selected mature T lymphocytes leave the thymus, migrating to the T cell-dependent areas of secondary lymphoid organs [for reviews, see 13, 14]. Although the understating of this migration process is improving [15, 16], many gaps are still present, particularly if we consider that thymocyte migration is a result of multivectorial stimuli [17], whose complete ligand-receptor pair chart has not been completed so far.

In any case, considering that (1) PrP^{C} binds laminin [18, 19], which is a relevant extracellular matrix in thymus physiology [15], (2) PrP^{C} is involved in peripheral T cell activation [6, 20] and (3) it is constitutively expressed in the thymus [1], it appeared plausible that PrP^{C} plays a role in thymus physiology. Herein, we evaluated thymocyte differentiation and migration parameters in animals expressing distinct levels of PrP^{C}. Moreover, changes seen in the thymus of PrP^{C}-overexpressing Tga20 mice were correlated with the diminished amounts of T cells seen in peripheral lymphoid organs.

Materials and Methods

Animals

Wild-type control mice from inbred C57BL6/J × Sv129 mixed lineage, PrP^{C}-null ($Prnp^{0/0}$) [21] mice from an inbred colony and PrP^{C} transgenic mice overexpressing PrP^{C} (Tga20), kindly provided by Dr. Charles Weissmann (Scripps Florida, USA) [22], were used in these experiments. $Prnp^{0/0}$ mice and Tga20 animals have the same genetic background of the wild-type mice. Most experiments were done using 4- to 6-week-old females. Some experiments were performed in newborn as well as middle-aged (36–40 weeks) females, whereas in others we used young adult males. In all cases, at least 5 animals were evaluated per group. Mice were handled according to procedures approved by the Ethical Committee for Animal Research of the Oswaldo Cruz Foundation (Rio de Janeiro, Brazil).

Thymic Microenvironmental Cell Lines

The expression of PrP^{C} was also evaluated in the TEC lines named 1.4C18, 1C6 and 2BH4, representatives of cortical, medullary and mixed phenotypes, respectively [23–25]. Additionally, we searched for PrP^{C} in the thymic endothelial cell line tEnd.1 [26]. All these cell lines have a C57BL/6 mouse genetic background. They were grown in RPMI 1640, supplemented with 10% fetal calf serum, 2 mM glutamine, 100 U/ml penicillin and 100 U/ml streptomycin (Gibco, UK).

Antibodies

For flow cytometry analyses, the following fluorochrome-labeled monoclonal antibodies (mAb) were used: anti-CD3-FITC, anti-TCRβ-PE, anti-CD4-PE and anti-CD4-PerCP, anti-CD8-APC and anti-CD8-PerCP, anti-CD44-PE, anti-CD25-FITC and antiCD25-APC, anti-CD90-FITC, as well as isotype-matched negative controls for each fluorochrome applied as specific antibodies (Becton Dickinson, San Diego, Calif., USA). We also used the mouse mAb anti-PrP^{C} SAF-32 (SPI-bio), the corresponding Ig

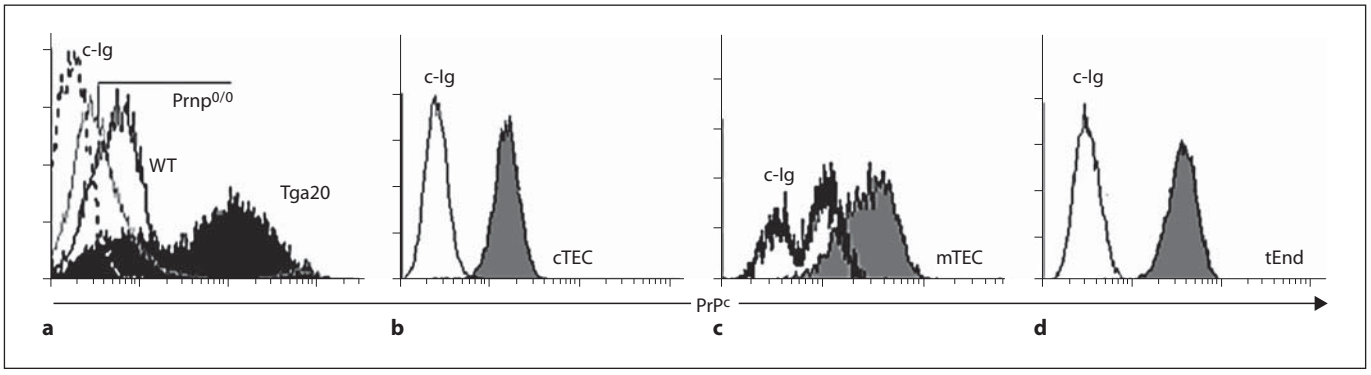

Fig. 1. Thymocytes and thymic microenvironmental cells constitutively express cellular prion protein. **a** PrP^C expression in total thymocytes from wild-type (WT), PrP-null ($Prnp^{0/0}$) and PrP^C transgenic (Tga20) mice. For comparison, the negative control using an unrelated antibody (c-Ig) is also shown (dotted line). **b**, **c** PrP^C expression in cortical (cTEC) and medullary (mTEC) thymic epithelial cell line. **d** PrP^C labeling in a thymic endothelial cell line (tEnd). PrP^C expression is shown in gray, whereas unrelated Ig (c-Ig) is represented by the open histograms.

negative control (Sigma Co., St. Louis, Mo., USA) and the secondary antibody Alexa-488-labeled goat anti-mouse (GAM-488) Ig serum (Amersham Biosciences, Piscataway, N.J., USA).

Flow Cytometry

Cells were isolated from thymus and subcutaneous lymph nodes, after organ maceration. The thymic epithelial and endothelial cell lines were detached from the culture flasks by using 10 mM EDTA. Once counted in a Neubauer chamber they were submitted to cytofluorometry for PrP^C detection.

Fluorochrome-labeled mAb staining was performed by incubating cells with murine normal serum for 15 min and subsequently with primary antibodies for 20 min. PrP^C staining was performed as follows: cells were first incubated with PBS/BSA 0.5%/sodium azide 0.05% during 15 min, and then subjected to the anti-PrP^C antibody for 20 min. After washing in PBS, PrP^C labeling was revealed with GAM-488 during 20 min. Cell suspensions were washed again in PBS, and fixed in 1% formaldehyde.

Annexin V staining was carried out using the appropriate buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$), in which cells were incubated with annexin V-FITC (Caltag/Invitrogen, USA) during 20 min at room temperature. Flow cytometry analyses were carried out on a FACSCalibur® device (Becton Dickinson) equipped with CellQuest software.

Cell Migration Assay

Thymocyte migratory activity was assessed in a transwell system, as previously described [27]. Briefly, 5-μm pore size transwell plates (Corning Costar, Cambridge, Mass., USA) were coated with 10 mg/ml of murine laminin (Sigma) for 1 h at 37°C, and later blocked with BSA 10 μg/ml. Thymocytes from pools of at least 3 animals (2.5×10^6 cells in 100 μl of BSA 1%/RPMI) were added in the upper chambers. After 4 h of incubation at 37°C in 5% CO_2 humidified atmosphere, laminin-driven migration was defined by subtracting the numbers of cells that migrated to the lower chambers containing only the migration medium (BSA 1%/RPMI).

Statistical Analysis

Unless stated otherwise, data were shown as means ± standard error. Results were statistically analyzed by one-way analysis of variance and subsequently by the Tukey post hoc test. Differences were considered statistically significant when the p values were <0.05.

Results

PrP^C Expression in Thymocytes and in Thymic Microenvironmental Cells

In normal mice, constitutive PrP^C expression was seen in thymocytes, comprising all CD4/CD8-defined subpopulations (fig. 1a). Interestingly, large densities of PrP^C were seen in all TEC lines analyzed, as well as in thymic endothelial cells (fig. 1b). As expected, we found increased PrP^C expression in thymocytes from Tga20 mice, compared to age-/sex-matched wild-type animals, whereas no significant PrP^C labeling was seen in thymocytes from $Prnp^{0/0}$ mice. Most importantly, overexpression of PrP^C correlated with a dramatic defect in intrathymic T cell development, comprising a significantly lower number of thymocytes in the organ, and accumulation of CD4–/CD8–cells, in parallel with very low numbers of the CD4+/CD8+ subset. Interestingly, this profile was already seen in newborn animals and remained in middle-aged mice (data not shown).

By contrast, the relative numbers of CD4/CD8-defined cell subsets in the thymus of $Prnp^{0/0}$ mice were similar to those seen in the wild-type animals, although a

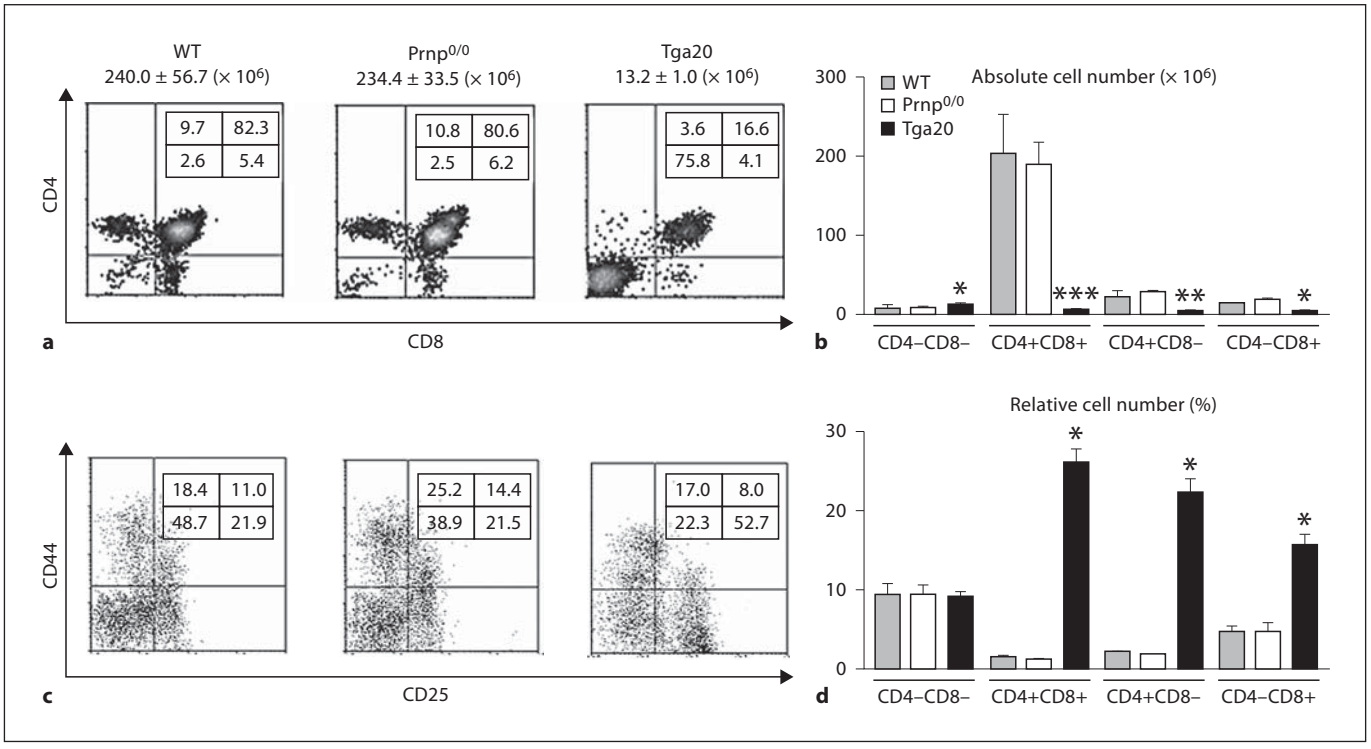

Fig. 2. Hypoplasia and partial arrest of thymocyte development in PrP^C transgenic mice. **a** Cellularity and CD4/CD8-defined thymocyte subsets in wild-type (WT), PrP^C-deficient ($Prnp^{0/0}$) and PrP^C transgenic (Tga20) mice. Total cell numbers, shown in the upper part of the flow cytometry profiles of each group, clearly demonstrated the severe decrease in thymic lymphocytes from Tga20, compared to WT or $Prnp^{0/0}$ mice ($p < 0.001$). Moreover, the cytofluorometric profiles show that PrP^C transgenic animals exhibit a large reduction of CD4+CD8+ cells, with a relative enhancement in CD4–CD8– thymocytes. Relative cell numbers for each CD4/CD8-defined thymocyte subset are shown inside the flow cytometry profile of each group. **b** In terms of absolute cells numbers, not only double-positive, but also CD4 as well as CD8 single-positive cells were largely reduced. **c** Differential expression of CD25 and CD44 cell markers within the CD4/CD8 double-negative compartment. In the Tga20 group, there is a clear reduction of the relative cell numbers of DN3 and DN4 cells (CD44–CD25+ and CD44–CD25–, respectively). **d** Part of the reduced thymocyte numbers seen in Tga20 mice is due to a significantly increased apoptosis (revealed by annexin V labeling), observed in CD4/CD8 double-positive and single-positive cells. Data are represented as means ± standard error of at least 6 animals. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared to WT mice.

slight decrease in the total thymocyte numbers was consistently seen in the absence of PrP^C.

Partial Arrest of Thymocyte Differentiation in Mice Overexpressing PrP^C: Relationship with Cell Death

Considering the high relative numbers of CD4–/CD8– in the thymus of Tga20 mice, we first evaluated whether these cells actually belonged to the T cell lineages. Using anti-CD90 as a pan-T cell marker, we could see that CD90 staining was similar in thymocytes from wild-type, $Prnp^{0/0}$ and Tga20 animals (data not shown).

We further analyzed the abnormal thymocyte development seen in Tga20 mice by studying the sequential stages of differentiation in the CD4–/CD8– compartment, as ascertained by CD44 and CD25 labeling. Such an evaluation clearly revealed that cells accumulate in the DN3 (CD25+CD44–) stage of differentiation, with significant decrease in the absolute numbers of downstream differentiation subsets, namely DN4 (CD25–CD44–), CD4+CD8+, as well as mature thymocytes, and CD4 and CD8 single-positive cells (fig. 2a–c).

Accumulation of DN3 thymocytes in PrP^C transgenic mice could be due to a deviation favoring the expansion of discrete CD4–CD8– thymocyte lineages, bearing the CD3/TCRαβ or the CD3/TCRγδ phenotype. Overall, we found an increase in the percentages of CD3+ TCRγδ+CD4–CD8– cells in the thymuses from Tga20 animals. Nevertheless, the absolute numbers of these

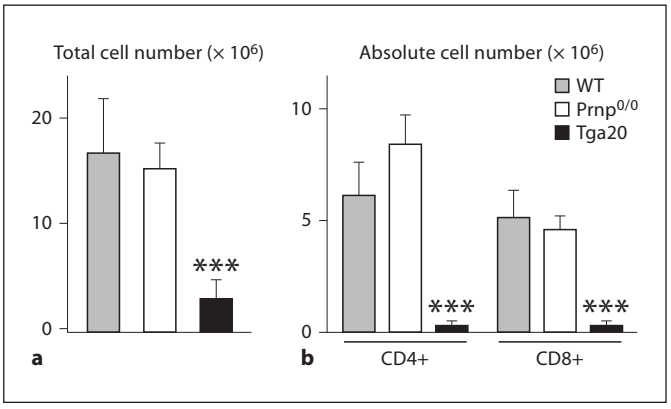

Fig. 3. Decrease in T cell numbers in subcutaneous lymph nodes from PrP^C transgenic mice. Total (**a**) and absolute (**b**) cell numbers of TCRβ+CD4+ and TCRβ+CD8+ lymphocytes in subcutaneous lymph nodes. Tga20 animals exhibit a highly significant decrease in T cells, compared to wild-type (WT) controls or PrP^C-deficient ($Prnp^{0/0}$) mice. Note that data are represented as means ± standard error in each mouse strain (n = 5). *** $p < 0.001$.

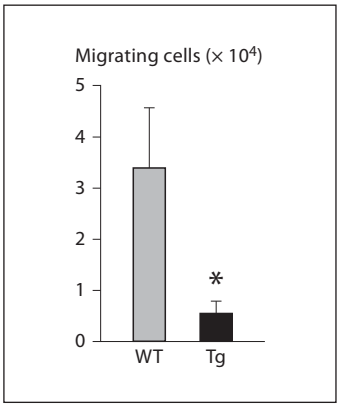

Fig. 4. Impaired laminin-driven cell migration in thymocytes from PrP^C transgenic mice. Thymocyte migration induced by laminin was largely impaired in PrP^C transgenic mice (Tg) compared to wild-type controls (WT). The numbers of laminin-driven migrating thymocytes were defined by subtracting the numbers of cells that migrated in the presence of the migration medium (BSA 1%/RPMI) alone. The figure represents the average of 3 independent experiments, each performed with a pool of 3 animals. * $p < 0.05$.

cells did not change, when compared to the wild-type or $Prnp^{0/0}$ counterparts (data not shown). These findings argue against a preferential expansion of the TCRγδ lineage, but rather point to a defect in the differentiation of CD3/TCRαβ cells.

One reason for such a defect could be an enhancement of cell death. We evaluated the levels of annexin V in freshly isolated thymocytes. The percentages of annexin V-positive cells were actually increased in Tga20 thymocytes, compared to wild-type controls. Such an increase was seen in immature CD4+CD8+ as well as mature CD4 and CD8 single-positive subsets (fig. 2d). This finding indicates that thymocytes from Tga20 mice are much more sensitive to in vivo proapoptotic signals. This is further supported by the fact that the relative numbers of apoptotic cells, after treatment with hydrocortisone, were higher in Tga20 mice than in controls (data not shown).

Decreased Numbers of Peripheral T Cells in PrP^C Transgenic Mice

Given the altered thymocyte differentiation seen in Tga20 mice, with very low numbers of thymocytes, increased cell death and accumulation of very immature cell subsets, we investigated if these animals also exhibited abnormal numbers of T cells in their peripheral lymphoid organs. In fact, we found a significant decrease in the relative and absolute numbers of both TCRαβ+CD4+ and TCRαβ+CD8+ T lymphocytes, as ascertained in subcutaneous lymph nodes (fig. 3). Interestingly, such a defect was again specific to the PrP^C overexpression condition, since $Prnp^{0/0}$ mice did not exhibit significant alterations in lymph nodes when compared to their wild-type counterparts (fig. 3). Of note, we observed an increase in TCRγδ T lymphocytes in spleen and mesenteric lymph nodes (data not shown).

Abnormal Laminin-Driven Thymocyte Migration in PrP^C Transgenic Mice

Considering the low numbers of T cells seen in peripheral compartments of PrP^C-Tg mice, together with the fact that PrP^C is a laminin-binding protein and that laminin is involved in thymocyte migration [3, 15, 27], it seemed plausible to raise the hypothesis that laminin-driven migration could also be altered in Tga20 mice. Ex vivo experiments in transwell chambers revealed that the migratory response of Tga20 thymocytes to laminin was absent or largely impaired, compared to the well-known haptotactic response seen with thymocytes from wild-type controls (fig. 4), revealing that, in addition to abnormal differentiation, thymocyte migration is altered in conditions of PrP^C overexpression.

Discussion

The precise role of PrP^C in the immune system is to be determined. Yet, the fact that PrP^C is constitutively expressed in primary and secondary lymphoid organs [1, 7–10] leads to the hypothesis of a relevant physiological role of this protein. Herein, we confirmed that PrP^C is expressed by the majority of thymocytes, comprising all CD4/CD8-defined subsets. Interestingly, PrP^C is also expressed by thymic microenvironmental cells. We showed that TEC lines representing cortical and medullary epithelial cells spontaneously express large amounts of PrP^C, and so does a thymic endothelial cell line. Interestingly, thymic dendritic cells also express PrP^C [29]. Taken together, these data indicate that PrP^C is expressed, not only by developing thymocytes, but also by key microenvironmental elements, responsible for positive and negative selection of the T cell repertoire, as well as transmigration across blood vessel walls. This is in keeping with the role proposed for PrP^C in transendothelial monocyte migration [28], which is likely linked to the recently reported binding of PrP^C to E-selectin [30].

We demonstrated herein that overexpression of PrP^C results in a severe disturbance in the thymus, with likely consequences for the generation and/or maintenance of the peripheral T cell pool. This concept was raised from studies performed in mice with distinct levels of PrP^C expression. Compared to wild-type age-matched controls, lack of PrP^C generated a discrete reduction in the total thymocyte numbers, whereas a severe decrease in thymus cellularity was seen in PrP^C transgenic mice. In this respect, our data confirm the findings recently reported by Jouvin-Marche et al. [9].

The thymic hypoplasia seen in Tga20 mice was mainly due to a severe decrease in the numbers of CD4+CD8+ thymocytes, with simultaneous accumulation of cells in the CD25+CD44– DN3 stage of thymocyte differentiation, when the primary event of β-selection of developing thymocytes occurs. This is in keeping with the findings showing that PrP^C influences the signaling efficiency of the TCR complex [5, 20, 31].

One might argue that the accumulation of double-negative cells could be partially due to increase in non-T cell lineage(s). However, this is not the case, since these cells were labeled by the pan-T cell marker CD90.

Several mechanisms may be involved in the thymic hypoplasia seen in Tga20 mouse thymus. Herein, we investigated whether thymocytes from these animals were more sensitive to apoptosis. This was actually the case, as we found an increase in thymocyte death, in the CD4/CD8 double-positive as well as single-positive subpopulations. Such increase could be explained by a more sensitized state to apoptosis, as it was demonstrated for the PrP^C-overexpressing HEK293 human kidney cell line, in which activation of caspase-3 has been seen [11]. Nevertheless, data from our group show that PrP^C exerts an antiapoptotic role in neurons [32], suggesting that the involvement of PrP^C in induction versus protection from programmed cell death may vary according to the tissue, or the cell type in a given tissue. In this respect, it is conceivable that the interaction of PrP^C with a given extracellular matrix component may define which signal (pro- or antiapoptotic) will be triggered. This issue is presently being investigated.

Another relevant aspect possibly linked to the thymic hypoplasia seen in Tga20 mice is related to peripheral T cell pool. Compared to age-matched controls, we found a decrease in both CD4+ and CD8+ T lymphocyte relative numbers in subcutaneous lymph nodes of Tga20 animals. This may be due to a defect in the homing of recent thymic emigrants to these lymph nodes. In this respect, we found a dramatic impairment in the laminin-induced migration of Tga20-derived thymocytes. Since laminin is known to be involved in thymocyte migration [16], it is likely that, in addition to the higher sensitivity to death signals, thymocytes from transgenic mice exhibit defects related to cell migration, thus raising the general concept that overexpression of PrP^C generates a multifaceted disturbance in the thymus. Whether or not other thymocyte migration-related molecules are modulated in transgenic animals is presently under investigation.

In any case, at least one of the consequences of such multifaceted intrathymic disturbance is the reduced numbers of T cells seen in the peripheral lymphoid organs of these animals. Experiments are now in progress to evaluate the ability of Tga20 peripheral T lymphocytes to mount an immune response. In this respect, the data discussed above indicate that PrP^C should be placed among those molecules that contribute to the shaping of the T cell repertoire as well as the population dynamics of T lymphocytes.

Acknowledgments

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