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DIFFERENTIATION INHIBITION OF REGULATORY T CELLS IN HIROSAKI HAIRLESS RAT DUE TO DELETION OF THE *LY49* FAMILY GENES EXPRESSED IN THYMIC T CELLS AND DENDRITIC CELLS

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Abstract The Hirosaki hairless rat (HHR) is a mutant strain derived from the Sprague-Dawley rat (SDR). The HHR thymus was markedly small and showed underdeveloped medulla. In the HHR thymus, CD4 levels were decreased, suggesting that differentiation of CD4-positive T cells is disturbed. Because naturally occurring regulatory T (nTreg) cells complete the differentiation in the thymus, the maturation status of the cells was examined in the HHR thymus. Real-time PCR revealed reduced expression of the *Foxp3* and *CD25* (*IL-2Rα*) genes, important for nTreg differentiation, and flow cytometric analysis showed decreased number of $CD4^+CD25^+Foxp3^+$ nTreg cells, indicating that mature nTreg formation is repressed. To explore genes responsible for the failure of nTreg differentiation, comparative genome hybridization array was performed using DNA from HHR and SDR. This analysis identified two regions deleted on HHR chromosome 4. One region contained the *Klra17* (*similar to Ly49si1*) gene and the other the *Ly49s3*, *Ly49s4*, *Ly49i3* and *Ly49i4* genes. RT-PCR of T cells and dendritic cells (DCs) isolated from the SDR thymus revealed that the *Klra17* gene was expressed in CD4-single positive (CD4-SP) cells and the *Ly49s3* gene in DCs, indicating that HHR loses expression of the *Klra17* and *Ly49s3* genes in thymic CD4-SP cells and DCs, respectively. The mixed culture of CD4-SP cells and DCs from the HHR thymus exhibited poor nTreg differentiation. Taken together, these results suggest that the *Klra17* and *Ly49s3* genes are responsible for the differentiation of nTreg cells.

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Key words: regulatory T cell; the *Ly49* family genes; Hirosaki hairless rat

Introduction

Lymphocyte differentiation is a highly orchestrated process tightly regulated by interaction between lymphocytes and the cells supporting their differentiation. In the thymus, T cells are positively and negatively selected via interaction with antigen-presenting stromal cells including thymic epithelial cells and dendritic cells (DCs)[1, 2)]. These processes are mediated by a wide variety of molecules produced by these cells such as TCR, CD4, CD8, MHC, costimulatory molecules, cytokines and chemokines[1-3)]. Disruption of the regulatory system for T cell differentiation results in inappropriate suppression or activation of effector functions of lymphocytes which lead to immunosuppressive or autoimmune states[4-6)]. A precise analysis of these immune disorders will improve understanding of the regulatory mechanisms of lymphocyte differentiation.

The Hirosaki hairless rat (HHR), spontaneously derived from the Sprague-Dawley rat (SDR) in our laboratory in 1985, is a mutant strain with a nearly bare phenotype which is inherited in an autosomal recessive manner[7)]. We have recently demonstrated a deletion of 80 kb of genomic DNA in the q36 region on chromosome 7 containing

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five basic keratin genes in HHR as the reason for the hairless phenotype[8]. The most famous hairless animals are nude mice and rats. In these animals, the *Foxn1* gene, encoding a transcription factor critical to the differentiation and survival of thymic and skin epithelial cells, is mutated and this mutation results in both of hairless phenotype and severe thymic defects leading to a loss of T cell development[9, 10]. Other than *Foxn1* mutation, defects of the genes like *IL-2R*, *Jak3*, *IL-7R*, *Rag1*, *Rag2*, and *ADA* have been reported as the reasons of severe combined immunodeficiencies in humans and animals[11]. Prompted by this line of knowledge, we assessed immunological features of HHR and obtained evidence indicating the lymphoid abnormalities in HHR. We here describe developmental failure of regulatory T cells in HHR.

The thymus is underdeveloped in HHR

In the HHR peripheral blood, white blood cell count (WBC) was decreased, while red blood cell count, hematocrit level and hemoglobin concentration were similar in comparison with those in the SDR peripheral blood. The morphological analysis of leukocytes revealed a decrease in lymphocytes in the HHR peripheral blood. Among the lymphoid organs, whereas no remarkable difference in appearance and weight of the spleens was observed between HHR and SDR, the thymus of HHR was markedly smaller than that of SDR. Histological analysis showed that the medulla, the site of T cell maturation, was underdeveloped in the HHR thymus. From these findings, it is speculated that differentiation of T cells is disturbed in the HHR thymus.

Differentiation of regulatory T cells is inhibited in the HHR thymus

Consistent with our speculation, flow cytometric analysis showed increased number of thymocytes with low CD4 levels and real-time RT-PCR analysis showed low expression of the *CD4* gene in the HHR thymus, suggesting that differentiation of $CD4^+$ cells is impaired in HHR. Among $CD4^+$ T cell subsets, naturally occurring regulatory T (nTreg) cells are known to complete their differentiation in the thymus[12]. We, therefore, examined the differentiation status of nTreg cells in the HHR thymus. Real-time RT-PCR showed decreases in abundance of *Foxp3* mRNA, encoding a master regulatory transcription factor for nTreg differentiation, and *CD25* mRNA, encoding IL-2 receptor α chain (Rα) important for nTreg differentiation, in CD4-single positive (CD4-SP) cells from the HHR thymus, suggesting that differentiation of nTreg cells is impaired in the HHR thymus. Indeed, flow cytometric analysis revealed that the number of $CD4^+CD25^+Foxp3^+$ nTreg cells was decresed (Fig. 1). Leukocyte infiltration, one of the features of autoimmune diseases, was actually observed in the dermis of HHR, supporting the idea that nTreg differentiation is impaired in the HHR thymus.

Expression of the *Ly49* family genes are lost in CD4-SP cells and DCs in the HHR thymus

The next interest is identification of the gene(s) for the failure of nTreg differentiation in the HHR thymus. To address this matter, comparative genome hybridization (CGH) array analysis was performed using genomic DNAs from HHR and SDR. This analysis revealed two regions deleted on HHR chromosome 4. One region contained the *Klra17* (*similar to Ly49si1*) gene and the other the *Ly49s3*, *Ly49s4*, *Ly49i3* and *Ly49i4* genes. The Ly49 family proteins were originally identified as receptors on the surface of natural killar (NK) cells for MHC class I molecules on the target cells. When they bound to MHC class I molecules, they transmit

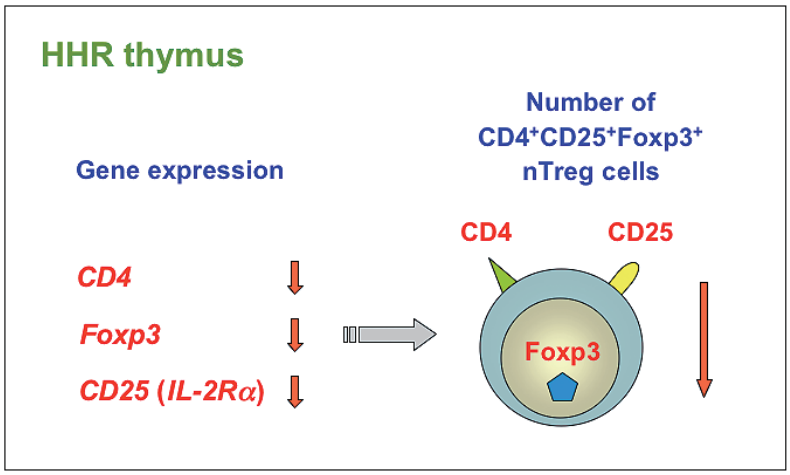

Figure 1. Differentiation inhibition of nTreg cells in the HHR thymus.
In the HHR thymus, expression levels of the *CD4*, *Foxp3* and *CD25* (IL-2Rα) genes, important for differentiation of nTreg cells, were lower than that in the SDR thymus. The number of $CD4^+CD25^+Foxp3^+$ nTreg cells was actually decreased in the HHR thymus.

inhibitory signals to prevent NK cells from mediating cytotoxicity[13]. It is known that some members of Ly49 family proteins are expressed on T cells and DCs[14-17]. The function of these members is supposed to be different from that of the members on NK cells. We examined whether the *Ly49* family genes deleted in HHR are expressed in the thymus of normal rat. RT-PCR analysis showed that the *Klra17* and *Ly49s3* genes were expressed in the SDR thymus. Further analysis with fractionated cells from the HHR thymus revealed that the *Klra17* gene was expressed in CD4-SP cells and the *Ly49s3* gene in DCs. It is, thus, indicated that HHR loses expression of the *Klra17* and *Ly49s3* genes in CD4-SP cells and DCs, respectively.

We previously demonstrated deletion of five basic keratin genes in HHR[8]. However, expression of these genes were hardly detected in the SDR thymus, suggesting that they have no correlations with T cell development and are not the causal genes of the differentiation inhibition of nTreg cells in the HHR thymus.

Co-culture of CD4-SP cells and DCs from the HHR thymus fails to differentiate into regulatory T cells

The above results prompt us to perform experiments to assess whether differentiation potential of CD4-SP cells and/or differentiation-promoting potential of DCs are altered in the HHR thymus. To this end, we isolated these cells and co-cultured them, and determined expression levels of the genes involved in nTreg differentiation and function. The results showed that expression levels of the *c-Fos*, *IL-2*, *CD25*, *CTLA4* and *PD-1* genes, markers for T cells, were lower in the co-culture of cells from the HHR thymus in comparison with that in the co-culture of cells from the SDR thymus. Addition of IFNα, usually functions as an activator of lymphocytes and DCs, further downregulated expression of these genes in the co-culture of the HHR cells, whereas upregulated expression of all genes except the *CTLA4* gene in the co-culture of the SDR cells. These results suggest that CD4-SP cells from the HHR thymus are

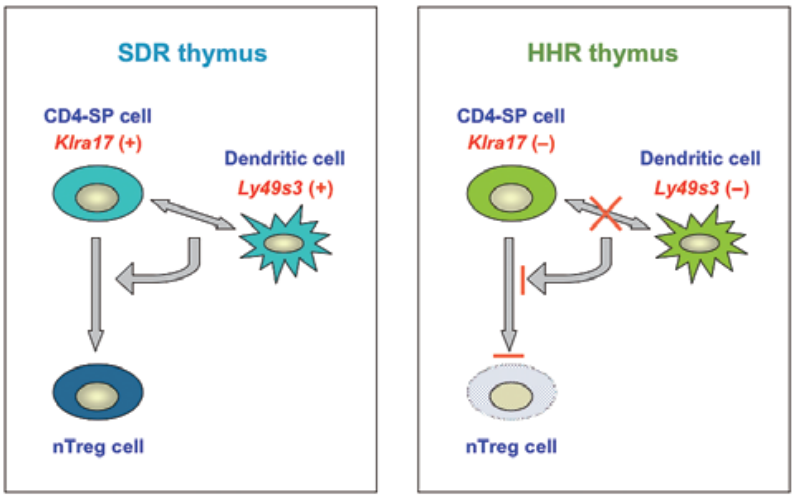

Figure 2. Loss of expression of the *Ly49* family genes as the reason of the differentiation inhibition of nTreg cells in the HHR thymus.

nTreg cells are differentiated in the thymus from *Klra17* (*similar to Ly49si1*)-positive CD4-SP cells through interaction with *Ly49s3*-positive DCs. In the HHR thymus, *Klra17*-negative CD4-SP cells cannot be differentiated into nTreg cells possibly through failure in interaction with DCs.

not differentiated effectively into regulatory T cells after stimulation with DCs from the HHR thymus. Given that the interaction between CD4-SP cells and DCs is important for nTreg differentiation and the Ly49 family members are cell surface receptors mediating cell-cell interaction, alterations in both cell types due to deletion of the *Klra17* and *Ly49s3* genes may lead to the differentiation inhibition of nTreg cells in the HHR thymus (Fig. 2).

Conclusion

In this article, we described immunological feature of HHR, showing low WBC count in the peripheral blood, small thymus, differentiation failure of nTreg cells in the thymus, loss of expression of the *Klra17* and *Ly49s3* genes in CD4-SP cells and DCs in the thymus, respectively, due to deletion of the genes. We also described decreased expression of the genes associated with differentiation and function of nTreg cells in the co-culture of CD4-SP cells and DCs from the HHR thymus. These results suggest that loss of expression of the *Klra17* and *Ly49s3* genes in CD4-SP cells and DCs, respectively, is responsible to differentiation inhibition of nTreg cells in the HHR thymus. Further studies to directly demonstrate this notion are being performed in our laboratory.

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