論 文 要 旨

Reduced Tim-3 expression on human T-lymphotropic virus type I (HTLV-I) Tax-specific cytotoxic T lymphocytes in HTLV-I infection

（HTLV-I 感染症における HTLV-I Tax 特異的細胞傷害性 T リンパ球上の Tim-3 発現の低下）

ナシュワ ハゼイン アブデルバリー リスク

【Introduction and Objectives】

Human T-lymphotropic virus type I (HTLV-I) is a retrovirus that preferentially infects $CD4^+$ lymphocytes in vivo. Although HTLV-I infection is life long, less than 1% of infected individuals develop HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP), a neurologic disease. HTLV-I proviral load and frequency of HTLV-I-specific $CD8^+$ cytotoxic T lymphocytes (CTLs) are increased in the peripheral blood of patients with HAM/TSP as compared to asymptomatic carriers. Although increasing evidence supports the hypothesis that such a strong CTL response could certainly contribute to the control of viral replication and disease development, the exact pathogenic role of the CTL responses remains unclear. T-cell immunoglobulin and mucin domain-containing molecule-3 (Tim-3) and programmed cell death-1 (PD-1) are T-cell exhaustion molecules and it remains unclear whether CTL function is impaired in HAM/TSP patients. In this study, we investigated Tim-3 and PD-1 expression in HTLV-I infection. In particular, we studied HTLV-I-specific CTLs and their degranulation activity in HAM/TSP patients and asymptomatic carriers as well as the role of Tim-3 and PD-1 in regulating their function.

【Materials and Methods】

Using the PBMCs of 32 HAM/TSP patients, 31 asymptomatic carriers (ACs) and 11 healthy controls (HCs), by the flow cytometer, we detected:

- Tim-3 or PD-1expression on CD3+CD4+, CD3+CD8+ and CD8+Tax tetramer+ cells.
- IFN-γ production in Tim-3+ and Tim-3– or in PD-1+ and PD-1– cells in both CD8+ and Tax tetramer+ cells.
- CTL cytolytic activity measured by CD107a degranulation assay in Tim-3+ and Tim-3– or in PD-1+ and PD-1– cells in Tax tetramer+ cells.
- Tim-3 or PD-1 expression on HTLV-I-infected CD4+ or CD8+ cells.

The quantitative PCR of HTLV-I proviral load for infected cases was done.

【Results】

- Low expression of Tim-3 on CD4+ and CD8+ T cells in HTLV-I infected individuals in comparison to

healthy controls.

- Low expression of Tim-3 on HTLV-I Tax-specific CTLs compared with CMV-specific CTLs in HTLV-I infection.
- There is no significant difference in frequency of Tim-3+ cells in HTLV-I Tax-specific CTLs in both HAM/TSP patients and asymptomatic carriers, although the mean fluorescence intensity (MFI) is higher in asymptomatic carriers than in HAM/TSP patients.
- There is no significant difference in PD-1 expression (neither frequency nor MFI) between HAM/TSP patients, asymptomatic carriers and healthy controls in either CD4+ or CD8+cells.
- Significant higher expression of PD-1 on HTLV-I Tax-specific CTLs compared with CMV-specific CTLs in HAM/TSP patients, and on Tax-specific CTLs in asymptomatic carriers than in HAM/TSP patients.
- Reduced IFN-γ production and cytolytic activity (CD107a expression) in Tim-3+, but not PD-1+, HTLV-I Tax-specific CTLs.
- No difference in the expression of Tim-3 or cytolytic activity between Tax-specific CTLs of HAM/TSP patients or asymptomatic carriers.
- The frequencies of Tim-3+ or PD-1+ cells in Tax-specific CTLs did not correlate with HTLV-I proviral loads, duration of illness, disease activity, age of the patients or serum HTLV-1 antibody titer in HAM/TSP patients.
- Low expression of Tim-3 on CD4+ and CD8+ HTLV-I-infected cells. Low expression of PD-1 on CD8+ HTLV-I infected cells.

【Discussion and Conclusions】

The decreased expression of Tim-3 in HTLV-I infection is a marked contrast to other chronic viral infections such as HIV and HCV infection, where Tim-3 expression is increased in T cells, including the virus-specific CTLs. As our and others' results proved that Tim-3 identifies a subset of CTLs with impaired production of cytokines and cytolytic activity. It strongly suggests that the Th1/Tc1 immune response is not negatively regulated by Tim-3 in HTLV-I infection. Rather, immune cells such as HTLV-I-specific CTLs may be resistant to cell death through the Tim-3/galectin-9 pathway. IFN-γ production was higher in CD8+ cells and HTLV-I Tax-specific CTLs that expressed PD-1, which also show higher CD107a expression as compared to their PD-1- counterparts in HAM/TSP patients. These results indicate that PD-1+ HTLV-I Tax-specific CTLs are capable of producing proinflammatory cytokines and have high cytolytic activity during HTLV-I infection. These results suggest that PD-1 and Tim-3 may have a distinct function in regulating immune responses in HTLV-1 infection. Tim-3 and CD107a expression in HTLV-I Tax-specific CTLs are not significantly different between HAM/TSP patients and asymptomatic carriers. Therefore, we concluded that Tim-3, but not PD-1, expression is reduced in HTLV-I infection and that the expression levels on HTLV-I Tax-specific CTLs are not different between HAM/TSP patients and HTLV-I carries. These results suggest that HTLV-I Tax-specific CTLs preserve their cytolytic activity, thereby controlling viral replication.

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論文審査の要旨

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Reduced Tim-3 expression on human T-lymphotropic virus type I (HTLV-I) Tax-specific cytotoxic T lymphocytes in HTLV-I infection

（HTLV-I 感染における HTLV-I 特異的細胞障害性 T リンパ球での Tim-3 発現の低下）

HAM は HTLV-I 感染者の 1%以下に発症する。HAM と無症候性 HTLV-I キャリアでの最も大きな違いは、HAM では HTLV-I ウイルス量および HTLV-I 特異的細胞障害性 T リンパ球（CTL）が多いことであり、ウイルス量が減少すれば HAM の病態は改善するものと考えられている。近年、免疫疲労関連分子である Tim-3 および PD-1 が同定され、HIV や HCV などの慢性ウイルス感染症における長期の抗原刺激の結果、CTL などの免疫細胞が疲弊し、ウイルス排除が上手く行えないことが指摘されている。そこで学位申請者らは、HAM およびキャリアを含めた HTLV-I 感染者において、HTLV-I 特異的 CTL が免疫疲労しているのか、また HAM の CTL はキャリアより疲労しているのかについて検討を行った。HAM およびキャリアの PBMC を用いて、CD8+細胞、CD4+細胞ならびに、テトラマーで同定された HTLV-I Tax 特異的 CTL における Tim-3 および PD-1 の発現を、フローサイトメトリーにて検討した。さらにサイトカイン産生能と CTL 活性を、ウイルス抗原刺激による IFN-γ産生ならびに CD107a 発現にて測定した。これらと、患者のウイルス量や臨床パラメーターとの関連を検討した。

その結果、本研究により以下の知見が明らかになった。

1）HAM および HTLV-I キャリア両群における Tim-3 の発現は、正常者と比べて、リンパ球全体ならびに HTLV-I Tax 特異的 CTL において低下していた。
2）HAM とキャリアでは、HTLV-I 特異的 CTL での Tim-3 陽性細胞率は差がなかった。
3）Tim-3 陽性細胞では、IFN-γ産生および CTL 活性は低下し、PD-1 陽性細胞では逆であった。
4）HAM とキャリアでは、HTLV-I 特異的 CTL での CD107a 発現は差がなかった。
5）HTLV-I 感染細胞では、Tim-3 ならびに PD-1 の発現は低下していた。

本研究は、HTLV-I 感染症では他の慢性ウイルス感染症と異なり、ウイルス特異的 CTL を含む免疫細胞は疲弊していないことを示し、HTLV-I 感染症の新たな特徴を明らかにした。さらに、HAM とキャリアでは、HTLV-I 特異的 CTL あたりの CTL 活性には差がないことを示し、HAM におけるウイルス量の増大は他の原因による可能性を示した点で、非常に興味深い。よって本研究は、学位論文として十分な価値を有するものと判定した。

最終試験の結果の要旨

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主査および副査の５名は、平成２２年１２月２４日、学位申請者 ナシュワ ハゼイン アブデルバリー リスク 君に面接し、学位申請論文の内容について説明を求めると共に、関連事項について試問を行った。具体的には、以下のような質疑応答がなされ、いずれについても満足すべき回答を得ることができた。

Q1) There is a speculation that HTLV-I-specific CTLs in HAM patients have low cytolytic activity than in carriers. How does the immune response in HAM lead to neural cell damage?
Answer: I found that the CTL activity in HAM patients is not different from that in carries. HAM patients have a higher frequency of the CTLs than carries. The strong CTL response may reduce the HTLV-I-infected cells, while such strong response may cause pathological tissue damage such as neural cell damage.

Q2) Are there any differences between HLA-A2 and A24 positive HAM patients regarding CTL activity?
Answer: No, I could not detect any differences between the two groups regarding cytolytic activity.

Q3) Which type is more common, HLA-A2 or HLA-A24, in Egypt?
Answer: I have no accurate data regarding HLA typing in Egypt.

Q4) Does Tax reduce Tim-3 expression, or does not a Tim-3 expressing cell express Tax protein?
Answer: I investigated whether Tax expression correlated with Tim-3 MFI or frequency, but I could not detect any correlations. However, some kinetics examinations would be needed.

Q5) In the Fig 1B and C, the percentages of the Tim-3 were reduced in the infected individuals, while the MFI was higher in carriers than in controls. What is your explanation?
Answer: I have no exact explanation. I speculate that HAM patients may have increased Th17 cells, which by nature express a lesser amount of Tim-3 than Th1 cells. This may cause the reduced MFI on the cells.

Q6) In the Fig 4A and B, why are the percentages of INF-γ+ cells different between in the histogram diagram and in the column chart?
Answer: To know the IFN-γ expression in Tim-3 positive or negative cells, we normalized the values in either Tim-3 -positive or -negative cell population, which are shown in the column chart.

最終試験の結果の要旨

Q7) You detected the CTL activity against whole PBMC. Why did not you evaluate the activity against HTLV-I-infected CD4+ cells?
Answer: The aim of this assay was to evaluate the maximum response of HTLV-I-specific CTLs, therefore, we exogenously added Tax antigen to the whole PBMC. The point is important, however, it is difficult to evaluate CTL activity against naturally infected CD4+ cells, because the CD4+ cells do not express viral antigens.

Q8) Do the CTLs act via either paracrine or autocrine mechanism?
Answer: CTLs usually function via paracrine mechanism, as they secrete many cytokines, such as INF-γ and degranulate vesicles containing granzyme B and perforin, to the target cells.

Q9) How about Tim-3 expression in ATL patients?
Answer: To my knowledge, there is no report regarding Tim-3 expression in ATL patients.

Q10) Why does HTLV-I mainly infect CD4+ cells?
Answer: In vitro, HTLV-I infects many cell types like CD4, CD8, DCs, epithelial cells and monocytes. I am not sure why it is mainly detected in CD4+ cells in vivo. I speculate that CD4+ cells may have a high proliferation capacity.

Q11) How did you quantify HTLV-I proviral load by PCR?
Answer: The quantitative PCR was carried out using HTLV-I Tax primers and β-actin primers. The copy numbers of tax and β-actin genes were determined by each standard curve. The proviral loads were corrected using the amount of β-actin.

Q12) How did you know that the degranulation assay only recognized the surface expression of CD107a, but not intracellular expression?
Answer: I detected CD107a on the cell surface during antigen stimulation by adding anti-CD107a antibody into the culture. In addition, I detected no CD107a without antigen stimulation and on resting cells.

Q13) How does Tim-3 or PD-1 affect the IFN-γ expression?
Answer: IFN-γ production is regulated by two signaling pathways from the T cell receptor and costimulatory receptor, via contact to antigen-presenting cells. Tim-3 and PD-1 may play a role in the pathways, but it is still unclear.

Q14) How did you select the controls in your study?
Answer: The main selection point is to be HTLV-1 negative.

最終試験の結果の要旨

Q15) As Tim-3 expression is reduced in both carriers and HAM patients, does it mean that it is not related to disease onset?
Answer: It would not a determinant for the disease development from the carrier state.

Q16) In the Fig 1A, why is the Tim-3 expression so different between in the gated total lymphocytes and in both CD4+ and CD8+ cells?
Answer: Tim-3 is also expressed by other types of cells including dendritic cells, natural killer cells, mast cells and monocytes. Some of them were included within the gated lymphocytes.

Q17) What is the percentage of infected cells in CD8+ cells?
Answer: The range of infected CD8+ cells in HAM patients was 0.4% – 5% and the mean was 2% of the total CD8+ population.

Q18) The total frequency of CD107a positive cells showed no difference between carriers and HAM patients within Tax tetramer+ cells. How do you explain the CTLs may contribute to HAM pathogenesis?
Answer: HAM patients have a higher frequency of the CTLs. Therefore, it seemed that the total CTL response is higher in HAM patients than in carriers, which may cause pathological tissue damage. However, more detailed analyses are needed.

Q19) Tim-3 is expressed by only 3% of HTLV-I-specific CTLs, how do you think that it plays a role in HAM pathology?
Answer: The reduced expression of Tim-3 suggests that the immune response in HAM patients is still strong or not so exhausted, which contrasts to other chronic viral infections.

Q20) Is there any speculation why Tim-3 expression is different between HTLV-I infection and HCV or HIV infection?
Answer: I have no exact reason. The question is interesting and would be a further study project.

Q21) Is there any negative correlation between the frequency of PD-1 positive cells and the frequency of virus specific T cells in HTLV-I infection?
Answer: I did not find a correlation between the PD-1 expression and HTLV-I-specific CTL number.

Q22) Did you examine correlation between the frequency of Tim-3 and markers for autoimmune diseases (such as ANA, RA factor, anti CI-Ab) ?
Answer: It is a very interesting idea and I hope to do it in a future study.

以上の結果から、５名の審査委員は申請者が大学院博士課程修了者としての学力・識見を有しているものと認め、博士（医学）の学位を与えるに足る資格を有するものと認定した。