博士（医学）　塩　浜　康　雄

学 位 論 文 題 名

Augmented induction of a novel cancer/testis antigen in demethylated cancer cells

（脱メチル化処理がん細胞を用いた新規がん抗原の同定に関する研究）

学位論文内容の要旨

Background and Objectives : Cancer immunotherapy including cancer vaccine has been considered as an attractive therapy for cancer patients because of the fewer side effects. CT (cancer/testis) antigens are widely expressed in various human cancers but their expressions in normal cells are tightly restricted in testis. Generally, it has been reported that cancer vaccine therapy using HLA class I-binding short peptide derived from CT antigens induced the antigen-specific CTLs and maintained the cancer patients at long stable disease. However, the vaccine therapy focused on only CTL activation appears to be suboptimal to conquer cancer. To overcome this problem, it is necessary to develop more efficient method to improve immunosuppression in cancer patients. It has been demonstrated that the introduction of Th1-dominant immunity in tumor-bearing host is essential for inducing fully activated CTLs and the subsequent memory T cell generation. 5-Aza-2′-deoxycytidine (DAC) is well known to induce gene expression of CT antigens by the demethylation of promoter CpG islands of the treated cancer cells. Therefore, we first used DAC-treated demethylated cancer cells to identify novel CT antigens in the present study and then tried identification of the helper peptide epitopes, applicable to development of more efficient cancer immunotherapy.

Methods : Human lung cancer cells, LC-OK, A549, and LC-MS cells were treated with or without DAC for 3 days. Gene expression levels of the A549 cells were evaluated by microarray analysis. The augmented gene in DAC-treated A549 cells were first selected as candidates, and the candidate gene expression levels in normal tissues were further evaluated by the multiple tissue cDNA panels including testis. C16orf73 gene, HP15 expressed in only testis, was selected as novel CT antigen in this study. HP15-specific $CD4^+$ T cells were induced from purified $CD4^+$ T cells by using HP15-overlapping peptides covering whole amino-acid sequence of HP15. HLA restriction of the HP15-specific $CD4^+$ T cells was further determined by the IFN-γ production in response to the peptide-loaded LCLs. HP15-specific T cells induced from PBMCs, were mixed with HLA-matched or mismatched lung cancer cell lines, which were pre-treated with or without IFN-γ and DAC in the presence or absence of anti-HLA class I and anti-HLA class II mAbs for 72 h. IFN-γ production by the established T cells was detected by ELISA. HP15 gene expression levels in various cancer tissues, primary cancer cells, and normal PBMCs were evaluated by quantitative- and RT-PCR.

Results: Gene expression levels of CT antigens, MAGE-A4, XAGE, and BAGE were augmented in human lung cancer cell lines, LC-OK, A549, or LC-MS cells after DAC-treatment. In addition, expression of a novel C16orf73 gene, HP15 was greatly enhanced in the demethylated lung cancer cells. The HP15 expression was observed in only testis but not other normal tissues, suggesting that HP15 was a novel CT antigen. HP15 gene expression was also detected in several cancer cell lines. Next, HP15-specific $CD4^+$ T cells were induced by using HP15-overlapping peptides. Then, helper

epitopes of HP15 CT antigen and the HLA restriction were identified by HP15-specific $CD4^+$ T cells and the overlapping peptides. As a result, almost all regions of HP15 CT antigen contained helper epitopes. Furthermore, it was confirmed that HP15-specific T cells established from PBMCs recognized HP15 antigen epitopes naturally processed and bond to HLA on human cancer cells in a HLA-class II dependent manner. In addition, the HP15-specific T cells did not respond to HLA-mismatched A549 cells, even if the cells were treated with DAC plus IFN-γ. HP15 gene was widely expressed in colon, lung, gallbladder, head and neck, and renal tissues from cancer patients. It was also confirmed that DAC-treatment enhanced HP15 gene expression level of primary cancer cells established from colon cancer patients but did not affect on that of normal PBMCs from healthy volunteers.

Discussion: In the present work, a novel CT antigen, HP15 was identified by DAC-treatment of human lung cancer cells. HP15 gene expression was observed in various cancer tissues of cancer patients as well as several cancer cell lines but not in normal tissues except testis. Therefore, the present strategy using demethylated cancer cells by treatment with DAC would be a useful tool to find novel CT antigens applicable to cancer vaccine therapy in human. It was also demonstrated that DAC-treatment caused the augmented induction of HP15 CT antigen in all cancer cells tested here as well as other CT antigens such as MAGE-A4 and XAGE, and BAGE in various cancer cells. These results suggested that DAC-treatment of cancer cells would increase their immunogenicity via augmented induction of CT antigens including our found HP15 CT antigen. To consider this possibility, helper epitopes of HP15 antigen were further identified, which can induce IFN-γ-producing $CD4^+$ T cells. In the present experiments, it was confirmed that HP15-specific $CD4^+$ T cells, induced by synthetic peptides, responded to the IFN-γ-treated and HLA class II-induced target cancer cells in a HLA class II- but not class I-dependent manner. These data suggest that such $CD4^+$ T cells would at least recognize the helper peptide epitopes naturally processed on HLA class II molecules of cancer cells. Recently, a clinical study for cancer patients revealed that DAC treatment induced NY-ESO-1 gene expression in the tumor tissues. In fact, HP15 gene expression was induced by DAC treatment in primary cancer cells from colorectal cancer patient. It was indicated that HP15 CT antigen was widely expressed in human cancer tissues in addition to human cancer cell lines. Taken together, the present results strongly indicate that DAC-treatment will become a novel strategy to induce immunogenic CT antigen, which facilitate the therapeutic efficacy of cancer vaccine treatment.

Conclusion: A novel CT antigen HP15 was identified in the present experiments and the gene expression was enhanced by DAC-treatment or spontaneously expressed in some cancer cells. In addition, it was confirmed that HP15 was expressed in various cancer tissues of patients. Therefore, demethylated cancer cells are useful tool to identify of novel CT antigens for cancer immunotherapy. Then, helper epitopes of HP15 antigen were determined by using HP15 overlapping peptides. In fact, HP15-specific $CD4^+$ T cells were successfully induced from PBMCs by HP15-overlapping peptides and the established T cells recognized HP15 and HLA-matched HLA-class II expressing cancer cells. These results indicate that HP15 is applicable to T cell based cancer immunotherapy. HP15 was widely expressed in colon, lung, gallbladder, head and neck, and renal tissues from cancer patients in addition to various cancer cell lines. Thus, these findings suggested that demethylation drugs such as DAC would be a useful tool not only for finding novel CT antigens but also for developing the combined therapy with cancer vaccine.

学位論文審査の要旨

主　査　　教　授　　上　出　利　光
副　査　　教　授　　西　村　孝　司
副　査　　教　授　　笠　原　正　典
副　査　　教　授　　瀬　谷　　司

学位論文題名

Augmented induction of a novel cancer/testis antigen in demethylated cancer cells

(脱メチル化処理がん細胞を用いた新規がん抗原の同定に関する研究)

申請者は、5-Aza-2 -deoxycytidine(DAC)処理がん細胞を用いて新規がん精巣抗原(CT)抗原C16orf73(HP15)を同定した。HP15抗原由来オーバーラッピングペプチドを用いてヘルパーエピトープ及びそのHLA拘束性を同定した。また、HP15由来オーバーラッピングペプチドを用いたHP15特異的$CD4^+$ T細胞の誘導実験より、HP15ヘルパーエピトープ及びHLA拘束性を同定した。HP15抗原はその全アミノ酸配列において多くのヘルパーエピトープを持ち、それらのHLA拘束性は多くの日本人に適応可能であることが明らかとなった。さらに、上記ペプチドを用いてヒト抹消血(PBMC)より誘導したHP15特異的T細胞は、HP15抗原及びHLAクラスII陽性がん細胞株を認識してIFN-γを産生した。様々ながん患者由来がん組織においてもHP15遺伝子の発現が認められ、その発現の見られない初代培養がん細胞においてもDAC処理によりHP15遺伝子の発現が起こることが明らかとなった。以上の結果は、HP15抗原を標的とした免疫治療及びDAC併用療法の有効性を示唆するものであった。

学位論文発表後、副査である瀬谷司教授より遺伝子発現の網羅的検討に用いたDAC処理がん細胞株及び初代培養がん細胞の由来に関する質問があり、それぞれのがん細胞の由来に関する回答があった。また、がん患者へのDAC投与の安全性に関する質問に、DACを用いた固形がんに対する臨床試験の報告などを例に、DACの安全性に関して回答を行った。さらに、HP15マウス相同遺伝子、発現部位及びKOマウスに関する質問があり、相同遺伝子は存在するがKOマウスの報告がないことから、今後検討したいとの回答があった。副査である笠原正典教授より、PBMC以外の正常組織のDAC処理によるHP15及びその他遺伝子の発現に関する質問があり、DAC処理繊維芽細胞に関する報告では遺伝子変化が少なかったことや、DACの作用機序には活発な細胞分裂を必要とすることから、正常細胞に与える影響は少ないのではないかとの回答があった。また、DAC処理がん細胞におけるその他の遺伝子発現については、がん抑制遺伝子等の増殖抑制に関する報告があるとの回答があった。加えて笠原教授より、腸の細胞などの活発に分裂している細胞の指摘があり、今後それらの細胞にDAC

が与える影響について検討したいとの回答があった。また、DAC 以外の脱メチル化試薬やクロマチンの脱メチル化等による HP15 遺伝子の発現変化について質問があり、他の試薬による検討は行ってないが、MAGE-1 遺伝子の発現制御を例に挙げ、DNA 特異的脱メチル化を引き起こす DAC が HP15 遺伝子の発現に重要ではないかとの回答があった。また、HP15 ヘルパーエピトープの HLA 拘束性と日本人 HLA 適合率の質問に、同定した HP15 ヘルパーエピトープ HLA 拘束性は多くの日本人に適応可能であるとの回答があった。 主査の上出利光教授より抗原探索に細胞株である A549 細胞を用いた根拠について質問があり、DAC に対して細胞死など副作用が少ない A549 細胞を選択し、脱メチル化条件下で発現する新規がん抗原を探索したとの回答があった。また、$CD4^+$ T 及び $CD8^+$ T 細胞の誘導効率に関する質問があり、用いたペプチドを含め $CD4^+$ T 細胞の誘導に適した培養条件のため、$CD8^+$ T 細胞の誘導効率が良くなかったのではないかとの回答があった。また、今回用いたペプチドの種類に関する質問に、キラーエピトープを含むロングペプチドを用いたが、将来はさらに当研究室で研究開発中の HK-HELP を応用したいとの回答があった。副査の西村孝司教授より、人体への IFN-γ投与による副作用について質問があり、IFN-γの投与は避け、生体内においては腫瘍局所或いは近傍リンパ節で抗原特異的に活性化 $CD4^+$ T 細胞より産生される IFN-γによる効果を期待しているとの回答があった。

この論文は、DAC 処理がん細胞を用いた新規がん精巣抗原 HP15 の同定を行ったのみでなく、HP15 ヘルパーエピトープを同定し、HP15 抗原のがん免疫療法への適応可能性を示した点で高く評価され、今後、DAC を含む様々なエピジェネティック治療薬と免疫療法の併用療法の開発に寄与することが期待される。

審査員一同は、これらの成果を高く評価し、これまでの研究活動における研鑽なども併せ申請者が博士（医学）の学位を受けるのに十分な資格を有するものと判定した。