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人類子宮頸癌腫瘤內浸潤淋巴球表現殺手細胞抑制受體免疫調控之研究(1/3)

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（一）中文摘要。（五百字以內）

關鍵詞：子宮頸癌，腫瘤內浸潤淋巴球，殺手細胞抑制受體，免疫調控

子宮頸癌是台灣女性癌症發生率的第一位。癌細胞可能經由各種不同之方式，包括分泌免疫抑制物質或改變表面抗原來逃避宿主免疫系統的控制，進而造成深部組織侵入與惡性轉移。在吾人先前之研究中利用機械式研磨萃取法(mechanical dispersal technique)來分離子宮頸癌腫瘤細胞與腫瘤內浸潤淋巴球（tumor-infiltrating lymphocytes, TILs），由此技術進行子宮頸癌腫瘤浸潤淋巴球之研究中發現，子宮頸癌腫瘤組織中存在大量之浸潤淋巴球，而這些免疫細胞均有受抑制之現象*(Sheu et al, Hum Immunol 1997; 56:39-48)*，而 TILs 與子宮頸癌腫瘤預後有極密切之相關 *(Sheu et al, Cancer 1999;86:1537-1543)*。進一步之研究發現，子宮頸癌細胞可能分泌免疫抑制物質來逃避宿主免疫系統之作用，進而造成深部組織侵入與惡性蔓延(*Cancer Research, 2001, 61:237-242)*。由於人類子宮頸癌腫瘤細胞可產生大量之細胞免疫抑制介質(immunosuppressive mediators)，而免疫抑制介質又與多種免疫細胞表面細胞激素受體(cytokine receptors)之作用有關，而免疫細胞表面殺手細胞抑制受體(killer-inhibitory receptors, KIRs)可能受細胞介質之影響，來調控免疫細胞與癌細胞表面組織相容性分子(histocompatibility complex molecules)之表現以及交互作用。本計畫研究主要是利用細胞免疫螢光染色及流體細胞儀分析子宮頸癌腫瘤中浸潤淋巴球表面殺手細胞抑制受體分布，深入探討免疫細胞表面殺手細胞抑制受體分布與癌細胞為何能抑制免疫反應之可能原因。**第一年之研究**主要利用細胞免疫螢光染色及流體細胞儀分析子宮頸癌腫瘤中浸潤淋巴球表面殺手細胞抑制受體分布。吾人預期可找出子宮頸癌之腫瘤細胞與宿主免疫系統間之可能互動關係，進而了解癌細胞於微環境中所產生免疫極化現象之可能機轉。

（二）英文摘要。（五百字以內）

Cytotoxic T Lymphocytes (CTLs) may obtain natural killer (NK)-like, human lymphocyte antigen (HLA)-independent activity and become cytolytic against certain HLA-class I-negative targets. It has previously been found that NK cells bear killer-cell inhibitory receptors (KIRs) which inhibit NK cell activity upon interaction with certain HLA class I molecules. These MHC-class I-specific KIRs are recognized originally on NK cells and later found on selected subpopulations of $CD8^+$ T lymphocytes, usually with activated ($CD69^+$)/memory ($CD28^-CD45RO^+$) phenotypes. Expression of these functional NK cell receptors (NKRs) on cytolytic T lymphocytes that recognize classical or non-classical MHC molecules has been first linked to inhibition of cytotoxic functions. The acquisition of both NK-like activity and expression of KIRs by these $CD56^+$ and/or $CD161^+$ $CD8^+$NK-T cells is parallel to prevent damage to normal host cells. KIRs are proposed to, not only restrain the T cell receptor (TCR)-mediated cytolysis, but also hold back the NK-like cytotoxicity implemented by activated $CD8^+$ CTLs in both *in vitro* human and mice models. However, the functional roles and mechanisms dictating the regulation of KIRs expression on effector CTLs still remain to be clarified.

There is also limited knowledge about the induction of KIRs expression *in vivo*. Up-regulation of KIRs has been linked to the modulation of the virus- and/or tumor-specific immune responses in animal models. Previously, we have demonstrated that human cancer cells may alter the functional composition of anti-tumor effector cells (e.g., polarity of $CD8^+$ cytotoxic T cells) within the tumor milieu ***(Sheu et al, Journal of Imunology, 2001)***. We have further illustrated that cancer-derived mediators are responsible for the immunosuppressive conditions of tumor-infiltrating lymphocytes (TILs) in human cervical cancer (CC) ***(Sheu et al, Cancer Research 2001)***. However, the expression of KIRs on TILs *in vivo* and the possible correlation of cancer-derived mediators within the tumor microenvironment remain to be stratified. In this study proposal, we will examine the expression of various NKRs on TILs derived from human CC. **In the first year project,** we directly examine the expressions of various NKRs on TILs derived from human CC by triple-color flow cytometry with combination of different surface markers. Our study will be important for the elucidation of possible tumor-host interaction in cervical cancer milieu and further utilization of adoptive immuno-therapy in adjuvant cancer treatment.

First year project:

It has previously been found that NK cells bear killer-cell inhibitory receptors (KIRs) which inhibit NK cell activity upon interaction with certain HLA class I molecules (1, 2). Expression of these functional NK cell receptors (NKRs) on cytolytic T lymphocytes that recognize classical or non-classical MHC molecules has been first linked to inhibition of cytotoxic functions (1, 2). These MHC-class I-specific KIRs are recognized originally on NK cells and later found on selected subpopulations of $CD8^+$ T lymphocytes, usually with activated ($CD69^+$)/memory ($CD28^-CD45RO^+$) phenotypes (1,3-8). HLA-specific NKRs are broadly categorized into two subgroups: the immunoglobulin (Ig)-like superfamily and the C-type lectin superfamily (9). The first group includes various members that specifically recognize groups of human leukocyte antigen HLA-C (p58), HLA-B (p70), and HLA-A (p140) alleles (4, 10-13). Ig-like superfamily is first seen as a kind of HLA-dependent inhibitory NKRs that does not sway its expression status once NK cells are matured from thymus. The second group is a type II trans-membrane protein that contains a C-type lectin domain (14-23).

In the first year project, we directly examine the expressions of various NKRs on TILs derived from human CC by triple-color flow cytometry with combination of different surface markers.

Case recruitment.

Primary cases of cervical neoplasia will be enrolled in this study. Tissue specimens will be obtained after total hysterectomy. Cervical tissues from cases of uterine fibroids will be included as normal controls. Each case of cervical cancer is evaluated for clinical parameters including grade, lymphatic or vascular permeation, lymph node metastatic status, and clinical stage. Clinical staging of each patient is defined according to the 1995 modification of the International Federation of Gynecologists and Obstetricians (FIGO) staging of carcinoma of the cervical cancer.

Mechanical dispersal technique

For separating cervical cancer cells and TILs, tissue specimens will be aseptically excised immediately after operation from at least four different tumor sites and two sites of normal cervix. Fragments of tissue are carefully washed with phosphate-buffered saline (PBS) for removal of contaminated blood and then weighed. Tissue specimens are cut, minced, and pressed gently through a 380 μm sieve and then a 45.7 μm sieve with RPMI-1640 medium

(Gibco, Life Technologies, Grand Island, NY, USA). The filtered solution is centrifuged, then layered over a Percoll discontinuous gradient (30%, 55%, and 100%) and centrifuged at 800 x g for 30 minutes. The enriched mononuclear cell suspension is collected from the interface of the 55% and 100% Percoll solutions and then washed twice with RPMI 1640 medium. The recovered cells are checked for viability with the Trypan Blue staining method and counted. Tumor cells are isolated from the interface of the 30% and 55% Percoll solutions and then transferred to serum-free culture medium. Normal cervical cells are separated by the same procedure as mentioned above. Venous blood of each patient is obtained before operation and transferred to test tubes containing heparin. Peripheral blood mononuclear cells (PBMCs) are isolated by Ficoll hypaque (1.077 density). The PBMCs of patients with cervical cancer are resuspended at $1x10^6$ cells/mL in RPMI medium. Purification of pan-T cells is performed by passage of the PBMCs through columns of nylon wool.

Flow cytometry analysis

Monoclonal antibodies labeled with fluorescein isothiocyanate (FITC), phycoerythin (PE), and peridinin chlorophyll protein (Per-CP) (all fluorescein-conjugated mAbs except for anti-CD94/NKG2A-PE, are purchased from Becton-Dickinson Immunocytometry System, Beckton-Dickinson, San Jose, CA) are used for trio-color flow cytometry. Surface staining will be done using following reagents: simultest anti-CD45-FITC + anti-CD14-PE, anti-CD3-FITC + anti-CD19-PE, and anti-CD3-FITC + anti-CD16+CD56-PE; anti-CD4-FITC, anti-CD8-FITC, anti-CD56-FITC; a mixture of PE-coupled NKR-specific mAbs: anti-CD94 (Immunotech, Marseille, France), anti-NKG2A (Immunotech), anti-CD158a (EB6), anti-CD158b (GL183), anti-NKB1 (NKB1); anti-CD161-PE, anti-CD69-PE, anti-CD8-PerCP, and anti-CD3-PerCP. Anti-mouse cytosol-IgG1-FITC + cytosol-IgG2a-PE will be used as negative control. Triple-color flow cytometry will be performed on FACSCalibur (BD Biosciences, Mountain View, CA). Data are acquired with CellQuest software (BD Biosciences) by the use of forward-scatter/side-scatter thresholds and analyzed with either CellQuest software or WinMDI software (Joseph Trotter, Scripps Research Institute).

Results

High Ratio of $CD8^+$ T Lymphocytes in Gated $CD3^+$ TILs

The viability of immunocytes used in the studies was over 95% at the completion of the isolation procedure in all cases. Higher ratio of $CD8^+$ T lymphocyte was noted in gated $CD3^+$

TILs than that in autologous PBMCs (53.60% [IQR=11.95] vs. 31.06% [IQR=13.80], n = 20, $P < 0.001$), which was compatible with our previous data (34).

Analyses of the Expression of Ig-like NKRs on CD4$^+$ and CD8$^+$ T Lymphocytes

The expression of Ig-like NKRs (CD158a, CD158b, and NKB1) was determined on either CD4$^+$ or CD8$^+$ T lymphocytes from both peripheral blood and NC or CC tissues. These Ig-like NKRs could be detected at variable levels on CD8$^+$ T lymphocytes. Triple fluorescence flowcytometric analyses showed that the expression ratios of all three Ig-like NKRs by CD8$^+$ TILs were higher than that of CD8$^+$ PBMCs (CD158a: 1.30% [IQR=1.67] vs. 0.26% [IQR=0.37], n=14, $P < 0.01$; CD158b: 2.38% [IQR=2.34] vs. 1.19% [IQR=0.81], n= 14, $P < 0.01$; NKB1: 0.81% [IQR=0.58] vs. 0.35% [IQR=0.59], n=14, P=0.012). After rating with the CD8$^+$ T lymphocytes ratio between TILs and PBMCs, the expression ratios of Ig-like NKR$^+$CD8$^+$ T lymphocytes were similar in gated CD8$^+$-autologous TILs and PBMCs (CD158a: 2.16% [IQR=2.22] vs. 2.44% [IQR=2.72]; CD158b: 6.09% [IQR=5.11] vs. 3.89% [IQR=4.82]; NKB1: 1.32% [IQR=2.13] vs. 1.56% [IQR=5.20], n=14, *no statistical significance*). Analyzing the CD4$^+$ T lymphocytes derived from either PBMCs or TILs, we found no or only minimal expression of CD158a, CD158b, and NKB1. Our data demonstrated that Ig-like NKRs were not expressed on CD4$^+$ T lymphocytes, but evenly expressed on both CD8$^+$-autologous TILs and PBMCs.

Analyses of the Expression of C-type lectin NKRs on CD4$^+$ and CD8$^+$ T Lymphocytes

The expression ratios of C-type lectin superfamily (CD94 and NKG2A) on CD4$^+$ and CD8$^+$ T lymphocytes from PBMCs and TILs were compared by triple fluorescence flow cytometric analyses. In all conditions tested, CD4$^+$ T cells express minimal levels of CD94 and NKG2A. On the contrary, CC-infiltrating CD8$^+$ T lymphocytes were found to express significantly more CD94 than either peripheral blood CD8$^+$ T cells or normal cervix-infiltrating CD8$^+$ T lymphocytes (16.83% [IQR=17.57] vs. 8.01% [IQR=11.24], n=16, $P < 0.01$). The expression ratio of NKG2A on CD8$^+$ TILs were also significantly higher than that in autologous peripheral blood CD8$^+$ T cells or CD8$^+$ T lymphocytes isolated from normal cervix (13.11% [IQR=16.23] vs. 3.02% [IQR=3.60], n=10, $P < 0.01$).

Reduced proportion of CD161$^+$CD8$^+$ NK-T lymphocytes in TILs

Unlike the expression patterns of the NKRs mentioned above, CD161$^+$ T lymphocytes (NK-T cells) were found in both CD4$^+$ and CD8$^+$ T cells at variable proportions. In the

present study, a significantly lower expression ratio of CD161 was demonstrated in $CD8^+CD3^+$ TILs than that in PBMCs (7.64% [IQR=15.79] vs. 20.53% [IQR=23.43], n=12, P=0.012), which indicated the compositional alterations of $CD161^+CD8^+$ NK-T cells in cancer milieu.

Discordant expression of CD94/NKG2A$^+$ on CD56$^+$ or CD161$^+$ CD8$^+$ NK-T cells within CC-infiltrating CD8$^+$ T Lymphocytes

Dual NKRs co-expression analyses showed the possible concordant and discordant expressions of CD94/NKG2A with CD56 and/or CD161 on the same lymphocytes in gated $CD8^+$ T lymphocytes. In PBMCs, concordant expression of CD94/NKG2A with CD56 and/or CD161 was observed in about one-third to over half of gated $CD8^+$ T lymphocytes. On the contrary, $CD8^+$ T lymphocytes derived from TILs showed significantly discordant co-expressions of CD94/NKG2A$^+$ and CD56 and/or CD161, which demonstrated that CD94/NKG2A were mainly expressed on $CD56^-CD161^-CD8^+$ TILs within cancer milieu, but not on $CD56^+$ and/or $CD161^+$ NK-T lineages.

Discussion

Cytotoxic T Lymphocytes (CTLs) may obtain NK-like, HLA-independent activity and become cytolytic against certain HLA-class I-negative targets. In both *in vitro* human and mice models, the acquisition of both NK-like activity and expression of KIRs by these $CD56^+$ and/or $CD161^+$ $CD8^+$NK-T cells is parallel to prevent damage to normal host cells. KIRs are proposed to, not only restrain the T cell receptor (TCR)-mediated cytolysis, but also hold back the NK-like cytotoxicity implemented by activated $CD8^+$ CTLs (1, 2, 24). Expression of inhibitory NKRs on effector CTLs has been linked to the reduction of tumor necrosis factor (TNF) secretion (25-27). However, the functional roles and mechanisms dictating the regulation of KIRs expression on effector CTLs still remain to be clarified. There is also limited knowledge about the induction of KIRs expression *in vivo*. Up-regulation of KIRs has been linked to the modulation of the virus- and/or tumor-specific immune responses in animal models (28-30). Regulation of KIR expression in human T cells has been proposed to as a safety mechanism that may impair cytolytic NK-T-cell responses (27). Functionally, CTLs that exhibit a low level of lytic activity against autologous tumor cells can be dramatically reversed upon KIR blockade (25, 26).

Previously, we have demonstrated that human cancer cells may alter the functional composition of anti-tumor effector cells (e.g., polarity of $CD8^+$ cytotoxic T cells) within the tumor milieu (31). We have further illustrated that cancer-derived mediators are responsible for the immunosuppressive conditions of tumor-infiltrating lymphocytes (TILs) in human cervical cancer (CC) (32-34). However, the expression of KIRs on TILs *in vivo* and the possible correlation of cancer-derived mediators within the tumor microenvironment remain to be stratified. It is conceivable that premature or abnormal up-regulation of KIRs on CTLs might be induced by cancer cells as yet another way to paralyze the anti-tumor immune defenses.

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