行政院國家科學委員會研究計畫成果報告

計畫名稱：活體外子宮頸癌細胞與自體免疫細胞作用機轉之研究

In vitro immune interaction models of cancer cells –autologous immunocytes in human cervical cancer

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In the third year project, we will try to identify the biophysical properties of the tumor-derived immunosuppressive mediators. For discriminating the immunosuppressive mediators derived from cancer cells to be either membrane-bound or soluble form, different culture conditions with trans-well permeabilizing membranes will be added into the culture wells of previously established autologous co-culture system. Empiric trials of possible protease inhibitors in titrated concentrations will be utilized in the MLTC system for blocking the immuno-suppressive effects of cervical cancer. We have proved the tumor-derived immuno-suppressive effect and establish an in vitro cancer-immunocyte interaction model (MLTC) in the first project. We focused our cytokine target on interleukin-2 receptor alpha, which was critical for lymphocyte proliferation. Besides, all the supernatants of co-culture will be collected for the measurement of soluble form of cytokines to rule out the possibility of increased cleavage. In our data, the cancer-drived immunosuppression may undergo a proteolytic cleavage pathway of cytokine receptors ***(Cancer Research,*** *2001;61;237-242)*. The results will shed new light on the understanding of tumor-mediated immunosuppression and provide a possible therapeutic potential for patients with cervical cancer.

Materials and Methods

Mechanical dispersal technique

For separating cervical cancer cells (CC) and TILs, tissue specimens were aseptically excised immediately after operation from at least four different tumor sites and two sites of normal cervical stroma. Fragments of tissue were carefully washed with phosphate-buffered saline (PBS) for removal of contaminated blood and then weighed. A mechanical dispersal technique as previously described was utilized for isolating immunocytes in this study[10]. Briefly, tissue specimens were 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 was 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 was collected from the interface of the 55% and 100% Percoll solutions and then washed twice with RPMI 1640 medium. The recovered cells were checked for viability with the Trypan Blue staining method and counted. Tumor cells were isolated from the interface of the 30% and 55% Percoll solutions and then transferred to serum-free culture medium. Normal cervical stromal cells (NC) were separated by the same procedure as mentioned above. Venous blood of each patient was obtained before operation and transferred to test tubes containing heparin. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll hypaque (1.077 density). The PBMCs of patients with cervical cancer were resuspended at $1x10^6$ cells/mL in RPMI medium. Purification of pan-T cells was performed by passage of the PBMCs through columns of nylon wool.

MLTC Autologous coculture assay

To discriminate trivial cancer-derived effects on cytokines/ cytokine receptors expression, we will partially activate PBMCs with 2 μg/mL of PHA in a 37°C, 5% CO_2 humidified incubator for 2 hours. All activation and coculture procedures outlined were performed in duplicate in 12 mm x 75 mm capped polystyrene test tubes. The PHA-activated T cells were then divided into two groups. In the experimental group (MLTC), $5x10^5$ activated T cells were cultured with $1x10^6$ autologous cancer cells (1:2) in 4 mL of culture medium. In the control group (MLNC), $5x10^5$ activated T cells were cultured with $1x10^6$ autologous normal cervical stromal cells. The immunophenotyping of activated T cells in both groups was analyzed by flow cytometry on days 1, 3, and 5 of coculture. The supernatant was collected at the same intervals and checked for the presence of cytokines / cytokine receptorsα by a standard enzyme-linked immunosorbent assay (ELISA) (Endogen Inc., Woburn, MA, USA).

Flow cytometry analysis

Monoclonal antibodies labeled with fluorescein isothiocyanate (FITC), phycoerythin (PE), and peridinin chlorophyll protein (Per-CP) (Becton-Dickinson Immunocytometry System; Beckton-Dickinson Inc., San Jose, CA, USA) were used for three-color flow cytometry. The following matchings were arranged: anti-CD45-FITC + anti-CD14-PE (leucogate), anti-CD3-FITC + anti-CD19-PE, anti-CD3-FITC + anti-CD4-PE, anti-CD3-FITC + anti-CD8-PE, anti-CD4-FITC + anti-CD25-PE + anti-CD3-PerCP, anti-CD8-FITC + anti-CD25-PE + anti-CD3-PerCP, anti-CD4-FITC + anti-HLA-DR-PE + anti-CD3-PerCP, anti-CD8-FITC + anti-HLA-DR-PE + anti-CD3-PerCP. A Simultest control (mouse IgG1-FITC + IgG2a-PE) was used as background control. Individual cell suspensions were incubated with monoclonal antibodies at 4°C for 30 minutes and then ished twice in PBS containing 2% fetal calf serum and 0.1% sodium azide. Three-color flow cytometry was performed on a FACScalibur (Beckton-Dickinson Inc., San Jose, CA, USA), by use of an argon ion laser with 15 mW at 488 nm excitation. Triggering was set on the forward scatter channel, and the threshold was adjusted to exclude debris. Ten thousand events acquired for lymphocytes were measured in each suspension. Leukogate was used to measure the proportion of lymphocytes in the sample being studied without any scatter gates.

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the control group (MLNC), $5x10^5$ activated T cells are cultured with $1x10^6$ autologous normal cervical stromal cells. The immunophenotyping of activated T cells in both groups is analyzed by flow cytometry on days 1, 3, and 5 of coculture. The supernatant is collected at the same intervals and checked for the presence of cytokines and receptors by a standard enzyme-linked immunosorbent assay (ELISA) (Endogen Inc., Woburn, MA, USA).

Purification of $CD8^+$ cytotoxic T lymphocytes

For isolating $CD8^+$ T cells from TILs, an indirect magnetic labeling system with magnetic activated cell sorter (MACS; Miltenyi Biotec, Gladbach, Germany) is utilized for negative depletion and purification. TILs and activated PBMCs are first passed through Percoll discontinuous gradients and 30 μm nylon mesh for removal of cell clumps. The immunocytes are re-suspended in buffer to a total volume of 80 μl per 10^7 total cells. The cell suspension is incubated in a cold room (6°-12°C) for 10 minutes with 20 μl of Hepten-Antibody Cocktail added (containing CD4, CD11b, CD16, CD19, CD36 and CD56 antibodies; Miltenyi Biotec, Gladbach, Germany). After repeated ishing, 20 μl of MACS anti-Hepten microbeads per 10^7 total cells is added. The suspension is further incubated for 15 min at 6°-12°C, then ished carefully by addition of 10-20X the labeling volume of the ishing buffer. The magnetically labeled cells are passed through a MACS separator in the magnetic field. The column is rinsed with an adequate volume of buffer and repeatedly eluted. The effluent is collected as negative fraction, representing the enriched $CD8^+$ T cell fraction. The cell purity is checked by flow cytometry after labeling with anti-CD8-FITC.

Competitive RT-PCR analysis

To study whether the decreased expression of cytokines/cytokine receptors is mediated at the transcriptional level, we measured the α mRNA expression in purified $CD8^+$ T cells by competitive RT-PCR. $CD8^+$ T cells are isolated from TILs, unstimulated PBMCs, and PBMCs

activated with PHA for 3 days. Total cellular RNA of MACS-purified $CD8^+$ T cells is extracted and reverse-transcribed (0.1-0.5 mg RNA) in the presence of cytokines/cytokine receptors oligonucleotide primers (Clontech Laboratories Inc., Palo Alto, CA, USA). First-strand cDNA is synthesized in a DNA thermal cycler (GeneAmp DNA thermal cycler 480; Perkin-Elmer Corp., Norwalk, USA), and the resulting cDNA is amplified by polymerase chain reaction (PCR). For competitive analysis of cytokines/cytokine receptors, another set of synthetic competitor (cytokines/cytokine receptors mimic primers) with a different length for amplification is utilized (Clontech Laboratories Inc., Palo Alto, CA, USA). For each sample, constant amounts of reverse-transcribed RNA are amplified together with a dilution series of cytokines/cytokine receptors synthetic competitor. A preliminary ten-fold serial dilution of PCR mimic is added to the first-strand cDNA samples. Human β-actin primers and PCR mimic are used as positive controls. The competitive PCR is performed in a DNA thermal cycler for 38 cycles of denaturation at 94°C for 40 sec, annealing at 55°C for 1 min, followed by polymerization at 72°C for 1 min. The PCR-amplified products are separated on an agarose gel and visualized by staining with 0.02 mg/mL of ethidium bromide. The ratio of cDNA/mimic is quantified by densitometric analysis. Differences in the amount of starting RNA as well as in reverse transcriptase efficiency in diverse samples are minimized by normalizing data in comparison with the expression of human β-actin, which is also evaluated by competitive RT-PCR.

ELISA for soluble cytokines measurement

Supernatants of MLTC and MLNC were collected in small aliquots. The cytokines and receptors was measured with a commercial ELISA kit (Endogen Inc., Woburn, MA, USA). All assays were performed in triplicate. A spectrophotometer set to 450-550 nm was utilized for measuring the optical density of each well of the 96-well polystyrene microtiter plate. The triplicate readings were averaged and expressed in units of pg/mL.

Preparation of protease inhibitors

Protease inhibitors (Boehringer Mannheim Biochemicals GmbH, Mannnheim, Germany and Calbiochem, La Jolla, CA) for a broad spectrum of inhibition were prepared. The selected protease inhibitors included antipain-dihydrochloride (papain, trypsin, cathepsin A and B inhibitor), bestatin (amino peptidase inhibitor), chymostatin (α-, β-, γ-, δ-chymotrypsin inhibitor), E-64 (cysteine protease inhibitor), leupeptin (serine and cysteine protease, plasmin, trypsin, papain, and cathepsin B inhibitor), pepstatin (aspartate protease), aprotinin (serine protease inhibitor, specific for plasmin, kallikrein, trypsin, and chymotrypsin), $TIMP^3$-2 (72 kD and 92 kD gelatinases inhibitor), and MMP-inhibitor I (a broad spectrum gelatinases inhibitor). All reagents were prepared in azide-free deionized H_2O. Titrated concentrations of individual protease inhibitors were prepared according to the recommended working formula of the reagent supplier (Boehringer Mannheim Biochemicals). Drug toxicity was tested by addition of titrated concentrations of reagents in lymphocyte culture for 24 hours, with more than 90% viable cells by the Trypan Blue staining method.

Protease inhibition assay

Different protease inhibitors were added to the established MLTC. For maximal expression of IL-2Rα on the surface of T cells, PBMCs were fully activated with 10 μg/mL of PHA for 24 hours prior to MLTC. Autologous cancer cells ($1x10^6$) were first cultured in 1 mL of culture medium containing protease inhibitors. The final concentrations of protease inhibitors as recommended in each tube were as follows: (a) antipain-dihydrochloride, 5 and 50 μg/mL; (b) aprotinin, 1 and 10 μg/mL; (c) bestatin, 4 and 40 μg/mL; (d) chymostatin, 6 and 60 μg/mL; (e) E-64, 1 and 10 μg/mL; (f) leupeptin, 1 and 10 μg/mL; (g) pepstatin, 1 and 10 μg/mL; (h) TIMP-2, 0.1 and 1 μg/mL; and (i) MMP-inhibitor I, 10 and 100 μM/mL (Calbiochem). Constant numbers ($5x10^5$ cells) of PHA-activated T cells were then added to

the MLTC. In the control group, which included only activated T cells and coculture of activated T cells with cancer cells, no protease inhibitor was added. The supernatant of each culture tube was collected on the third day of coculture, and the concentration of sIL-2Rα was checked by ELISA.

Effect of MMP-inhibitors on T-cell proliferation

PBMCs were fully activated with 10 μg/mL of PHA for 24 hours prior to MLTC. Autologous cancer cells ($1x10^6$) were first cultured in 1 mL of culture medium containing 1 μg/mL of TIMP-2 or 100 μg/mL of MMP-inhibitor I for 2 hours and cocultured with PHA-activated T cells ($5x10^5$ cells). In the control group, no TIMP-2 was added. Two days after MLTC, T cells were purified by MACS as previously described and cultured at constant numbers ($2x10^5$ cells/well) in the presence of titrated IL-2 concentrations (1 IU/mL, 3 IU/mL, and 10 IU/mL). Titrated thymidine ([^{3}H]TdR) was added during the last 18 hours of culture. Cells were harvested, and thymidine incorporation was counted by liquid scintillation. All assays were performed in triplicate.

Results

In the first experiment, PHA-activated PBMCs were cocultured with autologous CC cells or NC cells. The expression of IL-2Rα and HLA-DR on activated T cells was measured by flow cytometry. The kinetic expression of IL-2Rα on activated T cells in the MLNC was compatible with the known physiologic pattern, being highest on day 3 and having progressively decreased by day 5. Thus, the NC cells had no influence on the level of IL-2Rα expression in MLNC. In contrast, CC cells, appeared to affect the expression of IL-2Rα by T cells in MLTC. On day 1 post coculture, the mean fluorescence intensity (MFI) of IL-2Rα

expression on activated $CD4^+$ cells was similar in both MLTC and MLNC. It was noteworthy that the MFI of IL-2Rα on $CD4^+$ cells was significantly lower in MLTC than in MLNC on day 3 (170.67 ± 17.01 *vs.* 360.10 ± 14.65) and day 5 (94.14 ± 6.12 *vs.* 174.34 ± 23.63). A similar finding was obtained with $CD8^+$ cells (139.01 ± 8.86 *vs.* 340.04 ± 28.93 on day 3, and 64.41 ± 10.67 *vs.* 196.26 ± 28.67 on day 5, for MLTC and MLNC, respectively). However, there was no difference in the kinetic expression patterns of HLA-DR on activated T cells ($CD4^+$ or $CD8^+$) between MLTC and MLNC. The expression of HLA-DR on activated T cells of MLTC and MLNC was low on day 1 and elevated on day 3, and remained persistently high on day 5. Our previous study had shown that the expression of HLA-DR, and CD69 as well, on TILs isolated from patients with CC was not altered. Together, these findings indicate that CC cells can down-regulate IL-2Rα expression on encountered T cells in a restricted manner.

The possibility exists that the CC-associated down-regulation of IL-2Rα results from excessive shedding of IL-2Rα from T cells. To clarify this possibility, we analyzed the amounts of soluble IL-2Rα (sIL-2Rα) in the supernatants of MLTC and MLNC. The mean concentration of sIL-2Rα in MLNC supernatants was 1,045.11 ± 17.33 pg/mL and increased about 2- and 3- fold by day 3 and day 5, respectively. The cumulative sIL-2Rα concentration in the supernatants of MLNC was compatible with the pattern of natural shedding of IL-2Rα. However, the mean concentration of sIL-2Rα in the supernatants of MLTC was significantly higher than that in MLNC on day 3 and day 5 cocultures (increased more than 3- and 4- fold, respectively) ($P < 0.001$). It became evident that CC cells could enhance the release of sIL-2Rα from activated T cells into the surroundings, and that a proteolytic cleavage might mediate the process.

Next, we studied whether the decreased expression of IL-2Rα on TILs in CC was mediated at the transcriptional level. $CD8^+$ T cells were isolated with a purity of >97% from TILs, unstimulated PBMCs, and PHA-activated PBMCs. Expression of IL-2Rα mRNA was

measured by competitive RT-PCR. The amount of IL-2Rα mRNA in TIL-derived $CD8^+$ T cells was comparable to that in the activated $CD8^+$ T cells. In both TILs and activated $CD8^+$ T cells, the amount of IL-2Rα mRNA was about 100-fold that expressed in non-stimulated PBMC-derived $CD8^+$ cells. A deficiency in IL-2 protein and IL-2R expression despite adequate levels of IL-2 mRNA has also been shown in TILs from patients with breast cancer. Because the IL-2Rα mRNA was abundant in TILs, cancer-mediated suppression did not occur at the transcriptional level.

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Using the kinetic experiments of MLTC described above, we conducted a protease inhibition assay to determine whether specific inhibitors could reverse or limit the cancer-induced proteolytic cleavage of IL-2Rα. For achieving maximal expression of IL-2Rα, PBMCs were fully activated with 10 μg/mL of PHA for 24 hours prior to MLTC. After 3 days of coculture, the concentration of sIL-2Rα in the MLTC group was approximately 3-fold that in the T-cells-only group (6,001.0±679.6 pg/mL *vs.* 2,241.3±197.3 pg/mL, n=6, $P<0.001$). Protease inhibitors such as E-64, aprotinin, bestatin, leupeptin, pepstatin, chymostatin, and

antipain-dihydrochloride had no apparent activity in suppressing CC cell-mediated proteolytic cleavage of IL-2Rα.

TIMP-2 and MMP-inhibitor I significantly inhibited the CC cell-induced IL-2Rα cleavage in a dose-dependent pattern. At a concentration of 1 μg/mL of TIMP-2, cancer-induced IL-2Rα cleavage was totally inhibited. The concentration of sIL-2Rα in the TIMP-2-MLTC group was 1,953.1±81.2 pg/mL, compared with a concentration of 6,001.0±679.6 pg/mL in the MLTC group without inhibitor (n=6, P=0.004). At a concentration of 0.1 μg/mL of TIMP-2, partial inhibition existed (3,136.2±327.7 pg/mL *vs.* 6,001.0±679.6 pg/mL; P=0.011). At a concentration of 100 μM/mL of MMP-inhibitor I, IL-2Rα cleavage was also totally inhibited. The concentration of sIL-2Rα in the MMP-inhibitor I-MLTC group was 2,114.7±165.7 pg/mL, compared with a concentration of 6,660.1±640.5 pg/mL in the MLTC group without inhibitor (n=6, P=0.001). At a concentration of 10 μM/mL of MMP-inhibitor I, partial inhibition existed (3,264.2±226.2 pg/mL *vs.* 6,660.1±640.5 pg/mL; P=0.006). The finding indicates that an MMP-mediated proteolytic process is likely to be responsible for the IL-2Rα cleavage, and that MMP inhibitors block this process.

To examine whether TIMP-2 or MMP-inhibitor I added to cultures could restore the proliferative function of cancer-encountered T cells, we further performed an IL-2-promoted T-cell proliferation assay. In the MLTC experiments illustrated in Figure 2C, cancer-encountered T cells proliferated poorly in the presence of IL-2. However, at a concentration of 1 μg/mL of TIMP-2 or 100 μg/mL of MMP-inhibitor I, the proliferative ability of cancer-encountered T cells could be restored in an IL-2 dose-dependent manner, which indicated that MMP inhibitors are functionally capable of restoration of the T-cell proliferation function by blocking the cancer-induced IL-2Rα cleavage.