Abstract. Tumor-associated MUC4 mucin has considerable potential as an immunotherapy target for pancreatic cancer. In previous studies, we developed dendritic cell (DC) vaccines which elicited MUC4 antigen-specific cytotoxic T lymphocyte (MS-CTL) response against tumor cells in vitro. Due to the observation that MS-CTL apoptotic rate increased significantly when co-cultured with MUC4+ tumor cells compared with T2 cells, we investigated whether high expression levels of MUC4 in pancreatic cancer cells would have an effect on the significant increase of apoptosis rate of MS-CTLs. First, the adverse influence of regulatory T cells (Tregs) was eliminated by CD8+ T lymphocyte sorting before the induction of MS-CTLs. Then, we constructed clonal MUC4-knockdown HPAC pancreatic cancer sublines with different MUC4 expression for co-incubation system. By utilizing appropriate control to rule out the possible apoptosis-induced pathway of intrinsic activated cell-autonomous death (ACAD) and analogous antigen-dependent apoptosis of CTL (ADAC) in our study system, further analysis of the effect of MUC4 membrane-expression, supernatants and blockade of CTL surface Fas receptor on MS-CTL apoptosis was carried out. The results demonstrated that the level of MUC4 membrane expression strongly positively correlated with MS-CTL apoptosis and the influence of supernatants and Fas-blockade did not significantly correlate with MS-CTL apoptosis. This evidence suggested that there may be a novel counterattack pathway of pancreatic cancer cells, which is a MUC4-mediated, cell contact-dependent and Fas-independent process, to induce CTL apoptosis. Therefore, further exploration and understanding of the potential counterattack mechanisms is beneficial to enhance the efficacy of MUC4 specific tumor vaccines.

Introduction

It is well known that pancreatic cancer is a lethal disease that is difficult to treat. Surgical resection remains the only potential curative approach to a small proportion in early stage (1). There is a need for the continual development of multimodality treatments including immunotherapy to improve patient prognosis (2).

As a member of the transmembrane mucin family (3,4), MUC4 is considered to have considerable potential as an immunotherapy target for pancreatic cancer (5,6). MUC4, in particular, is aberrantly expressed in pancreatic ductal adenocarcinoma (PDAC) and precancerous pancreatic intraepithelial neoplasias (PanINs), but not in benign or normal parts of pancreas (7-9). The level of MUC4 expression correlates significantly with a poor prognosis of PDAC (9,10). Previous studies suggested that cancer cells could use MUC4 for invasion, metastasis and resistance to chemotherapy (11-14). MUC4 antigen-pulsed vaccines are promising to eradicate focus in early stage and eliminate the minimal residual lesion remaining after surgical resection with minimizing damage to normal cells. Thus, the development in MUC4-antigen related vaccines is favorable for prevention of tumor recurrence and the approaches posses safety and tolerability with less substantial toxicity.

In our previous studies (15-17), we developed MUC4-antigen related dendritic cell (DC) vaccines which elicited MUC4 antigen-specific cytotoxic T lymphocyte (MS-CTL) response against MUC4-expressing tumor cells in vitro.
During examination of the cytotoxic activity of the vaccine in vitro, we observed the apoptosis of CTLs and noted that the apoptotic rate of CTLs co-cultured with MUC4+ tumor cells was significantly increased compared to those co-cultured with T2 cells of pulsed MUC4-epitope peptide, suggesting MUC4+ tumor cells could counterattack MS-CTLs via some pathway during the cytolytic process.

MUC4-bearing tumor cells lose their polarity, allowing MUC4 to be uniformly expressed all over the cell surface (18). With a larger size (ranges between 1.1 and 2.1 µm, depending on the length of the central tandem repeat domain) above the cell surface, MUC4 could mask most cell surface molecules (not exceeding a length of 35 nm) and interrupt their functions on cell–cell interactions (18). On the other hand, the aberrant overexpression or effusion of MUC4 mucin, including distinct types protein (secreted or membrane-associated forms), confers on tumor cells potential ligands for interaction with other receptors at the cell surface, and they might inactivate immune effector cells through receptor-ligand interactions (19). Thus, we postulated that MUC4 might contribute to the enhanced apoptotic rate of MS-CTLs during the killing process.

In the present study, we first isolated pure CD8+ T cells before the induction and amplification of MS-CTLs to eliminate the killing influence of Tregs. Secondly, we fixed basic parameter and defined precise gating template for apoptosis measurements by flow cytometry in order to investigate the nature of the apoptosis of MS-CTLs in co-incubation system. Thirdly, we repeated comparative analysis of the apoptosis rate of MS-CTLs incubated with MUC4+ tumor cells and pulsed-peptide T2 cells to confirm the previous observations. Then, we addressed whether high levels of MUC4 by pancreatic cancer cells would have an effect on the significant increase of apoptosis rate of MS-CTLs: i) clonal MUC4-knockdown HPAC sublines with different MUC4 expression were selected for co-incubation system and further analyses were performed; ii) the total apoptosis-induced effects of supernatants on MS-CTLs were compared among distinct co-cultured groups; iii) analyses of the blocking effect of Fas on MS-CTL surface were applied to further elucidate whether the Fas-FasL pathway might be involved in MS-CTL apoptosis in co-incubation system.

**Materials and methods**

**Cell lines, cytokines, culture medium, epitope peptide.** T2 cells (HLA-A2+/MUC4-, TAP deficient) were a generous gift from Weifeng Chen (Immunity Department, Peking University). The three human HLA-A2+/MUC4+ tumor cell lines were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA), including HCT 116 (colorectal carcinoma), CFPAC-1 and HPAC (pancreatic adenocarcinoma). HCT 116 cells were grown in McCoy’s-5A medium (Sigma-Aldrich, St. Louis, MO, USA). Other lines were cultured in DMEM (Gibco, Auckland, New Zealand), supplemented with 10% heat-inactivated fetal calf serum (Gibco, Carlsbad, CA, USA), 2 mM glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin at 37°C in 5% CO2.

Recombinant cytokines of human GM-CSF, IL-4, TNF-α, IL-2 were purchased from PeproTech (Rocky Hill, NJ, USA).

The HLA-A2-restricted and MUC4-specific CTL epitope peptides (LLGVGTFVV) were synthesized by Shanghai Sangon Biological Engineering Technology (Shanghai, China).

**HLA-A2+ volunteers.** Three healthy HLA-A2+ donors (male, average age 24-30 years) from Jiangsu Provincial Blood Center (Nanjing, China) were screened from 45 volunteers, and the verification of HLA-A2 subtype was performed as previously described (16,20). Samples were collected after obtaining patient informed consent and the present study was approved by the Ethics Committees of The First Affiliated Hospital of Nanjing Medical University (reference no: 2011-SRFA-100).

**Induction of human HLA-A2-restricted and MUC4-specific CTL.** Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats with Ficoll-Hypaque (TBD Science, Tianjin, China), as previously described (16). CD8+ T lymphocytes in the PBMCs separated from the blood samples were isolated by CD8 Dynabeads (Daynal Biotech, USA), according to the manufacturer’s instructions. The isolated CD8+ T cells were analyzed by flow cytometry with anti-CD8-FITC antibodies (BD Pharmingen, San Jose, CA, USA).

DCs were induced by IL-4 and GM-CSF cocktail as previously described (16). On day 5, 1,000 U/ml of tumor necrosis factor-α (TNF-α) was added to induce final maturation. After 7 days of culture, DCs were harvested and were confirmed with mature DC-specific phenotype by FACS. Matured DCs (1x106 cells/well) were pulsed with 20 µg/ml epitope peptide (LLGVGTFVV) for 2 h, washed, and irradiated by Co60 (2 Gy/min for 15 min). The irradiated, peptide-loaded DCs were mixed with 1x105 cells/well CD8+ T cells at a final DC: CD8+ T cell ratio of 1:10. The co-cultures were seeded in U-bottom 96-well plates and incubated for 3 days at 37°C, 5% CO2. IL-2 (10 U/ml) was added on day 4 and the same amount of loaded DCs was added on day 7 and day 10. On day 14, cells were harvested as MS-CTLs and used for subsequent MS-CTL/tumor cell interaction experiments.

**Selection of clonal MUC4-knockdown HPAC sublines with different MUC4 expression**

Construction of clonal MUC4-knockdown HPAC sublines. The target DNA sequences corresponding to MUC4-shRNA were previously reported (21,22). The selected sequences are present in all splice variants of MUC4 characterized thus far, as follows: forward oligo: 5'-cgcggAACGCAAGCATCGGACTTC-3', reverse oligo: 5'-aattcaaaaaAACGCAAGCATCGGACTTCACctcgagGTGAAGTCCGATGCTTGCGTTtttttg-3'; EcoRI restriction enzyme sites (5' and 3' ends, respectively).

The target DNA was ligated to the plKO.1-TRC cloning vector (23) (Addgene ID #10879), which was supplied by PlusGene Center of Nanjing Medical University (Nanjing, China) and verified by sequencing. The verified recombinant vector plasmid (pLKO.1/MUC4-shRNA), the packaging plasmid p8.2 and pSV-G were co-transfected into 293T cells by Lipofectamine™ 2000 reagent (Invitrogen, Carlsbad, CA, USA). The supernatant of the cultured 293T cells was collected to infect the HPAC cells. The pLKO.1-shmRNA vector (Addgene ID #1864; negative control vector containing scrambled shRNA insert) was used to package virus and infect
HPACs as control. Stable clones were then selected in a medium containing puromycin (5 µg/ml; Sigma). MUC4 expression in the derived sublines was confirmed via real-time RT-PCR, western blot analysis and flow cytometry.

Identification of clonal MUC4-knockdown HPAC sublines.

Quantitative real-time PCR. Total RNA was extracted from the selected MUC4-knockdown HPAC sublines with TRIzol reagent (Invitrogen). The reverse transcription using iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA) and quantitative real-time PCR using TaqMan Gene Expression assays (Applied Biosystems, Foster City, CA, USA) were performed as previously described (9). Each quantification PCR was performed in triplicate. The mRNA concentration was defined as the ratio of target mRNA copies relative to GAPDH mRNA copies.

Western blot analysis. The HPAC-derived clones were processed for protein extraction and western blotting using standard procedures. Cell lysates were prepared as previously described (24). Protein concentrations were determined by using the Bradford assay and proteins (20 µg/lane) were resolved on 4-20% Mini-Protean TGX™ precast gels (#456-1093; Bio-Rad Laboratories). Resolved proteins were transferred onto the polyvinylidene difluoride (PVDF) membrane and blocked in 5% non-fat milk in PBS for 2 h and subjected to the standard immunodetection procedure using specific antibodies. The following primary antibodies were used for labeling of the membranes: 1 µg/ml anti-MUC4 mouse monoclonal antibody (ab60720; Abcam, Cambridge, UK) for MUC4 immunodetection; 1:1,000-diluted anti-GAPDH mouse monoclonal antibody (AG019; Beyotime Institute of Biotechnology, Haimen, China) for GAPDH immunodetection. The membranes were incubated at 4°C overnight followed by six 10-min washes in TBST [50 mmol/l Tris-HCl (pH 7.4), 150 mmol/l NaCl, and 0.05% Tween-20]. Membranes were then incubated with 1:2,000-diluted horseradish peroxidase (HRP)-labeled goat anti-mouse IgG (A0216; Beyotime) for 1 h at room temperature followed by six 10-min washes with TBST. The blots were developed with an enhanced chemiluminescence kit (Amersham Biosciences, Freiburg, Germany). PageRuler™ Plus Prestained Protein Ladder (#26619/SM1811, 10-250 kD; Fermentas) were used as internal molecular weight standards. Each blot was repeated three times. The value of optical density (OD) was measured by the software (Quantity One v462; Bio-Rad Laboratories) and the relative protein expression levels of MUC4 were calculated with the following ratio: (OD value of MUC4 - OD value of background) / (OD value of GAPDH - OD value of background).

FACS analysis of surface expression of MUC4. Cells (1x10⁶) were suspended in 100 µl of PBS containing 1% BSA and incubated on ice for 30 min with 5 µg/ml monoclonal anti-MUC4 (clone Ig8) antibodies (mouse IgG1; Invitrogen). After washing twice with 2 ml of cold PBS, the cells were incubated on ice for 30 min with 1:100 diluted fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG antibody (eBiosciences), washed twice with PBS, and fixed in 1% paraformaldehyde and, finally, 10,000 gated events were analyzed using a FACScalibur flow cytometer (BD Biosciences). Each measurement was repeated three times. Geometric mean fluorescence intensity (GMFI) values of equal quantity cells only labeled primary antibodies were calculated as background values. GMFI values of equal quantity cells only labeled second antibodies were calculated as negative control (NC). Fluorescence index (FI) was calculated with the following formula: FI = (GMFI sample - GMFI background) / GMFI background.

MS-CTL/tumor-cell co-incubation experiments

Fixed basic co-incubation condition and standardizing measurements. Co-incubated system with basic condition and fixed parameter: MS-CTLs suspended in 10% FCS RPMI-1640 medium were seeded into a round-bottomed 96-well plate at 5x10⁵ cells/100 µl/well in triplicate. The prepared target cells were added into the corresponding wells at 1x10⁵ cells/100 µl/well to make the effector/target ratios equal 5:1, respectively. At the same time 10% FCS RPMI-1640 medium without target cells was added into MS-CTLs suspended at a final volume of 200 µl/well as background control. The plates were spinned at 1,000 rpm for 10 min and then incubated for 4 h at 37°C, 5% CO₂, the same as the standard procedures of ³²Cr release assay (cytotoxicity assays) (25). After incubation, the plates were spun again at 1,000 rpm for 20 min; 100 µl supernatant were harvested for subsequent tests and 100 µl co-incubated cells were harvested for the following apoptosis measurements of CTLs.

FACS-apoptosis measurements of CTLs from the co-incubated system: the co-incubated cells at a volume of 100 µl were harvested into tube and labeled with 5 µl of anti-CD8-APC antibodies (Invitrogen) for 20 min at room temperature, protected from light. Then, cells were resuspended in buffer and were stained with an Annexin V-FITC kit (Invitrogen Systems). Cell suspensions were evaluated using a FACScalibur flow cytometer (BD Biosciences) within 1 h and the results were analyzed using CellQuest version 3.1 software (BD Biosciences). At least 10,000 events per sample were collected with debris and aggregates excluded using low forward and orthogonal light scatter values. Compensations were established using single color controls. The gates for CD8 positive cells were set to calculate CTL (CD8⁺) apoptosis. Cells that stained positively with Annexin V were considered to be apoptotic. Fig. IC shows that CTLs were sorted out easily from co-incubated cells by CD8-APC positive gate and were calculated accurately.

Co-incubation experiments. A series of experiments was carried out based on basic condition (see above) as follows: i) the apoptosis rates of MS-CTLs incubated with MUC4⁺ tumor cells (HCT 116, CFPAC-1 and HPAC) and pulsed-peptide T2 cells were compared to confirm the previous observations; ii) clonal MUC4-knockdown HPAC sublines with different MUC4 expression were selected as target cells and further MS-CTL apoptosis was examined; iii) the harvested supernatant of distinct groups, the same as ii), were used to incubate MS-CTLs for 4 h and following apoptosis measurement for the analysis of the influence of supernatant; iv) effect of blockade of Fas on MS-CTL surface. We hypothesized that the Fas-FasL pathway might contribute to MS-CTL apoptosis as one of the effect factors. Before co-incubating with target cells, also the same as ii), MS-CTLs were cultured for 1 h at 37°C in the presence of antagonistic mouse anti-human
Fas, ZB4 (Beckman Coulter, Brea, CA, USA; 2 µg/ml). The effective blocking dose was defined according to lymphocyte counting using tetrazolium bromide (MTT). After incubation, cells were collected for the detection of apoptosis.

Statistical analysis. Results are expressed as means ± standard error (SEM). Data were analyzed using one-way analysis of variance between groups (ANOVA). Correlations between CTL apoptosis rates and determined factors were analyzed using linear regression analysis. The variations in blocking effect of Fas on CTL surface were analyzed by 2-way ANOVA. Statistical analysis was performed with SPSS 17.0 software. Differences were considered statistically significant at P<0.05.

Results

Generation of mature DCs and MS-CTLs. After 7 days of culture in medium containing GM-CSF, IL-4 and TNF-α, FACS analysis showed that DCs from blood samples of three HLA-A*0201 volunteers displayed typical mature phenotypic characteristics. The frequency of expression of the phenotypic markers CD14, CD86, CD1a, CD11c and HLA-DR was 1.32±0.12, 89.13±3.76, 90.24±8.63, 97.32±1.23 and 96.55±2.59%, respectively (one representative FACS histogram is depicted in Fig. 1A). CD8+ T cells (purities >99%, as shown in Fig. 1B) were induced into human HLA-A2-restricted and MUC4-specific CTLs by co-culture with epitope peptide-
loaded DCs. The specific cytotoxic activity of MS-CTLs is consistent with our previous reports (16) (data not shown).

The apoptosis rate of MS-CTLs increases significantly when co-cultured with MUC4+ tumor cells compared to the background control. Induction of MS-CTLs using blood sample from volunteer 1; compared to the background control group (CTLs without target cells added) or T2 cell group, the apoptosis rate of MS-CTLs in MUC4+ tumor cell (HCT 116, CFPAC-1 and HPAC) groups increased significantly, P<0.05, respectively (Fig. 2). Using blood samples from volunteers 2 and 3, the changing trend of MS-CTL apoptosis rate was similar, although the changing extent had a slight difference (Fig. 2).

Selection of MUC4-shRNA HPAC sublines with different MUC4 expression. Seven stably transfected clones were identified via real-time RT-PCR. The expression level of MUC4 normalized to that of GAPDH is shown in Fig. 3A. Compared to HPAC (100%), the MUC4 expression levels were 96.23±19.76, 20.30±1.05, 67.59±7.16, 75.49±9.17, 6.85±0.17, 13.07±3.62 and 43.35±3.15% in the stably transfected empty vector clone of HPAC (HPAC-EV), six stably transfected MUC4-shRNA clones of HPAC, i.e. HPAC-sh-#1-6, respectively.

Three clones, i.e. HPAC-sh-#3, HPAC-sh-#4 and HPAC-sh-#6, with a wide range of different MUC4 expression levels were selected for subsequent confirmation via western blot analysis and flow cytometry. Fig. 3B shows that the relative protein expression levels of MUC4 were normalized to that of GAPDH. The expression levels were 0.68±0.10, 0.66±0.04, 0.40±0.03, -0.0008±0.03 and 0.21±0.01 in HPAC, HPAC-EV, HPAC-sh-#3, HPAC-sh-#4 and HPAC-sh-#6, respectively.

Cell membrane surface expression of MUC4 was determined by FACS analysis. Fig. 3C shows that the fluorescence index (FI) on behalf of relative surface expression levels of MUC4 was 17.56±0.69, 0.97±0.09, 8.61±0.51 and 0.10±0.01 in HPAC, HPAC-EV, HPAC-sh-#3, HPAC-sh-#4, HPAC-sh-#6 and NC (negative control), respectively. This is consistent with the results of the western blot analysis. These results demonstrated higher, medium and lower reduction of MUC4 expression in HPAC-sh-#4, HPAC-sh-#6 and HPAC-sh-#3, and were therefore termed HPAC-sh-Higher...
MUC4 expression on HPAC surface contributes to MS-CTL apoptosis, while the influence of supernatants does not significantly correlate with MS-CTL apoptosis. Induction of MS-CTLs using blood sample from volunteer 1: compared to background control (group 1), the apoptosis rate of MS-CTLs in HPAC group (group 2), HPAC-EV group (group 3), H-sh-L group (group 4), and H-sh-M (group 5) increased significantly, $P<0.05$, respectively (Fig. 4A). Compared to group 2, MS-CTL apoptosis rate in groups 4, 5 and H-sh-H (group 6) decreased significantly, $P<0.05$, respectively (Fig. 4A). Meanwhile, as shown in Fig. 4B, a strong positive correlation ($r^2=0.852$, $P<0.0001$) was observed between CTL apoptosis rate and the surface expression levels of MUC4 on HPAC sublines indicated by mean fluorescence index (MFI). Using blood samples from volunteers 2 and 3, the variation of data presented a similar general trend, although the changing extent was different (Fig. 4B and D). Therefore, these results demonstrated that surface expression of MUC4 on HPACs contributed to MS-CTL apoptosis.

After MS-CTLs were re-cultured using the supernatants of groups 1-6 with fixed basic condition, one-way ANOVA demonstrated MS-CTL apoptosis had no statistically significant differences ($P>0.05$) among distinct groups (Fig. 4E). The result showed that the total effects of supernatants which might hold various soluble factors were not clearly produced on MS-CTL apoptosis.

Fas-blockade has no significant effect on MUC4-mediated MS-CTL apoptosis. Although high levels of MUC4 expres-
sion on HPAC surface were verified to have an effect on the significant increase of apoptosis of MS-CTLs during tumor cell-CTL interaction, whether the classical Fas-FasL pathway is actively involved in this process or not remains to be determined. As shown in Fig. 5, two-way ANOVA indicated that Fas-blockade/non had no statistically significant effect on MS-CTL apoptosis (P>0.05). At the same time, the effect of interaction of the factors (Fas-blockade and MUC4-expression) was non-significant. These results indicated that the Fas-FasL pathway did not participate in the process of MUC4-mediated MS-CTL apoptosis.

Collectively, the results suggested that during the cytolytic process, MUC4-bearing tumor cells could counterattack MS-CTLs via cell surface MUC4-mediated MS-CTL apoptosis, indicating a novel Fas-independent counterattack pathway, although the underlying mechanism may be diverse.

Discussion

As a class of molecules with significant roles in cell-cell communication, tumor-associated mucins, including human mucin4 (MUC4), might affect the immune response in several ways: they might provide an impenetrable barrier for immune effector cells, preventing an antitumor response; they might inactivate immune effector cells through receptor-ligand interactions; or they might sequester cytokines or other compounds that suppress a tumor immune response (19).

Although the exact mechanisms of immune regulation by MUC4 are not yet clear, our observations support the hypothesis that MUC4 may be employed by MUC4-bearing cancer cells to counterattack immune cells for their enhanced survival. Our data clearly indicate that the apoptosis of MS-CTLs can be mediated by membrane-associated MUC4 on the surface of pancreatic cancer cells during the cytolytic process. However, our data showed that supernatants (which could contain various soluble factors generated by the interaction of cancer cells and CTLs) have non-significant effects on the apoptosis of MS-CTLs. Moreover, in light of previous reports, three soluble factors, TNF-α (CD120a,b) (26,27), TNF-related apoptosis-inducing ligand (TRAIL) (28,29) and secreted forms of MUC4 (sMUC4) (30), could be involved in the apoptosis induction and function inhibition of activated T cells. We also quantitatively determined the levels of these three soluble apoptosis-related factors in supernatants with standard ELISA kits. The concentrations of three factors appeared to be in the picomolar or nanomolar ranges, respectively, but there was no significant difference in TNF-α, TRAIL or sMUC4 levels among distinct groups and MS-CTL apoptosis did not correlate with these soluble factors (unpublished data). Thus, these two aspects clearly show that MUC4-mediated apoptosis of MS-CTLs is contact-dependent of effector cell with target cell.

Currently, it is considered that the Fas-FasL pathway can result in T-cell suicide and fratricide, known as activation-induced cell death (AICD) (31-33). In the present study, a small degree of apoptosis (~20%) of background control (CTLs without target cells added) was observed, which could occur related with that it took two weeks to induce MUC4 specific CTLs from peripheral blood in vitro and cell-culture medium containing IL-2 might induce the FasL expression on CTL surface and contribute to the AICD effect. FasL expression on tumor cells is considered the ‘counterattack’ of tumor to Fas-expressing effector T lymphocytes, which leads to the occurrence of immune evasion (33-35). However, our data demonstrated that blockade of Fas receptor by pretreatment with the antagonistic Fas antibody (ZB4) did not significantly inhibit the occurrence of MS-CTL apoptosis during the control experiments and the parallel processing systems, suggesting that mainly the cell death stimulus is initiated through a Fas-independent pathway; it might be possible that the interaction of MUC4 (functionally characterized domains)
with other receptors on T cells results in a series of apoptosis signal transductions.

Additionally, we utilized CTL self control group or CTL added with wild tumor cell group to rule out the possible effect of intrinsic activated cell-autonomous death (ACAD) (32) and analogous antigen-dependent apoptosis of CTL (ADAC) (36) in our study system, which gave indirect evidence to the localization of extrinsic MUC4-mediated apoptosis of MS-CTLs. Generally, the pathway of the T-cell apoptosis is considered the intrinsic and extrinsic apoptotic pathway, meanwhile, the absence of appropriate survival signals can cause ACAD (32). On the other hand, ADAC appears only when there is both excessive antigen density and a sufficiently high avidity in the TCR-MHC/peptide interaction (36). However, in the present study, MS-CTL apoptosis did not agree with ACAD and ADAC, since the induction process of MS-CTL is fixed and standard by using appropriate amounts of MUC4 epitope peptide. Moreover, the presence of extrinsic MUC4-mediated apoptosis of MS-CTLs can be verified by implementing the appropriate controls.

In addition, regulatory T cells (Tregs, CD4+CD25+) are considered to suppress effector T-cell activation, proliferation, and cytokine production (37,38). In view of the fact that Treg cells are capable of killing autologous CD8+ T cells, CD8+ T lymphocytes were isolated from the PBMCs before the induction and amplification of MS-CTLs in order to eliminate the effect of Tregs in this study.

As shown in Fig. 6, the present study may provide a first step towards the elucidation of this mechanism in the apoptosis of MUC4-specific CTLs, suggesting that high levels of MUC4 molecules on cancer cell surface as a ligand might induce apoptosis of MS-CTLs by binding to undiscovered receptor on T cells to initiate a signal transduction cascade of apoptosis. Muc4/MUC4 was confirmed to be a ligand/modulator for the receptor tyrosine kinase ErbB2, regulating its phosphorylation and the phosphorylation of its partner ErbB3, with or without the involvement of the ErbB3 ligand neuregulin, which induces signaling related to growth, motility, or differentiation properties of the cell (39-41). It has been speculated that the EGF-like domain of MUC4 specific domains plays a role in the above receptor-ligand interactions (42,43). Meanwhile, the other unique domains present in the MUC4 mucin but not found in other membrane-bound mucins are NIDO, AMOP, and vWD domains (44). Although there is no direct evidence in the literature yet, the homology and evolution analysis hints at a role for both NIDO and AMOP domain in cell-cell interaction and adhesion to the extracellular matrix (45-48). Whether MUC4 itself can establish novel interactions with receptor protein on the surface of CTLs via functionally characterized domain is yet to be confirmed. Further studies on the signaling pathways downstream of CTL apoptosis are presently being carried out by means of construction of functionally primed human CD8+ MUC4-specific CTL clones model system, target molecule screening, domain deletion mutant, function-blocking and other techniques in our laboratory.

In summary, several lines of evidence suggest that there may be a novel counterattack pathway of pancreatic cancer cells, which is a MUC4-mediated, cell contact-dependent and Fas-independent process, to induce CTL apoptosis and attenuate the antitumor effect of CTLs (Fig. 6).

MUC4 is aberrantly expressed in a variety of human epithelial malignancies, particularly in pancreatic cancer (48), hence, the development and implications of anti-MUC4-specific immunotherapeutic strategies are important. Therefore, further exploration and understanding of the potential counterattack mechanisms and the intention to develop strategies to prevent or bypass apoptosis of CTLs is highly advantageous in enhancing the efficacy of MUC4-specific tumor vaccines.

Acknowledgements

The present study was partially supported by the National Natural Science Foundation of China (81101802, 81001079), the Natural Science Foundation of Jiangsu Province (BK2011845), the Program for Development of Innovative Research Team in the First Affiliated Hospital of NJMU, the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD, JX10231801), and the Research Special Fund for Public Welfare Industry of Health (201202007).

References

Cloning of a disintegrin MUC4


