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Cytotoxic T-lymphocytes response in vitro activated by dendritic cells pulsed with heat shock protein 70 derived from human bladder tumor cell lines of EJ*

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Abstract ***Objective:*** To investigate whether human dendritic cells (DC) derived from peripheral blood mononuclear cells (PBMC), which were pulsed by heat shock protein 70 (HSP70) isolated from human bladder tumor cell lines of EJ, were able to induce peptide specific cytotoxic T-lymphocytes (CTL) response *in vitro* and give the experimental foundation for the future clinical trials of immunotherapy in bladder tumor. ***Methods:*** The EJ-derived HSP70 co-cultured with DC from the healthy volunteers' PBMC, along with the crude lysate (the supernatant before HSP70 purification) from EJ cells were used as the experimental groups and DC not pulsed by any tumor cells antigen were the blank control. The autologous T-lymphocytes were added into the above various DC groups, and after incubation, the stimulation indexes (SI) and interferon-γ (IFN-γ) were detected to evaluate the immune activities of various DC groups. The killing effects of CTL to target cells, EJ and Hela cells, were determined with ^{51}Cr releasing test. ***Results:*** Both DC/HSP70 and DC/the crude lysate could effectively activate CTL *in vitro* and kill target cells EJ. The killing effect of DC/HSP70 to EJ was much stronger than DC/the crude lysate (the supernatant before HSP70 purification) ($P < 0.05$). DC without any tumor cell antigens had a lower killing power to EJ. Meanwhile, DC/HSP70 had little killing power to Hela non-relevant to bladder tumor histopathologically as compared with EJ cells ($P < 0.05$). ***Conclusion:*** The DC pulsed by HSP70 derived from the autologous tumor cells could induce a peptide complexes specific CTL response to tumor cells, and the CTL response induced by the DC/HSP70 was stronger, which display the basis of the possible clinical application of DC/HSP70 for bladder tumor.

Key words heat shock protein 70 (HSP70); dendritic cells (DC); cytotoxic T-lymphocytes (CTL); bladder tumor

Bladder tumor is the most common tumor of the urinary system with 132 432 deaths worldwide. A sampling investigation showed that bladder tumor was chiefly primary transitional cell carcinoma (TCC) and had one of the ten highest morbidity rates for malignant tumors. Bladder tumor represented two percent of all malignant tumors and is the fifth most common malignancy in men in Europe and North America. It is estimated that there are 52 000 new bladder tumor cases and 10 000 deaths in the United States per year [1]. The major obstacle in the management of bladder tumor is the low sensitivity to chemotherapy and the high recurrence of the carcinoma after operation, which represents approximately 40% bladder tumor patients [2].

It is thus important to establish novel therapeutic strategies and consequently improve the survival of patients with advanced disease. It was reported that the tumor-derived, heat shock protein 70 (HSP70)-rich cell lysate was able to activate dendritic cells (DC) and provoke strong anti-tumor immune response [3]. HSPs as chaperon, in which HSP70 is a chief family member, are a group of highly conserved proteins found in nearly all cells and induced by various stress factors including some tumor proteins. HSP70 with relative molecular weight around 70 kDa and ATPase activity is located in all prokaryotes and in most compartments of eukaryotes and plays essential roles in protein metabolism regardless of stress or non-stress conditions, including functions in the other proteins folding, membrane translocation and degradation of mis-folding proteins. Elevated HSP70 expression results in higher cell survival rate after exposure to cytotoxic stimuli, such as tumor proteins [4, 5]. HSP preparations, including HSP70, GRP94/gp96 derived from tumor cells and virus-infected cells, succeed in eliciting cellular

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immunity. The immunogenicity of HSP70 preparations has been ascribed peptides bound to HSP70. It is reported that HSP70 proteins chaperone antigenic peptides into antigen-presenting cells, potentially allowing peptides to enter the MHC class I pathway for loading onto MHC class I molecules, where they can be presented to cytotoxic CD8+ T cells [6]. DC as professional antigen presenting cells (APC) with potent antigen presenting ability can take up and process tumor associated antigen (TAA) and transfer TAA to specific CD8+ T-lymphocytes to kill tumor cells [7]. This provides a strategy for immunotherapy against tumor by using DC pulsed with HSP70 that are isolated from tumor cells or tissues. Up to now, there is little information regarding immunotherapy to bladder tumor with DC pulsed by HSP70. So, we investigated the data in this field and preliminarily probed its fundamental mechanism.

Materials and methods

Cell culture

The EJ cell lines of human bladder carcinoma, and Hela (kindly supplied by the Institute of Urology, Peking University, China) were cultured in RPMI-1640 medium (Invitrogen, USA) containing 10% fetal calf serum (FCS, Invitrogen, USA), 100 U/mL penicillin (Invitrogen, USA) and 100 μg/mL streptomycin (Invitrogen, USA) in 5% CO_2 air environment at 37 ℃. RPMI-1640 was replenished in every two or three days to maintain cell growth. The cells were resuspended in fresh RPMI-1640 medium to provide a cell density of 1×10^6 cells/mL before experiment. Cell viability was evaluated by a trypan blue exclusion test.

Preparation of HSP70

The HSP70 were purified adapting the method from Peng *et al* [8]. EJ cells (1×10^7) were homogenized in hypotonic buffer without detergent (10 mmol/L Tris-Ac, 10 mmol/L NaCl, and 0.2 mmol/L phenylmethylsulfonyl fluoride, pH 7.2) and centrifuged at 100 000 × g. The supernatant (partly as crude lysate used in pulsing DC) was incubated with Active Blue 2 Sepharose beads (Sigma, USA) for 30 min, after which the buffer was changed to buffer D (20 mmol/L Tris-Ac, 20 mmol/L NaCl, 15 mmol/L β-mercaptoethanol, 3 mmol/L $MgCl_2$, and 0.5 mmol/L phenylmethylsulfonyl fluoride, pH 7.5). The sample was loaded onto an ADP- or ATP-agarose column (Sigma, USA). HSP70 was eluted with 3 mmol/L ATP-containing buffer D, after which the buffer of the eluate was changed to buffer A (20 mmol/L sodium phosphate and 20 mmol/L NaCl, pH 7.0), and the eluate was loaded on a DEAE-Sephasel column (Sigma, USA). HSP70 was eluted with 1.5 mL fractions with buffer A1 (130 mmol/L NaCl in buffer A). The protein content in the different fractions was measured with the Bradford assay (Bio-Rad, USA) with bovine serum albumin (BSA, Bio-Rad, USA) as standard and according to the manufacturer's instructions.

SDS-PAGE and Western blot

The quality of HSP70 protein was monitored by Western blot as described [9]. Samples of the purified HSP70 (50 μg) and the crude lysate from the supernatant were run on SDS-PAGE 12.5% homogenous gels. The gel was silver stained after partial transfer to polyvinyl difluoride membrane (Bio-Rad, USA). The blot was blocked in PBS containing 0.1% Tween –20 and 5% skim milk (Sigma, USA) at 37 ℃ for 1 h. The membrane was then incubated in mouse anti-human HSP70 mAb (Santa Cruz, USA) against HSP70 (1:200) at 4 ℃ overnight, followed by a peroxidase-conjugated sheep anti-mouse secondary antibody (DAKO, Denmark) (1:1000). Immunoreactive proteins were detected by chemiluminescent ECL reagent (Amersham, USA), and the chemilluminescent signal on the blot was exposed to an X-ray film.

Preparation of DC from peripheral blood mononuclear cells (PBMC)

Based on the protocol as described [10] and a little changed, PBMC from the volunteers' blood was isolated by Ficoll-Hypaque (Pharmacia, Sweden) density gradient centrifugation, washed twice, and allowed to adhere 24-well plates (Nunclon, USA) at a density of 3×10^6/cells per dish in 1 mL of complete culture medium. After 2 h in 5% CO_2 air at 37 ℃, the non-adhere cells were removed and the adhere cells were cultured in a complete culture medium containing 1000 U/mL GM-CSF (Minneapolis, USA) and 500 U/mL IL-4 (Promega, USA). On day 3, 300 μL of medium was removed and fresh serum-free medium with full dose of the above cytokines was added. TNF-α (1000 U/mL, Minneapolis, USA) was added into on day 6 to induce the maturation of DC and cells were harvested on day 9 to be used in the evaluation of DC cells and the induction of CTL.

Flow cytometry

DC was washed in 0.5 mL PBS and incubated for 30 min at 4 ℃ with mAbs as follows: anti-CD80, APC-conjugated (BD PharMingen, USA), anti-CD83, APC-conjugated (BD PharMingen, USA), anti-CD86, Cychrome-conjugated (BD PharMingen, USA), anti-HLA-DR, APC-conjugated (BD PhaMingen, USA). Then, the populations were analyzed by fluorescence activated cell sorting (FACS) through the FAC440 (Becton Dickinson, USA).

Preparation of pulsed DC

HSP70 were added into DC (1×10^6/mL) at the concentrations of 6, 8, 10 and 12 μg/mL on day 6 and co-cultured to day 9. The uptake rate of DC to HSP70 after DC

being co-cultured with HSP70 was evaluated with FACS, in which FACS detected HSP70 in DC at the concentrations of 6, 8, 10 and 12 μg/mL, respectively with mouse anti-human HSP70 mAb (Santa Cruz, USA) conjugated by second antibody, sheep anti-mouse IgG-FITC (DAKO, Denmark). The cultured DC was divided into three groups with 1×10^6 cells/mL per group, into which group added with 10 μg/mL HSP70 to prepare pulsed DC, group treated with the supernatant crude lysate and the other group without any antigen used as blank control. In addition, the morphology for DC to take up HSP70 was observed under confocal microscope (Leica SP2, Germany) in fixed cells incubated with HSP70 for 72 h, in which cells were stained with mouse anti-human HSP70 mAb and then conjugated by sheep anti-mouse IgG-FITC.

Mixed lymphocytes reaction

The autologous T-lymphocytes (2×10^5) were co-cultured with pulsed DC and non-pulsed at the 5:1, 10:1 and 20:1 ratio of T cells to DC cells, respectively, in which various DC were pretreated with 25 mg/L mitomycin (Sigma, USA) in 37 ℃, 5% CO_2 air for 45 min, then washed three times with PBS. The above mixed cells were cultured in 37 ℃, 5% CO_2 for 96 h in 96-well plates (Nunclon, USA). Then, 10 μL MTT (5 mg/mL, Sigma, USA) was added in medium, mixed well-homogeneously, and cultured for 4 h. The supernatants were removed and used as IFN-γ assay, performed by using ELISA kit (Jingmei Biotech Co, China) according to the protocol of manufacturer. DMSO (Sigma, USA) was added into the mixed media, dissolved completely, and then, the relative OD values were determined at 490 nm. The stimulated effects were showed as stimulation indexes (SI): (experimental OD / effector T cells control OD) × 100%.

Induction of CTL

T-lymphocytes derived from the autologous peripheral blood [11] were used as effector cells and divided into three groups, that was, treated with DC/HSP70, DC/the crude lysate (the supernatant before HSP70 purification) and DC without any antigens as blank control. T-lymphocytes (2×10^6) were cultured with three kinds of DC groups (4×10^5, pretreated with 25 mg/L mitomycin) in 24-well plates and incubated in 37 ℃, 5% CO_2 for 96 h and were harvested for killing test.

^{51}Cr-release test for CTL to kill target cells

The cytolytic activity of the induced CTL was determined by a standard ^{51}Cr-release assay. 12 target cells were labeled with 50 μCi ^{51}Cr-labeled sodium chromate (Amersham, USA) in RPMI-1640 with 10% FCS for 2 h at 37 ℃ and then washed twice with plain RPMI-1640. Target cells (5×10^3) were cultured with CTL by 1:10, 1:20, and 1:40 ratio of target cells / effector cells in 96-well round-bottom plates in a final volume of 200 μL RPMI-1640 with 10% FCS, centrifuged to promote cell contact and incubated at 37 ℃ for 4 h. Then 100 μL of supernatant in each well was harvested manually. Radioactivity released into the supernatant was measured in a γ-counter, and the percent specific release was calculated from the mean of duplicate cultures according to the following formula: percent specific release = [(experimental release – spontaneous release) / (maximal release (1% NP-40) – spontaneous release)] × 100%.

Statistical analysis

SPSS 10.0 analysis of variance and Mann-Whitney (used in the comparison of means between two groups) were used to evaluate the experimental data given in the way of mean ± standard deviation (mean ± SD). All values quoted were two-sided, and those below 0.05 were judged as being statistically significant.

Results

Purification of HSP70

The silver staining of SDS-PAGE displayed a good purity of HSP70 derived from EJ cells and the specificity was ascertained by HSP70-specific antibody through Western blot analysis (Fig. 1). About 63.51 ± 23.38 micrograms of HSP70 were extracted from 1×10^7 EJ cells.

Culture and maturation of DC

DC from PBMC showed the sphere-like pattern smooth in surface and became greater than ever on day 3 during culture. On day 6, most cells dispersed in the medium, in which DC broke out magnificently in surface as dendritic typically. FACS analysis showed that DC expressed maturation markers, such as CD80 (86.12 ± 5.23%), CD83 (81.23 ± 3.56%), CD86 (89.62 ± 3.36%) and HLA-DR (82.69 ± 2.97%), on day 9 (Fig. 2). About 31.35% viable DC from the initial population of PBMC could be obtained.

DC pulsed with HSP70

DC was able to take up effectively HSP70 and the uptake rate of DC to HSP70-peptide complexes increased following the antigen concentration increasing from 6 μg/mL to 12 μg/mL based on the FASC detection. However, more than 10 μg/mL of the antigen concentration, the uptake rate did not increase (Fig. 3). Therefore, DC was pulsed with the concentration of 10 μg/mL HSP70-peptide complexes. Furthermore, the green fluorescence, representing HSP70-peptide complexes taken up by DC, could be obviously seen in the maturation cells of DC under confocal microscope (Fig. 4).

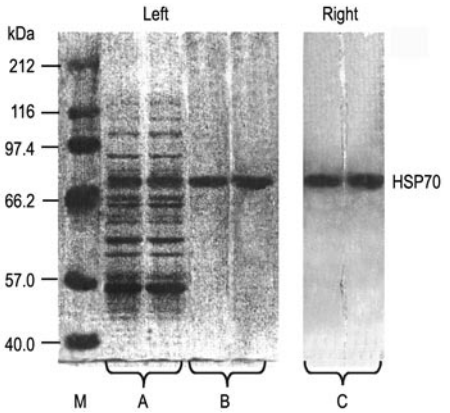

Fig. 1 Purification of HSP70. Left panel was SDS-PAGE (silver staining), and right panel was Western blot. M: molecular weight markers; A: the supernatant crude lysate on SDS-PAGE including various proteins band; B: HSP70 on SDS-PAGE; C: immunoblot band with HSP70-specific mAb

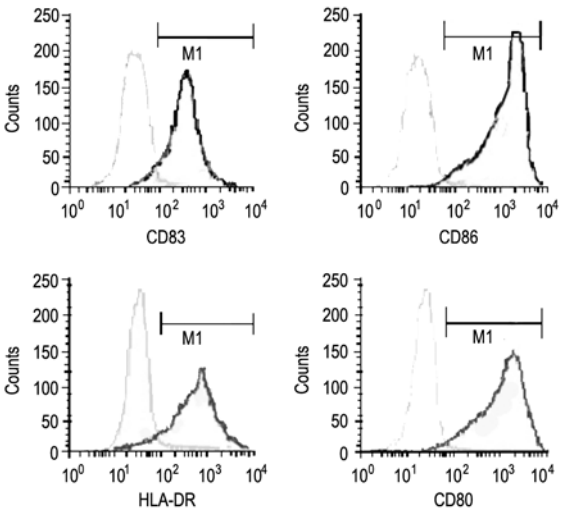

Fig. 2 Expression of activation markers on the surface of DC detected by FACS. Expression rates were as follows: CD83: 81.23 ± 3.56%; CD86: 89.62 ± 3.36%; HLA-DR: 82.69 ± 2.97%; CD80: 86.12 ± 5.23%

Immune activity of pulsed DC

The autologous MLR displayed that a few DC pulsed by tumor cells antigen could activate CTL effectively, and the ability of HSP-70 peptide complexes to activate immune response was more than the crude lysate ($P < 0.05$). But, the immunogenicity of DC alone was much lower regardless of the ratio of DC to effector T cells (Fig. 5).

Killing test to target cells

The killing effects of CTL activated by DC/HSP70 to EJ cells were stronger than DC/the crude lysate ($P < 0.05$). The group of DC alone had much lower killing rate to EJ cells as compared with DC/HSP70 ($P < 0.05$) and DC/the crude lysate ($P < 0.05$), respectively. However, the DC/HSP70 and DC/the crude lysate could elicit few killing

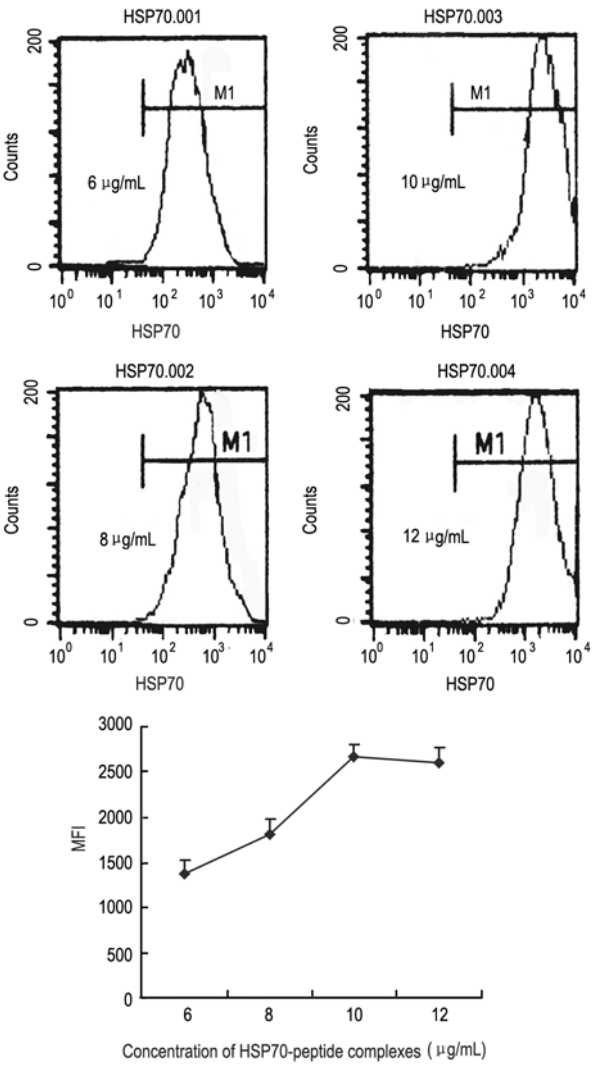

Fig. 3 The DC uptake rate to HSP70 following different concentration by FACS. DC was co-cultured with HSP70 at the concentrations of 6, 8, 10 and 12 μg/mL for 72 h. DC was stained with mouse anti-human HSP70 mAb, then, conjugated by sheep anti-mouse IgG-FITC. The upper figures were FACS results. MFI was mean fluorescence intensity representing the uptake rate of DC to HSP70. DC taking up HSP70 did not increase at 10 μg/mL. N = 3, repeated three times

reactions to Hela cells not relevant histopathologically with bladder tumor (Fig. 6). These data suggested that the anti-tumor immune response, induced by DC/HSP70, was specific to tumor cells, in which HSP70 assisted DC as a professional APC to transfer tumor associated antigen (TAA) onto CTL.

Discussion

HSP70 is an abundant soluble intracellular molecule, which functions as chaperone proteins to help the other proteins to change conformation, fold, transfer trans-

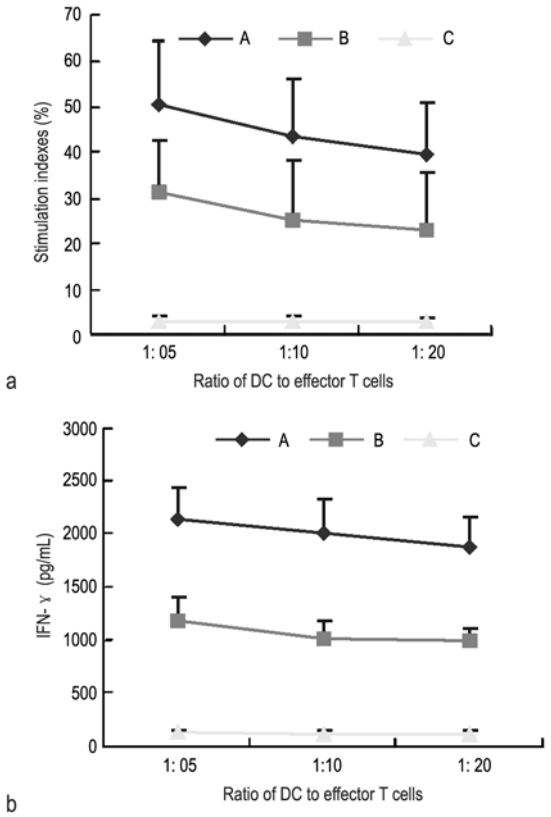

Fig. 5 Mixed lymphocytes reaction for evaluating various pulsed DC immunogenicity. A: DC/HSP70; B: DC/the crude lysate; C: DC. The (a) line chart was stimulation indexes (SI) at various ratio of DC to T cells, and the (b) was IFN-γ assay. As to SI, the interactions were not found between groups and the ratio of DC to T cells ($P > 0.05$). Furthermore, there were statistical differences between A, B and C ($P < 0.05$), but no statistical differences were found between the ratio of DC to T cells ($P > 0.05$). The results of IFN-γ assay were the same as SI. N = 3 wells, repeated three times

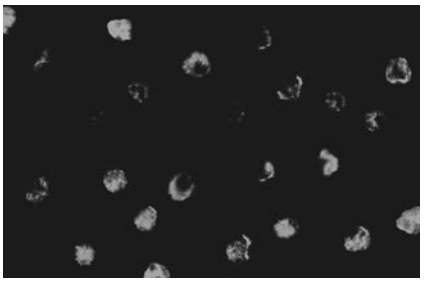

Fig . 4 The uptake of DC to HSP70 was observed with confocal microscopy (400 × original magnification) after DC was incubated with HSP70 for 72 h at the concentration of 10 μg/mL. DC was stained with mouse anti-human HSP70 mAb, then, conjugated by sheep anti-mouse IgG-FITC

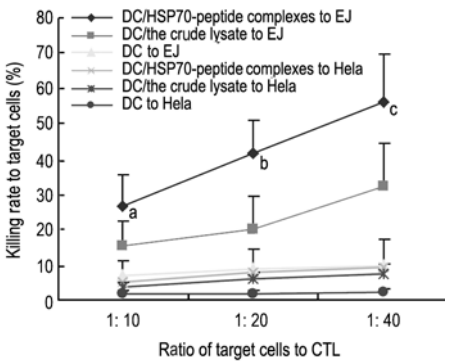

Fig. 6 CTL killing effects to target cells were determined by ^{51}Cr-release test. The interactions between groups and ratio were not seen ($P > 0.05$). As to Hela, no statistical differences were found regardless of groups or ratio ($P > 0.05$). As to EJ, there were statistical differences between groups ($P < 0.05$). Moreover, ^{a} $P < 0.05$, ^{b} $P < 0.05$, ^{c} $P < 0.05$, vs DC/HSP70 to Hela respectively. N = 3 wells, repeated three times

membranously, locate correctly and for denatured proteins to degrade while HSP70's ability to stimulate immune responses is not expected under physiological conditions. However, under the presence of stress factors, such as heavy mental ion, hyperthermia, tumor proteins, etc., HSP70 over-expression was able to help the cells to survive the above harmful circumstances [4]. It was reported that colon, renal, breast, endometrial and prostate carcinomas over-expressed HSP70 and that individual chaperone proteins, such as HSP70 and GRP94/gp96, had been shown to deliver partial maturation signals to DC to stimulate some cytokines expression, thus inducing Th1-type immune response [5, 6]. DC as an important APC is pivotal for the initiation of primary immune response of both helper and cytotoxic T lymphocytes [7, 12, 13].

Our previous investigations have shown that the over-expression of HSP70 in primary bladder TCC was found and correlated with tumor stage and grade [14], which laid some basis of applying HSP70-peptide complexes for pulsing DC. It was documented [3, 15] that HSP70 could not only transfer immunogenic signals to CD8+ T cells and/or CD4+ T cells with the help of MHC-I and/or MHC-II molecular pathway, but also present directly antigen to CD8+ T cells as antigen presenting molecule. Our data displayed that DC/HSP70 and DC/the crude lysate induced activated T-lymphocytes to produce IFN-γ, but the former was stronger than the latter. Meanwhile, CTL activated by DC/HSP70 could more strongly kill EJ cells than CTL activated by DC/the crude lysate. However, DC alone has few killing power to EJ cells. Furthermore, the killing ability induced by DC/HSP70 to EJ cells was much stronger than Hela cells non-relevant to bladder tumor histopathologically, which implied the specificity of immune reaction to tumor cells mediated by HSP70. It was reported that the reason of specificity might lie in that HSP70-peptide complexes inducing the maturation of DC to cause effective CTL reaction was dependent on the recognition of a specific MHC-restricted antigen peptides [12, 16].

On the other hand, the uptake of DC to HSP70 did

not increase when the concentration of HSP70 exceeded a certain point through FACS analysis, suggesting that the inter-reaction between DC and HSP70 might have a saturability. It was documented that the internalization of HSP, such as HSP90, gp96, etc., needed a receptor on the surface of DC cells [16]. Based on the above point of view, it was presumably believed that the reason of the uptake of DC to might lie in the saturation existing between the receptor on the surface of DC cells and ligand.

In conclusion, our investigations showed that HSP70 derived from human bladder tumor cell lines of EJ could exert a stronger specific trigger of anti-tumor cells CTL response. Though such a complete, distinct mechanism remains to be investigated, the ability of HSP70 to activate anti-tumor cells immune response profiles an active role of HSP70 as a candidate vaccine in bladder tumor patients.

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