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Novel vaccines for the treatment of chronic HBV infection based on mycobacterial heat shock protein 70

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Abstract

Immunogenic peptide-based vaccines can raise significant cellular immune responses. Although cytotoxic T lymphocytes (CTL) peptide epitopes are generally poor immunogens, heat shock protein 70 from *Mycobacterium tuberculosi*s (TBhsp70) can overcome this problem since it is a potent adjuvant that links innate and adaptive immune responses. Our goal is to use TBhsp70 as an adjuvant for development of therapeutic vaccines for chronic Hepatitis B virus infection (HBV). To this end, we genetically fused the HBV core 18–27 peptide ($HBcAg_{(18–27)}$) as a CTL epitope to the C-terminus of TBhsp70 and expressed the resulting protein in methylotropic yeast *Pichia pastoris* GS115. At the same time, the TBhsp70-$HBcAg_{(18–27)}$ peptide complex was reconstituted in vitro. We investigated whether TBhsp70–peptide complex and TBhsp70–peptide fusion protein could generate antigen specific CTL responses in vitro. Dendritic cells (DC) from HLA-$A2^+$ chronic HBV infection and healthy control pulsed with two vaccines were studied phenotypically by FACS analyses and functionally by cytokine release, and HBV-specific CTL response. Our results demonstrate that two vaccines can activate DC of chronic HBV infection and healthy control by upregulation CD40 and CD86, high production of IL-12p70 and TNF-α. Furthermore, autologous T cells with DC stimulated by two vaccines can produce IFN-γ and generate HBV-specific CTL response. However, capacity for CTL response and cytokines production from HBV infections remained inferior to that of healthy controls. Thus, the strategy of utilizing TBhsp70 may provide a novel design for the development of prophylactic and therapeutic vaccines.
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Keywords: Heat shock protein 70; Cytotoxic T lymphocytes; HBV; Dendritic cell

1. Introduction

Hepatitis B virus is a serious health problem with over 300 million people worldwide being affected by chronic HBV infection [1,2]. Chronic HBV infection incurs a serious sequelae such as liver cirrhosis and hepatocellular carcinoma. Preventive vaccines using HBsAg alone or combined with other antigens allow for the generation of neutralizing antibodies, which effectively prevent infection in immunocompetent individuals. Many studies show that major histocompatibility complex (MHC) class I-restricted cytotoxic T lymphocytes (CTL) can play a central role in the prevention, control, and clearance of HBV infections [3,4]. Therefore, therapeutic approaches focusing on cell-mediated immunity for viral elimination have been part of the research agenda for many years.

Recent advances in cellular immunity have greatly increased the potential of peptides as immunogens for CTLs. These include the demonstration that CTLs can recognize small antigenic peptides of 8–10 amino acids in length, which complex with MHC class I molecules and express on the surface of infected or cancer cells to be presented to T-cell receptors [5,6]. Targeting of dominant epitopes may be an effective way to overcome CTL tolerance [7] and to allow the immune response to focus on highly conserved epitopes [8]. How-

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ever, a major problem of peptide-based vaccines has still to be overcome since their minimal CTL epitopes are not immunogenic per se and generally require the addition of T helper epitopes and adjuvants for efficient stimulation of CTL. At this time, no potent immune adjuvants that induce effective cellular immunity are effectively approved for human use. Therefore, there is a need for well-tolerated, immune adjuvants that induce effective T-cell responses. Heat shock proteins (hsp) are among the candidates for such immune adjuvants, which induce T-cell response and maturation of DCs [9,10].

Srivastava et al. [11] have demonstrated that hsp strongly enhance the immune reaction to tumor-associated antigens. Several studies showed that hsp70 or gp96 preparations isolated from tumor cells in vivo or reconstituted cells in vitro were able to induce specific CTL responses capable of protecting against tumor growth and virus infection [12–16]. Many microbial components have been found to activate DCs and induce CTL response in a CD4 Th-independent manner. In this regard, *Mycobacterium tuberculosis* hsp70 (TBhsp70) is an especially powerful Ag containing multiple B- and T-cell epitopes [17,18]. In fact, Suzue et al. [19–21] found that TBhsp70 fused to ovalbumin (OVA) 161–276, elicited CTL responses specific for K^b-restricted OVA 257–264. In addition, they also produced and purified a recombinant HIV-I p24-hsp70 fusion protein and demonstrated that it could elicit both humoral and cellular responses against HIV-1 p24 in the absence of adjuvant. Roman et al. [22] reported that synthetic peptide such as influenza A virus nucleoprotein [residues 206–229 (pNP)] with TBhsp70 could elicit a peptide-specific T-cell response in vivo. The generation of CTL by MHC class I ligands fused to hsp70 was reported by Udono et al. [23,24]. Wang et al. [25] suggested that CD40 was a cellular receptor for TBhsp70. The CD40/CD40L interaction also leads to production of inflammatory cytokines, such as TNF-α, IL-1, IL-6, IL-12 and RANTES. Indeed, recent studies demonstrated a central role for ligation of CD40 on macrophages and DC in the induction of MHC class I-restricted antigen-specific CD8+ T-cell responses and protective immunity [26].

Chronic HBV infection is known to be associated with T-cell hyporesponsiveness and tolerance as well as DC dysfunction [27]. To overcome the deficiencies of presently available vaccines for the immunotherapy of chronic HBV infection, we design vaccines by using TBhsp70 as an adjuvant to treat HBV infection. Here, the HBV 18–27 core peptide is selected as the CTL epitope since it is conserved in the vast majority of HBV viral isolates, binds HLA-A2.1 with high affinity, and also is cross-reactive with several other common HLA-A2 subtypes [28,29]. We therefore have designed TBhsp70-HBcAg$_{(18–27)}$ fusion protein and TBhsp70-HBcAg$_{(18–27)}$ complex. We present here our initial results, which support the basic design of our molecular structure as well as the use of TBhsp70 as a molecular adjuvant.

2. Materials and methods

2.1. Construction of expression vectors

The DNA fragment containing the TBhsp70 coding sequence was synthesized by PCR using DNA purified from *M. tuberculosis* H37Rv as a template. One upstream primer (5′-CGCTCGAGAAAAGA ↓ gctCGTGCGGTCGGGATC-3′) contained an *Xho*I site that overlapped a yeast signal cleavage site factor without the AUG translation initiation codon of the hsp70 gene. The downstream primer (5′-GCGAATTCT CActtG GCCTCCCGGCCGTC-3′) contained an *Eco*RI site after the translation stop codon. The yeast shuttle plasmid vector pPIC9-hsp70 was created by subcloning the TBhsp70 gene into the expression vector pPIC9 at the *Xho*I and *Eco*RI sites. Plasmid pPIC9K-hsp70 was created by subcloning the TBhsp70 gene from pPIC9-hsp70 into the *Sac*I and *Hpa*I sites of pPIC9K (Invitrogen, USA) (Fig. 1). All plasmids were propagated in *Escherichia coli* [30]. For the fusion protein of TBhsp70 and the HBcAg$_{(18–27)}$ CTL epitope, the mini-gene encoding this CTL epitope was incorporated with a reverse primer containing a 3′*Eco*RI restriction site, synthesized by PCR using plasmid pPIC9-hsp70 as a template. Then, the pPIC9K-hsp70–HBcAg$_{(18–27)}$ construct was designed as described above.

2.2. Protein expression and purification

The constructs were integrated by electroporation into the genome of the yeast strain, *Pichia pastoris* GS115 (Invitrogen, USA). Transformants with the phenotype His^+Mut^+ were tested by PCR and selected for studies of gene expression. Culture conditions and expression were maintained according to the manufacturer's instructions. The cultures were grown to an OD600 of 1.0 and recombinant protein production was induced by addition of methanol (0.5% (v/v)) to the culture. After induction for 3 days, the cultures were centrifuged at 17,000 × *g* for 30 min to remove cells and precipitates. The supernatants were then concentrated by size exclusion using Microcon 50 with a molecular weight cutoff of 50 kDa. The concentrated supernatants were applied to an ATP–agarose column (Sigma, USA) and eluted with 1 mM MgATP preadjusted to pH 7.0. EDTA was added to the eluate to a final concentration of 2 mM [20]. The eluate, which contained essentially pure hsp70 or hsp70 fusion protein, was dialyzed against phosphate buffer.

2.3. Protein analysis

Sample aliquots were resuspended in Laemmli buffer and subjected to SDS-PAGE. Protein was visualized by Coomassie staining. For Western blotting, proteins were transferred from the gel to a bio-blot PVDF membrane (Costar, Cambridge, MA, USA) and probed with Abs. The anti-TBhsp70 mAb was purchased from MBL Co. (Japan),

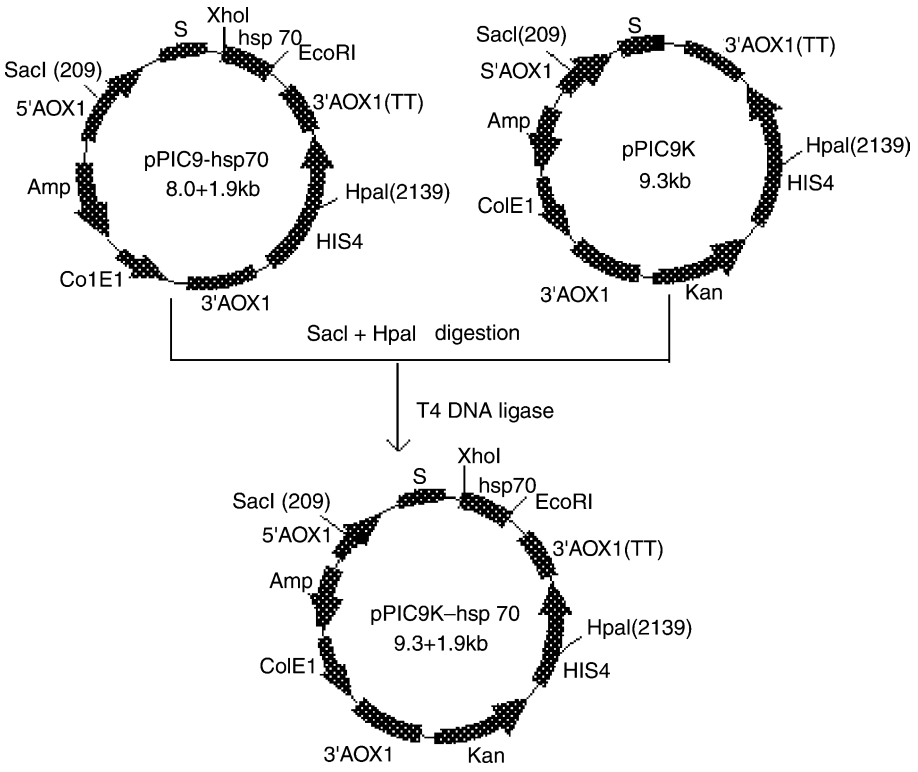

Fig. 1. Construction of recombinant plasmid pPIC9K-hsp70. The pPIC9K vector allows selection for production of *P. pastoris* GS115 that contain more than one copy of the genes of interest. The $HBcAg_{(18–27)}$ CTL epitope can be subcloned into the plasmid vector pPIC9K/hsp70 and epitopes fused to hsp70 can be expressed and easily purified.

and the goat anti-TBhsp70 Ab was obtained from Boster Co. Protein concentrations were determined by the Bradford assay.

2.4. hsp70–peptide binding and competition assay

Peptide binding to hsp70 is tightly regulated by adenosine nucleotide and ATP hydrolysis [31,32]. Hsp70–ADP interaction can stabilize and promote peptide binding to hsp70. Based on this feature, peptide can be easily loaded onto hsp70. Ten microgram each of hsp70 and biotinylated peptide $HBcAg_{(18–27)}$ (Bioasia, Shanghai) were incubated with the binding PBS buffer supplemented with 1 mM ADP and 1 mM $MgCl_2$ at 37 °C for 1 h, in a total volume of 20 μl. For some subsequent assays, free peptides had to be removed by size exclusion using Microcon. For competitive-binding assays, non-biotinylated peptide was added to the reaction mixture at varying concentrations. The hsp70–peptide complexes were then separated by non-denaturing 10% PAGE. After electrophoresis, the complexes were transferred to PVDF membranes for Western blotting. Blots were washed with PBS (0.1 M, pH 7.4) and then incubated with streptavidin conjugated to horseradish peroxidase (25 g/ml, Sigma) for 1 h at room temperature in PBS containing 2% dried skim milk powder. After further washing, peroxidase activity was determined with 3,3′-diaminobenzidine dihydrochloride [22].

2.5. Dendritic cell FACS analysis and cytokine production

HLA-$A2^+$ volunteers aged (18–45) years who had chronic active Hepatitis B [CAH, elevated alanine aminotransferase (ALT) for longer than 6 months, seropositivity for makers of HBV infection and positive HBV-DNA by hybridisation assay or quantitative PCR] were divided into five groups. Each group consisted of six subjects. As control, six HLA-$A2^+$ healthy HBV-naive and blood donors (seronegative for HBsAg, anti-HBs and anti-HBc). Human PBMCs were separated from HLA-$A2^+$ peripheral blood by centrifugation on Ficoll–Hypaque gradients and used as fresh samples. Monocytes were obtained after a 2 h adherence of PBMC to plastic. The nonadherent cells were washed off and collected as autologous lymphocytes. Monocytes isolated from human PBMCs were cultured for 5 days at 37 °C in a humidified 5% CO_2 atmosphere in medium containing rhGM-CSF (1000 U/ml) and rhIL-4 (500 U/ml). DCs were plated in 24-well plates at a cell density of 1×10^6 cells/ml and were loaded on Day 5 with hsp70, hsp70 fusion protein, hsp70–peptide complex, or peptide alone and culture medium serving as a control (10 μg/ml). FACS analysis to assess DC maturation was performed on Day 8, including evaluations of specific CD1a, and function-related molecules CD40, CD86 and HLA-DR with anti-FITC monoclonal antibody and isotype controls IgG1, IgG2a and IgG2b (Farmingen). The medium supernatants

were removed after 48 h to assay for IL-2, IL-12p70, TNF-α released by cultured DCs as measured with ELISA kits according to the manufacturer's instructions (Jing Mei Co., China). DCs were cultured as above, then washed and resuspended at 10^5 DC/well in 100 μl of medium and incubated for 72 h with autologous lymphocytes (10^6/well) in 96-well flat-bottom microculture plates for the IFN-γ assay (Jingmei Co., China).

2.6. CTL assay

The cytolytic activity of induced CTL was determined by a standard ^{51}Cr-release assay. DCs were pulsed with hsp70, hsp70–peptide complex and hsp70–peptide fusion protein (10 μg/ml) from Days 5 to 8. Autologous lymphocytes were co-incubated for a further 7 days as effector cells (E). Target cells (T) were HepG2.2.15, which expressed HBsAg and HBeAg antigens. Whilst HepG2 was used as control. Target cells (2×10^6 cell) were labeled with 100 μCi ^{51}Cr-labeled sodium chromate in RPMI with 20% FCS for 1 h at 37 °C and then washed with plain RPMI. Target cells (1×10^4) were added to a titration of CTL effectors in 96-well round plates in a final volume of 200 μl RPMI with 10% FCS for 4 h at 37 °C. Then 100 μl of supernatant in each well was harvested manually. Radioactivity released into 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 = 100 × (experimental release − spontaneous release)/[maximal release(0.1 M HCl) − spontaneous release]. Spontaneous release was <15% of maximum release in all experiments.

2.7. Statistical analysis

Data are expressed as the mean values ± S.D. of triplicate samples. The statistical significance of the differences was determined by the unpaired two-tailed Student's *t*-test. Differences were considered statistically significant for $p < 0.05$.

3. Results

3.1. Expression and purification of recombinant proteins

The *P. pastoris* expression system was developed to secrete TBhsp70 protein and fusion protein. The DNA sequence coding for the fragment of interest was cloned in the *P. pastoris* pPIC9K plasmid inframe with the yeast α-mating factor signal sequence for protein secretion (Fig. 1). The pPIC9K/hsp70 or pPIC9K/hsp70 fusion protein plasmid was then transformed and targeted to the *P. pastoris* genome by means of homologous recombination. The presence of the hsp70 or hsp70 fusion gene coding sequence in the genomic DNA of the transformants obtained was confirmed by PCR (Fig. 2). When using this *P. pastoris* expression system, recombinant protein expression was controlled by the alcohol oxidase 1(AOX1) promoter, which can be induced by the addition of methanol. Induction of protein expression of the transformants at a final methanol concentration of 0.5% resulted in the secretion of active TBhsp70 and TBhsp70 fusion protein. The resulting proteins of about 70 kDa, which were not present in the control strain, were clearly visible by SDS-PAGE analysis (Fig. 3) and Western blotting (Fig. 4). Cells were induced by growth in methanol for 72 h and production yields were estimated to be 120 mg/l of cell culture of and thus exceeding by far the production yield of hsp70 when expressed by *E. coli*. Although *P. pastoris* secreted few autologous proteins, additional purification of TBhsp70 and fusion

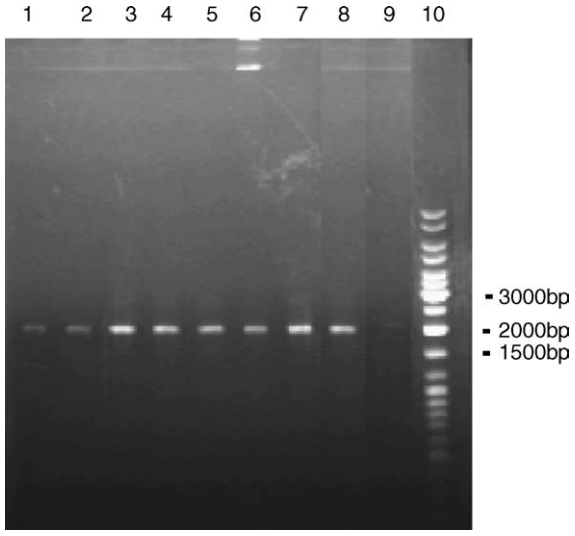

Fig. 2. Cloning of yeast PCR (TBhsp70). The presence of the hsp70 or hsp70 fusion gene coding sequence in the genomic DNA of the transformants obtained was confirmed by PCR. Lanes 1–8 depict eight yeast clones (PCR primers used in the above-mentioned Section 2); lane 9 represents GS115 without the gene of interest; lane10 indicates a 10 kb marker.

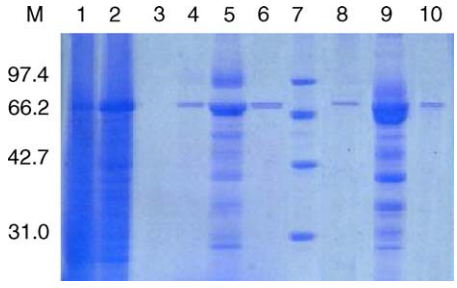

Fig. 3. SDS-PAGE of recombinant proteins. The culture media of methanol-induced *P. pastoris* cells and purified proteins were examined by 10% SDS-PAGE and proteins were visualized with Coomassie blue stain. Lane 1, cell lysate of GS115/pPIC9K; lane 2, cell lysate of GS115/pPIC9K-hsp70; lane 3, supernatant of GS115/pPIC9K; lane 4, TBhsp70 supernatant; lane 5, concentration of TBhsp70; lane 6, after purification; lane7, protein marker; lane 8, fusion protein supernatant; lane 9, concentrated fusion protein; and lane10, after purification.

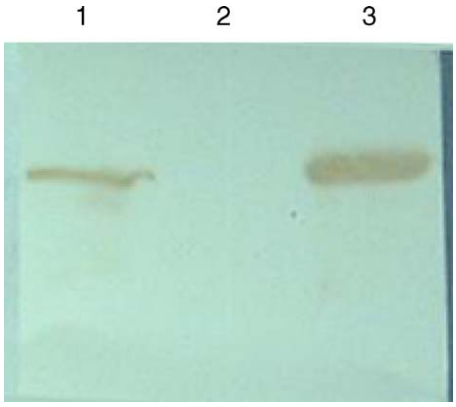

Fig. 4. Western blot analysis of expression products. Lane 1, supernatant of GS115/pPIC9K-hsp70; lane 2, supernatant of GS115/pPIC9K; and lane 3, supernatant of GS115/pPIC9K-hsp70 fusion protein.

protein was needed to determine further biological activities. To that end, culture supernatant proteins were concentrated by Microcon 50 and purified by ATP affinity chromatography. The purity of the recombinant proteins was assessed by SDS-PAGE.

3.2. Binding of CTL peptides to hsp70

The $HBcAg_{(18–27)}$ CTL epitope peptide (biotinylated-FLPSDFFPSV) was used for initial studies. Incubation of hsp70 with biotinylated peptide in the binding buffer was found to be sufficient to generate stable hsp70–peptide complexes as judged by Western blotting (not shown). When reconstitution is successful, a brown band, corresponding to the position of hsp70 and biotinylated peptide, will be visible after staining.

The binding of non-biotinylated $HBcAg_{(18–27)}$ CTL epitope peptide to hsp70 was tested by a competition assay as described in Section 2. In vitro reconstitution of complexes was done with different doses of peptide complexed to the same amount of hsp70 (10 μg (140 pmol)) varying from 1, 2, 4, 6, 8, 10 μg (668, 1336, 2672, 4013, 5344, 6689 pmol), respectively. It was clear that almost complete displacement of biotinylated peptide was achieved at 4 μg of peptide and 10 μg of hsp70, in which the molar ratio of peptide and hsp70 was approximately 20:1, indicating that the combination with peptide attains saturability. For subsequent experiments we used complexes containing 4 μg peptide and 10 μg of hsp70.

3.3. Maturation of monocyte-derived DC by hsp70, hsp70 fusion protein and hsp70–peptide complex

The differentiating but not yet mature DC cultured in GM-CSF and IL-4 were studied by FACS after 8 days. DC from healthy controls revealed a strong expression of the surface markers CD1a ($52.25 \pm 8.05\%$ positive cells), whereas DC from HBV infection was $1.04 \pm 0.5\%$. The percentage of DC expressing CD40 and CD86 was significantly lower in HBV infection in comparison to healthy controls. Interestingly, HLA-DR on DC generated from patients and controls showed high expression without significant differences (Fig. 5, Table 1).

After exposure to hsp70, hsp70 fusion protein and hsp70–peptide complex (10 μg/ml) from Days 5 to 8, DCs undergo further maturation. The percentage of cells expressing CD40 and CD86 was significantly increased in the patients and healthy controls (Fig. 5, Table 1), but CD40 and CD86 expression showed no significant differences in both groups. From the data, we can see hsp70 fusion protein and hsp70–peptide complex have more effects on DC expression CD40 than hsp70. But it is not apparently different in costimulatory molecule CD86 expression stimulated by three antigens. HLA-DR expression was not significantly affected on DC from normals or patients in response to the above antigens. HBV-DC showed increased expression CD1a. The results indicate that hsp70, hsp70 fusion protein and hsp70–peptide complex can induce DCs to become functionally mature.

3.4. Cytokine production of DC stimulated by hsp70, hsp70 fusion protein and hsp70–peptide complex

Supernatants of 48 h DCs stimulated with hsp70, hsp70 fusion protein and hsp70–peptide complex were analyzed for IL-1β, IL-12p70 and TNF-α secretion (Fig. 6). Cytokine detection showed that spontaneous IL-1β, IL-12p70 and TNF-α releases from HBV-DC and control DC loaded above antigens were significantly higher than those such as medium culture and peptide alone. Interestingly, we found that DCs from patients secreted lower amounts of IL-1β, IL-12p70 and TNF-α than DCs from controls. Cytokines secretion of healthy DC and HBV-DC pulsed with hsp70 fusion protein and hsp70–peptide complex were significantly higher than hsp70. The observed cytokine profile suggests a Th1 bias, as IL-12p70 is known to induce Th1 polarization.

To assess the influence of DCs from HBV infection and healthy control on Th subset differentiation, supernatants were collected from DC mixed with autologous lymphocytes to analyze IFN-γ levels (Fig. 7). A similar trend for IFN-γ levels could be detected in both groups, but the increase was significantly less in patient group compared with healthy group. Hsp70 fusion protein and hsp70–peptide complex can secret higher levels of IFN-γ than hsp70 and peptide alone. From the data, there is upregulation of peptide-specific T-cell proliferation.

3.5. Induction of HBV-specific CTL response by hsp70, hsp70 fusion protein and hsp70–peptide complex

To determine the ability of the above antigens to elicit CTL responses in vitro, DCs obtained from volunteers were cultured and stimulated with 10 μg/ml of each antigen. Mixed

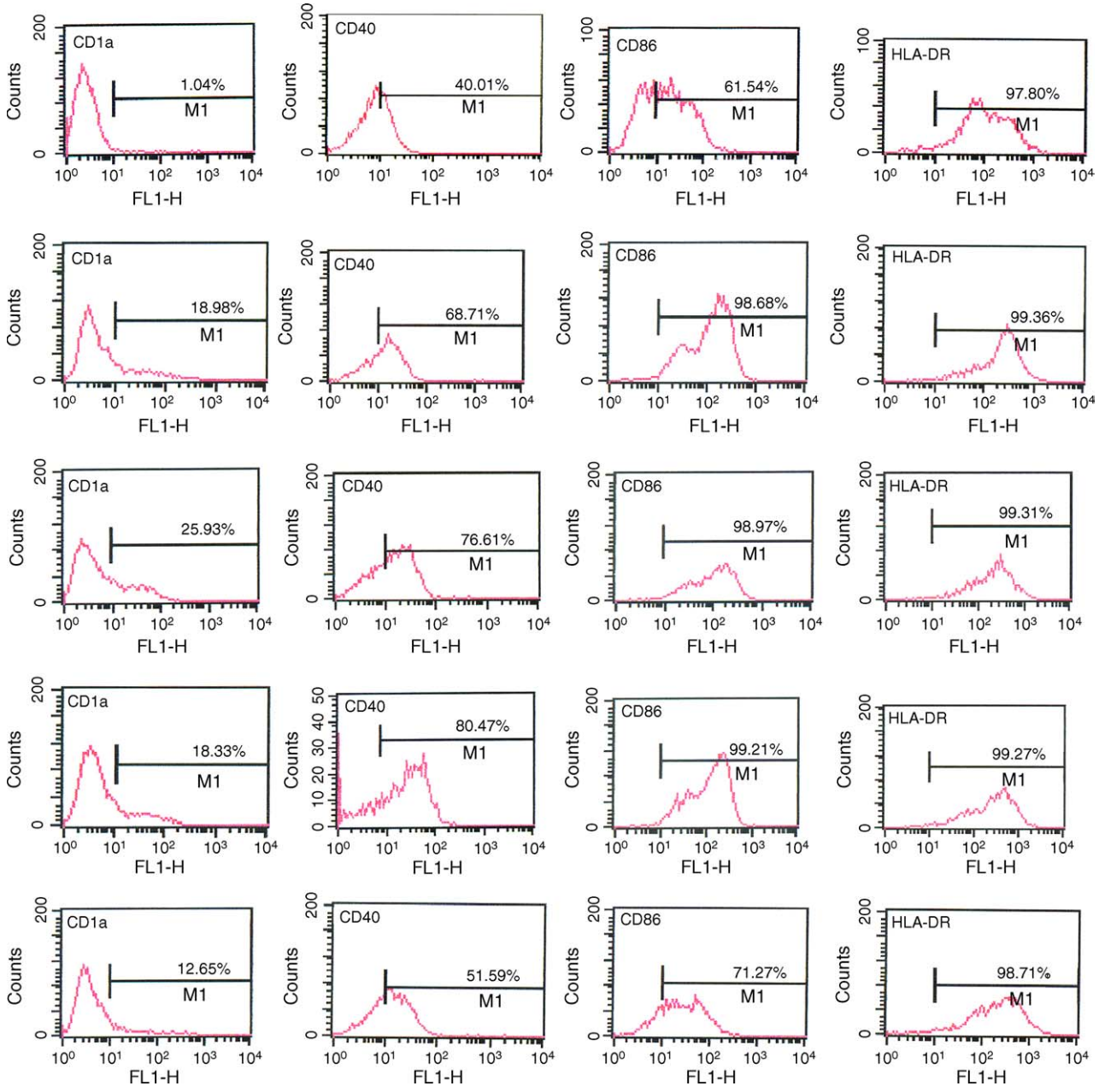

Fig. 5. FACS analysis of CD1a, CD40, CD86 and HLA-DR expression on HBV-DC pulsed different stimulus. Row 1, culture medium; row 2, hsp70; row 3, hsp70 fusion protein; row 4, hsp70–peptide complex; and row 5, $HBcAg_{(18–27)}$ peptide alone. A representative histogram plot is shown for each group with value depicting the mean for six patients.

Table 1
Expression of costimulatory molecules on DC from chronic active Hepatitis B (CAH) and healthy HBV-naive control (CTR) by different stimuli, calculated as percentage positive cells (mean ± S.D.)

Group	CD40 (% positive)		CD86 (% positive)		HLA-DR (% positive)	
	CAH ($n = 6$)	CTR ($n = 6$)	CAH ($n = 6$)	CTR ($n = 6$)	CAH ($n = 6$)	CTR ($n = 6$)
Medium	40.01 ± 7.12	60.00 ± 4.25*	61.54 ± 5.04	80.50 ± 6.80*	97.80 ± 1.00	98.54 ± 1.20
TBhsp70	68.71 ± 5.12	72.08 ± 2.10	98.68 ± 1.25†	99.25 ± 0.25†	99.36 ± 0.55	99.55 ± 0.10
TBhsp70 fusion protein	76.61 ± 7.70†	78.55 ± 3.81†	98.97 ± 1.00†	99.12 ± 0.38†	99.31 ± 0.45	99.38 ± 0.32
TBhsp70 complex	80.40 ± 4.45†	83.31 ± 2.54†	99.21 ± 0.55†	99.09 ± 0.71†	99.27 ± 0.28	99.66 ± 0.20
Peptide	51.59 ± 5.00	63.54 ± 3.01	71.27 ± 3.60	84.57 ± 5.20	98.71 ± 0.33	99.01 ± 0.78

† $P < 0.05$ compared with medium.
* $P < 0.05$ compared with CAH.

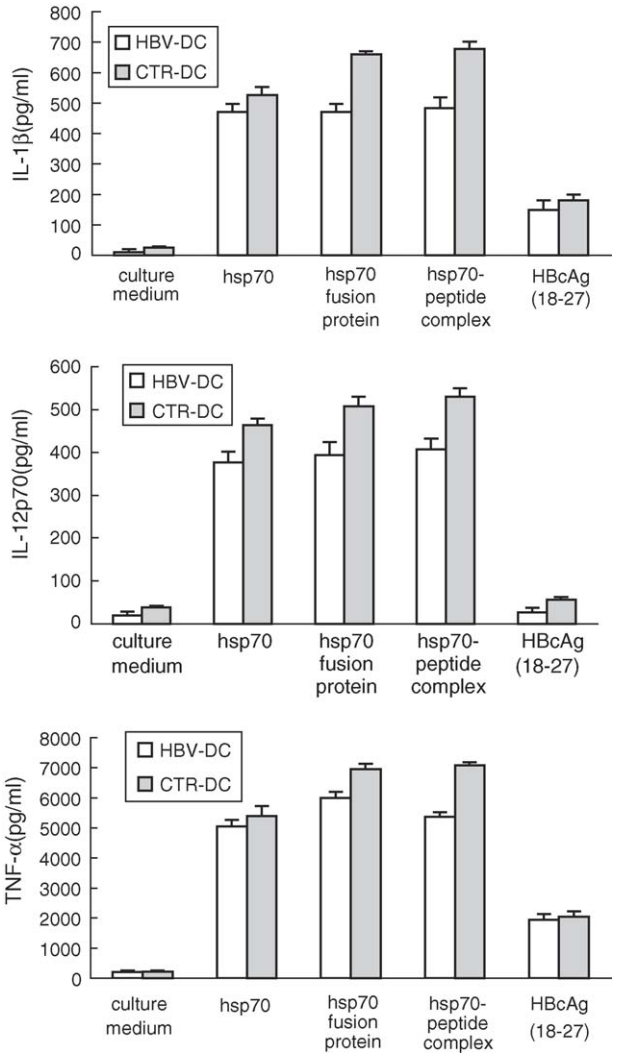

Fig. 6. Cytokine secretion by cultured DCs incubated with hsp70, hsp70 fusion protein, hsp70–peptide complex, respectively. DCs were plated in 24-well plates at a cell density of 1×10^6 cells/ml and on Day 5 were loaded with hsp70, hsp70 fusion protein, hsp70–peptide complex, peptide alone or culture medium as a control (10 μg/ml). The medium supernatants were removed at 48 h to assay for IL-1β, IL-12p70 and TNF-α released by cultured DCs. (□) chronic active HBV infection and (▨) healthy HBV-naive control. Results are representative of three separate experiments.

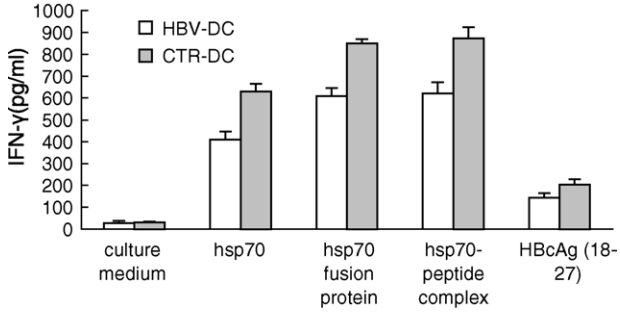

Fig. 7. Production of IFN-γ by DCs. Cultures were set up in parallel for IFN-γ production. DCs were resuspended at 10^5 DC/well in 100 μl of medium and incubated with autologous lymphocytes (10^6/well) in 96-well flat-bottom microculture plates. Supernatants (100 μl) were removed from each well after 72 h and assayed for IFN-γ production using an ELISA kit specific for IFN-γ. Results are representative of three experiments.

autologous lymphocytes with DC-pulsed culture medium, hsp70, hsp70 fusion protein, hsp70–peptide complex or peptide alone were used as effector cells (E), while HepG2.2.15 served as target cells (T) and HepG2 as control. CTL responses were investigated at different E/T ratios. It was evident from Fig. 8 that the E:T ratio had impacts on the lysis of target cells. As shown in Fig. 8, CTL from both hsp70 fusion protein and hsp70–peptide complex were able to lyse HepG2.2.15 but unable to lyse parental cells such as HepG2 (not shown). Peptide alone was capable of generating weak CTL lysis. Hsp70 showed almost no target cell killing, indicating that the induction of CTL response were HBV-specific response. CTL of HBV infection was less than that of healthy control, consistent with cytokines release. The data reflected dysfunction of DC and T cell in HBV patient compared to the healthy. Interestingly, there was no significant difference in hsp70 fusion protein and hsp70–peptide complex. These results indicate that hsp70 could provide immunological adjuvant effects for the HBV core 18–27 peptide irrespective of whether the CTL epitope was fused to the C-terminus or the complex reconstituted in vitro.

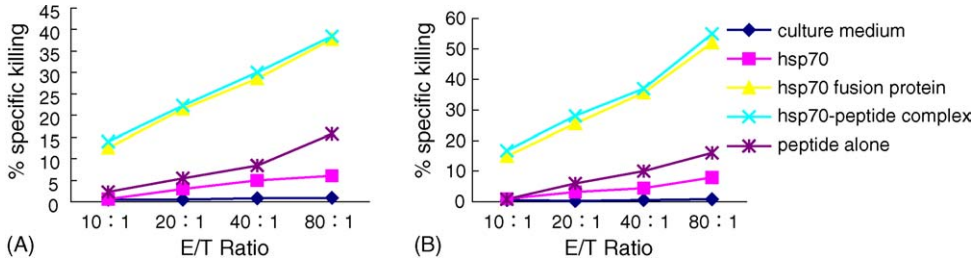

Fig. 8. CTL lysis of target cells at different E:T ratios. Mixed lymphocytes with DC pulsed culture medium, hsp70, hsp70 fusion protein, or hsp70–peptide complex were used as effector cells (E) with HepG2.2.15 serving as target cells (T): (A) DC and lymphocyte from chronic active HBV infection and (B) DC and lymphocyte from healthy HBV-naive control. Data are the mean of three experiments.

4. Discussion

The principal finding in this study is that we utilize *P. pastoris* yeast to express high level of TBhsp70 and fusion protein. In fact, this approach allows any gene or epitope of interest to be subcloned into the plasmid vector pPIC9K/hsp70. Epitopes fused to hsp70 can be easily expressed and purified compared to *E. coli*, recombinant proteins in *E. coli* contain trace levels of endotoxin, whereas *P. pastoris* yeast will not produce endotoxin. Furthermore, the pPIC9K vector also allows selection for production of *Pichia* strains that contain more than one copy of the gene of interest. We also demonstrate that TBhsp70-HBcAg$_{(18–27)}$ complex can be easily generated in vitro in the presence of ADP.

Another important finding is that recombinant hsp70, hsp70 fusion protein and hsp70–peptide complex can activate DCs. Dendritic cells are the most potent antigen presenting cells and play a central role in the induction of antiviral immune responses. Our FACS analysis revealed a significantly reduced expression of CD40, CD86 and CD1a on HBV-DC. These immature phenotypes are in concordance with Tavakoli et al. [33] and Wang et al. [34]. While HLA-DR expression on HBV-DC and healthy DC is more strong than 97%, which is contrary to Tavakoli et al. and similar to Beckebum et al. [35]. However, the reduced expression of costimulatory molecules of immature HBV-DC was reversed after exposure to hsp70, hsp70 fusion protein and hsp70–peptide complex, accompanying with high-level expression CD40 and CD86. The similar phenomena appeared in healthy control. As shown in Table 1, hsp70 fusion protein and hsp70–peptide complex show strong influence on CD40 expression. These results mean that there is no significant difference in costimulatory molecules in both patient DC and control DC after stimulated with mentioned antigens except some phenotype such as CD1a.

The key cytokines provided by APC determine the induced Th cell response towards a Th1 or Th2 cell. In HBV infection group and control group, TBhsp70 and other two vaccines interaction with DC led to increased secretion of cytokines IL-1β, IL-12p70 and TNF-α. IL-12 is essential for inducing Th1 polarization, thus the cytokines profile seen here suggests bias toward a Th1 response. Induction of IFN-γ also shows a polarization toward the Th1 cells. This was further supported by our observation of HBV-specific CTL response. However, to be effectively presented by DCs, the antigen peptide must be associated with TBhsp70 irrespective of whether the CTL epitope was fused to the C-terminus or the complex reconstituted in vitro, as TBhsp70 alone or peptide alone failed to elicit HBV-specific CTL response. Together these findings demonstrate the potent Th1 polarizing adjuvant effect of TBhsp70. Interestingly, cytokines production and CTL responses of HBV patients showed lower levels than those of healthy controls, suggesting that DC from HBV patients cannot respond fully to maturation signals, leading to a cascade effect, thereby lymphocytes are not adequately activated to respond to specific antigen.

The third important finding is that hsp70 fusion protein and hsp70–peptide complex became almost equally susceptible to lysis by peptide-specific CTL in a standard 4 h cytolytic assay, indicating that DC could generate small peptide from hsp70 fusion protein and hsp70–peptide complex and load them onto MHC class I molecules. However, what is the mechanism by which TBhsp70 exerts its adjuvant carrier effect? Analogous to mammalian gp96 and hsp70, the adjuvanticity of TBhsp70 is most likely due to its ability to activate DCs. Mammalian hsp70 induces DC maturation through receptors CD91 and toll-like receptor 4 [36,37]. However, TBhsp70 specifically binds CD40. Binding of TBhsp70 by CD40 leads human DC to release CC-chemokines such as RANTES. In our experiments, CD40 expression on HBV-DC showed a reduced level compared to control DC, after stimulated by TBhsp70 and two vaccines, upregulated expression without significant difference in the patients and controls. Signaling through CD40 can upregulate antigen presentation, increase expression of costimulatory molecular CD80, CD86, and CD58 [38], and make DC competent for CTL priming [39]. Thus, the ability of DC to stimulate T cells and induce CD40L expression and of CD40 cross-linking to modulate APC function is likely to result in a positive feedback loop. Recent studies demonstrated that CD40 ligation on DC triggers IL-12 production and induces MHC-I restricted antigen-specific $CD8^+$ T-cell response [40]. Why CTL response of the controls showed strongly than that of the patients, maybe related to the dysfunctional CD4 T cells or DCs in chronic Hepatitis B [41]. Yonca et al. discover that TBhsp70 induce NF-κB activation and signal through toll-like receptor 2 and 4 [37]. And its mechanisms of action deserve to be further explored. A better understanding of the recognition of TBhsp70 and its role in immune response could help in the rational design of more effective vaccines to treat HBV infections as well as HIV, human papillomvirus (HPV).

Taken together, our data demonstrate that TBhsp70 is crucial for the development of effective immunotherapy for HBV vaccines. However, the fusion protein has several advantages over hsp70–peptide complex. Since (1) the hsp70 fusion protein is easy to produce in *P. pastoris* GS115 at a high production and low cost compared to hsp70–peptide complex; (2) the hsp70 fusion protein has an equimolar amount of hsp70 and accompanying CTL peptide. In contrast, hsp70–peptide complex consists of a pool of nonidentical molecules with variable epitope density, the immune responses to an epitope can be strongly affected by differences in the molar ratio of epitope and carrier, the mode of epitope and carrier, and the position of B- and T-cell epitopes [42–44]. Therefore, hsp70 fusion proteins reduce the variables associated with hsp70–peptide complex. In addition, this expression system and purification scheme can both easily be transferred for industrial application and will overcome the difficulties and complexities of hsp–peptide complex.

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