

Full Length Research Paper

Leukemia: Derived heat shock protein gp96-peptide complex contribution to T cell and dendritic cell activation

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The molecular chaperone, heat shock protein gp96 (HSP-gp96), has been shown to have roles in the synthesis, processing and transport of tumor antigens. Therefore, the capacity for HSP-gp96 to induce dendritic cells (DCs), thymus-dependent lymphocytes (T lymphocytes), natural killer (NK) cells and cytotoxic T lymphocytes (CTLs) was investigated. Recombinant adenovirus (AD) containing HSP-gp96 (AD-gp96), as well as gp96-peptide complex from the human leukemia cell lines, K562, HL-60 and U937, was prepared. Purified gp96-peptide complex was found to stimulate the proliferation of T lymphocytes, increase the activity of NK cells and CTLs and induce the secretion of cytokines, compared with AD-gp96. In the latter case, levels of IFN- γ and TNF- α were found to increase and levels of IL-12(P70) and IL-10 decreased. In combination, these results indicated that the gp96-peptide complexes derived from the tumor cells contributed to the activation of lymphocytes and increase the presentation of tumor antigen. Furthermore, the chaperone function of gp96 promoted the maturation of DCs, enhanced the antigen presentation function of DCs and induced the secretion of cytokines by DCs. Therefore, gp96-peptide complex derived from the tumor cells potentially represents an immunization therapy for the elimination of residual leukemia cells.

Key words: Leukemia, heat shock protein gp96, dendritic cells, cytotoxic T lymphocytes.

INTRODUCTION

Leukemia is a malignant clonal disease in hematopoietic stem cells that is typically treated with chemotherapy and radiotherapy. However, these treatments are not been able to completely eliminate all leukemia cells from patients. In addition, transplantation of hematopoietic stem cells is also associated with a 30% recurrence rate. The ability to eliminate residual leukemia cells has therefore, been the focus of many studies in various countries. Currently, immunotherapy represents a promising approach for the treatment of leukemia.

Heat shock protein 96 (HSP-gp96) has a molecular

weight of 96 kDa and has been shown to be a chaperone protein that localizes to the endoplasmic reticulum in eukaryotes (Qu et al., 1994; Linderoth et al., 2000; Zheng et al., 2001). HSP-gp96, also known as GRP94, shares 50% sequence homology with heat shock protein 90 (HSP90) and correspondingly, is a member of the HSP90 family of proteins. HSP-gp96 has been shown to participate in the synthesis of tumor antigens, as well as their assembly, processing and transport. Therefore, HSP-gp96 can act as a chaperone protein and stimulate T-lymphocytes. In particular, the work by Tamura et al. (1997) has demonstrated that, HSP-gp96 is an effective treatment for lung cancer, colon adenocarcinoma, fibrosarcoma melanoma and spindle cell tumors induced by ultraviolet rays in rat models (Rivoltini et al., 2003; Hoos et al., 2003). In addition, there have been reports that a

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phase II clinical trial will include gp96-based vaccines for the treatment of melanoma and renal cell carcinomas (Castelli et al., 2004; Janetzki et al., 2000). However, independent of whether HSP-gp96 represents a tumor antigen, it is important to determine whether HSP-gp96 has the capacity to stimulate cytotoxic T lymphocytes (CTLs) and facilitate the complete cure of leukemia patients.

Therefore, the goal of this research was to compare the function of HSP-gp96 purified from three different leukemia cell lines. Specifically, the purified proteins were compared regarding their ability to stimulate lymphocytes, facilitate the transport of tumor antigen and enhance the transport of antigen and cytokine secretion in dendritic cells (DCs). In combination, these studies provide a foundation for further investigation of HSP-gp96 as a target in an immunotherapy approach to cure leukemia.

MATERIALS AND METHODS

Reagents

Leukemia cell lines K562, HL-60 and U937 were purchased from SIBCB (Shanghai institute of Biochemistry and Cell Biology). ConA-Sepharose and ion exchange columns were purchased from Pharmacia (NS, USA). Molecular weight markers for SDS-PAGE and bovine serum albumin (BSA) were purchased from Zhongshan Biological Technology (Beijing, China). PHA (phytohaemagglutinin), ammonium persulfate and α -pyran-1-methyl mannose were purchased from Fluka (MO, USA). A monoclonal antibody against HSP-gp96 was purchased from Santa Cruz Biotech (CA, USA). Glycine, acrylamide and ammonium sulfate were purchased from Sigma (MO, USA). Cytokine kits for IL-12 (p70), IFN- γ , TNF- α and IL-10 were purchased from Boster Biological Technology, Limited (Wuhan, China). Flow cytometry reagents and antigens were purchased from BD Pharmingen (Suzhou, China). LDH release kit was purchased from Promega Biological Technology (Shanghai, China).

The construction of HSP-gp96 recombinant adenovirus (Ad-gp96)

The gene fragment of human gp96 was inserted into the p-shuttle CMV shuttle vector, BJ5183-AD-1, which was already converted to facilitate homologous recombination in thallus [the adenovirus (AD) framework plasmid, pAdEasy-1, had already been pre-converted]. Recombinant AD plasmids containing the correct sequence were linearized for the transfection of AD293 cells. Virus produced in AD293 cells was then concentrated using cesium chloride (CsCl) ultracentrifugation to produce high-titer recombinant AD-gp96.

Isolation and characterization of gp96-leukemia antigen peptide complex

Gp96-leukemia antigen peptide complexes were purified as previously described (Meng et al., 2002; Dai et al., 2003). Briefly, human leukemia cell strains, K562, HL-60 and U937, were infected with AD-gp96 at an MOI of 100. One week later, cells were lysed and centrifuged for 30 min at $2000 \times g$ at 4°C . After the supernatants were aspirated, samples were centrifuged for an additional 15 min at $15000 \times g$ at 4°C . After the supernatants were aspirated, the samples were precipitated using 50%, and then 70% saturated

ammonium sulfate. The resulting precipitate was resuspended in 5 times its volume in equilibrium liquid (20 mmol / L Tris-Cl, pH7.4; 200 mmol / L NaCl; 2 mmol / L CaCl_2 , 2 mmol / L MgCl_2), added into a concanavalin affinity column and eluted using 10% α -pyran-1-methyl mannose. The eluant was then further purified using a DEAE ion-exchange column and eluted using a gradient of 300 to 1000 mM NaCl. Fractions containing the gp96-leukemia antigen peptide complex were identified using 10% SDS-PAGE and western blot. Protein concentrations of the samples were determined using Bradford assays and the protein purity achieved was 84%.

The preparation and identification of T cells and DCs

Conditions for the culture of DC cells have previously been described (Zhu et al., 2001; Tong et al., 2004; Tong et al., 2005; Tong et al., 2008). Briefly, peripheral blood mononuclear cells (PBMCs) were obtained from healthy volunteers and suspended into 5 ml culture flasks with RPMI1640 containing 10% fetal calf serum (FCS). After the cell concentration was adjusted to 1×10^6 cells/ml, it was incubated at 37°C with 5% CO_2 for 2 h. Non-adherent cells were collected as T cells and the adherent cells were treated with GM-CSF (500 U/ml) and IL-4 (500 U/ml) before being divided into two separate DC populations. Both cell populations were cultured for 7 days, with half of the growth media changed every 4 days. While one population of cells was stimulated with purified gp96-leukemia peptide complex (1 $\mu\text{g}/\text{ml}$) and the other was not, both cell groups were treated with 50 $\mu\text{g}/\text{ml}$ TNF- α . On the 10th day, suspension cells were collected. Direct immunofluorescence labeling of the cells was performed to phenotypically characterize the cell populations present. Cells were stained for CD3 $^+$, CD14 $^+$, CD19 $^+$, CD83 $^+$, CD1a $^+$, CD40 $^+$, CD54 $^+$, CD80 $^+$, CD86 $^+$, HLA-DR $^+$ and HLA-ABC $^+$ to identify the DCs present (Romani et al., 1996; Sallusto et al., 1994).

MTT assays

T cells ($2 \times 10^5/100$ ml) were inoculated into 96-well U-bottom tissue culture plates prior to the addition of purified gp96-peptide complex at three different concentrations (1, 2 and 5 $\mu\text{g}/\text{ml}$) in triplicate. After 5 days, 10 μl MTT (5 mg/ml) was added to each well and 4 days later, 100 μl DMSO was added. OD values at 550 nm were recorded using an ELISA plate reader (Multiscan MK3, Thermo Labsystems Oy, Finland) and the average values for each treatment group were reported.

Detection of NK cell activity

Conditions for the culture of NK cells have previously been described (Naofumi Shinagawa et al., 2008). Target cells included K562, HL-60 and U937 cells at ratios of 20:1, 10:1 and 5:1 to NK cells stimulated with purified gp96-peptide complex at 1, 2 and 5 $\mu\text{g}/\text{ml}$. Control effector cells were untreated NK cells. LDH release was detected by colorimetry and the average of three experiments was taken as the result.

Detection of CTL activity

Conditions for the culture of CTLs have previously been described (Naofumi et al., 2008). CTL activity was assayed for T cells and DCs incubated with either purified gp96-leukemia peptide complex or tumor antigen peptide complex for 7 days. K562, HL-60 and U937 were used as target cells in ratios of 40:1, 20:1, 10:1 and 5:1

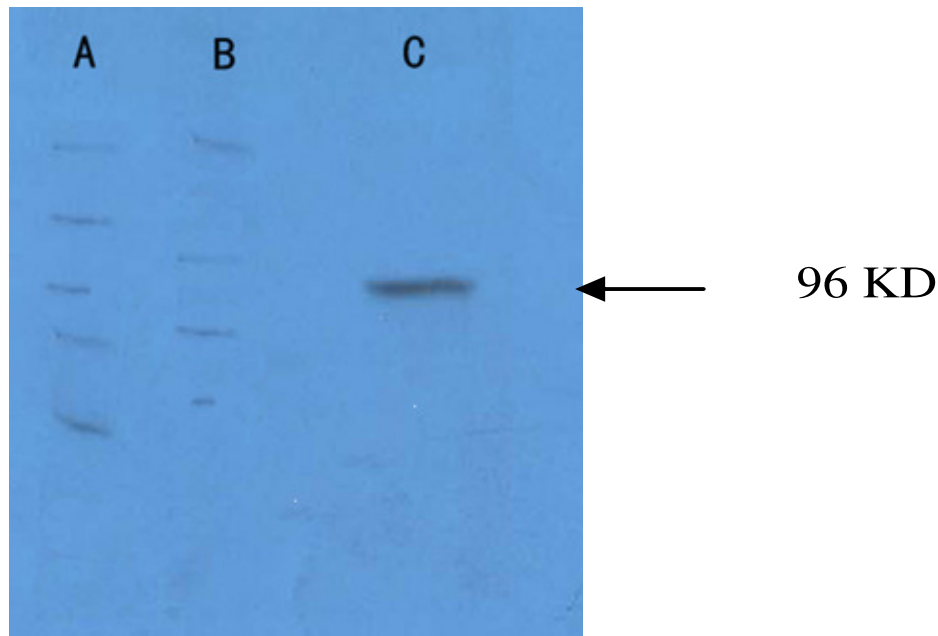


Figure 1. Characterization of the purified gp96-leukemia antigen peptide complexes. (A), Western blot of gp96-leukemia antigen peptide complexes from K562 cell; (B), K562 leukemia cell proteins; (C), purified eluted fraction from K562 cell DEAE-sepharose anion exchange chromatography.

to the effector cells. LDH release was detected by colorimetry and the average of three experiments was taken as the result.

Cytokine detection

A sandwich ELISA method was used to detect the expression levels of IL-12, IFN- γ , TNF- α and IL-10 in the cell supernatants according to the manufacturer's directions (Boster Biological Technology, Wuhan, China). Assays were performed in triplicate and the average values were reported.

Statistical analysis

Data were presented as the average \pm standard deviation ($\bar{x} \pm s$). The statistical software, SPSS7.0, was used for all analyses, with comparisons between groups made using Student's t-test. A P-value less than 0.05 were considered significant.

RESULTS

Isolation and effects of gp96-leukemia peptide complex on allogeneic normal T lymphocytes

Gp96-leukemia antigen peptide complexes were purified from human leukemia cell strains, K562, HL-60 and U937 that were infected with AD-gp96 (MOI = 100). Western blotting was used to confirm the size and purity of the isolated Gp96-leukemia antigen peptide complexes (Figure 1). To confirm the function of the gp96-leukemia peptide

complex isolated from the three different sources, T cells ($2 \times 10^5/100$ ml) were incubated with either purified gp96-peptide complex at three different concentrations (1, 2 and 5 $\mu\text{g/ml}$) or antigen peptide, in triplicate. After 5 days, MTT assays detected the proliferation of the treated T cells. Compared with the T cells incubated with antigen peptide, T cells incubated with the gp96-peptide complexes exhibited a marked increase in proliferation at each concentration, with the greatest difference in proliferation observed at the 5 $\mu\text{g/ml}$ concentration (Figure 2).

The influence of the gp96-leukemia peptide complex on NK cells

To evaluate the effects of the gp96-leukemia peptide complex on NK cells, K562, HL-60 and U937 cells were incubated with NK cells at ratios of 20:1, 10:1 and 5:1 respectively. Untreated NK cells were the control effector cells. Co-cultures were stimulated with 1, 2 and 5 $\mu\text{g/ml}$ purified gp96-peptide complex. The result showed that, except that the results of gp96-leukemia peptide complex causing HL-60 and tumor antigen, peptide were not significantly different at the concentration of 2 $\mu\text{g/ml}$, 1 $\mu\text{g/ml}$ and E: T at 5:1 ($P > 0.05$). The NK cells activity stimulated by gp96-peptide complex causing other three leukemia cells with different concentration and at the E: T of 20:1, 10:1, 5:1 was noticeably higher than that of antigen peptide ($P < 0.05$). For example, gp96-peptide complex caused different leukemia cells and tumor antigen peptide

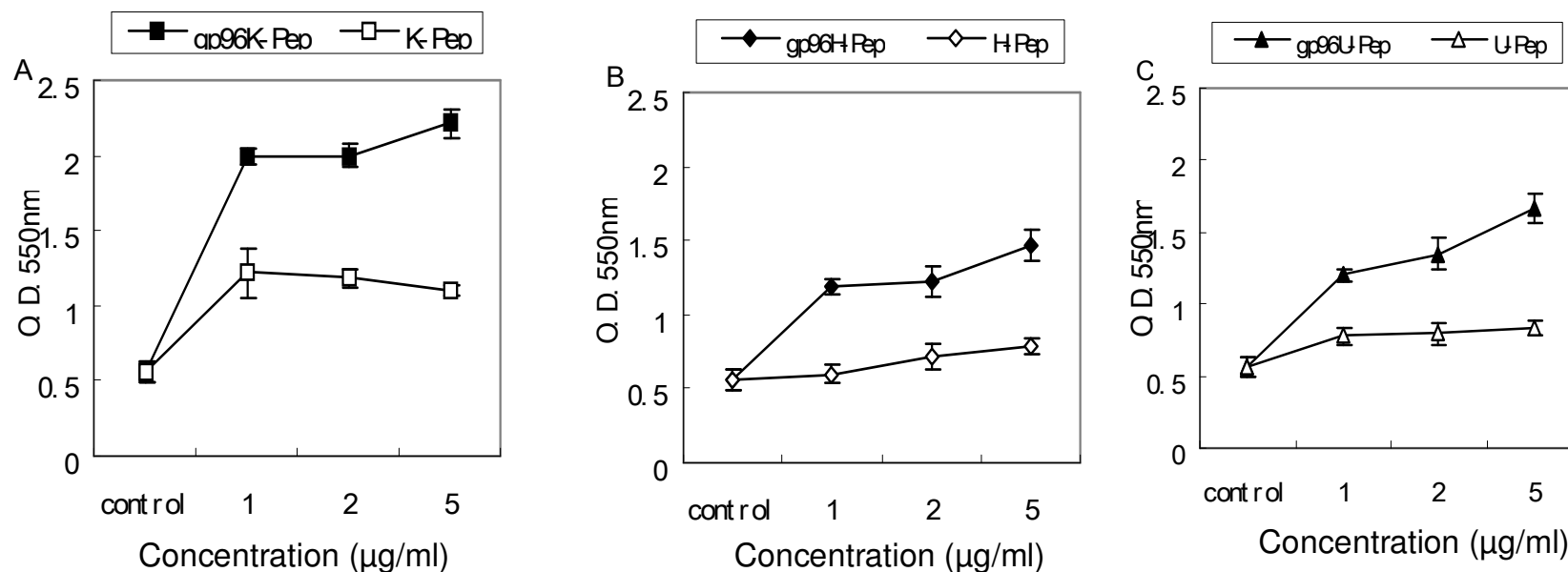


Figure 2. Proliferation assays of leukemia cell strains treated with purified gp96-peptide complexes. MTT assays were performed for: (A) K562 source GP96-leukemia antigen peptide complexes (gp96K-Pep) vs. K562 source antigen peptide (K-Pep), (B) HL-60 source GP96-leukemia antigen peptide complexes (gp96H-Pep) vs. HL-60 source antigen peptide (H-Pep), and (C) U937 source GP96-leukemia antigen peptide complexes (gp96U-Pep) vs. U937 source antigen peptide (U-Pep).

with the concentration of 2 µg/ml and target cells in the ratio of 10:1 (Figure 3).

The influence of the gp96-leukemia peptide complex on CTL activity

The day 7 DCs expressed high levels of CD11c, HLA-DR, CD80 and CD86, showing the characteristics of DCs. Cytotoxicity was assayed for T cells and DCs incubated with either the purified gp96-leukemia peptide complex or tumor antigen peptide complex for 7 days. K562, HL-60 and U937 were used as the target cells in the ratios of 40:1, 20:1, 10:1 and 5:1 to effector cells.

The result showed that compared with the

antigen peptide and except that the results of gp96-peptide complex originating U937 cells and tumor antigen were not significantly different when targeted: effector ratio was at 5:1 ($p > 0.05$). The gp96-peptide complex that caused different leukemia cells noticeably increased CTL activity at ratios 40:1, 20:1, 10:1 and 5:1 ($p < 0.05$) (Figure 4).

The dendritic cells stimulated by gp96-peptide complex causing HL-60 and U937 cells and tumor antigen peptide together with the dendritic cells were not significantly different at the ratio of 5:1 ($P > 0.05$). The CTL activity which was stimulated by the dendritic cells stimulated by gp96-peptide complexes causing different leukemia cells as target: effector ratios of 40:1, 20:1, 10:1, 5:1 was obviously higher than that stimulated by the

tumor antigen ($P < 0.05$) (Figure 5).

Cytokine expression by PBMCs and DCs treated with gp96-leukemia peptide complex

Sandwich ELISAs were used to detect the expression levels of IL-12, IFN- γ , TNF- α and IL-10 in the cell supernatants of PBMCs and DCs that were cultured with and without K562, HL60 and U937 cells expressing gp96-peptide complexes. Compared with the untreated PBMCs and DCs, the presence of leukemia cell strains expressing varying levels of gp96-peptide complex was found to markedly improve the secretion of TNF- α , IL-10, IL-12 (P70) and IFN- γ from PBMCs and DCs

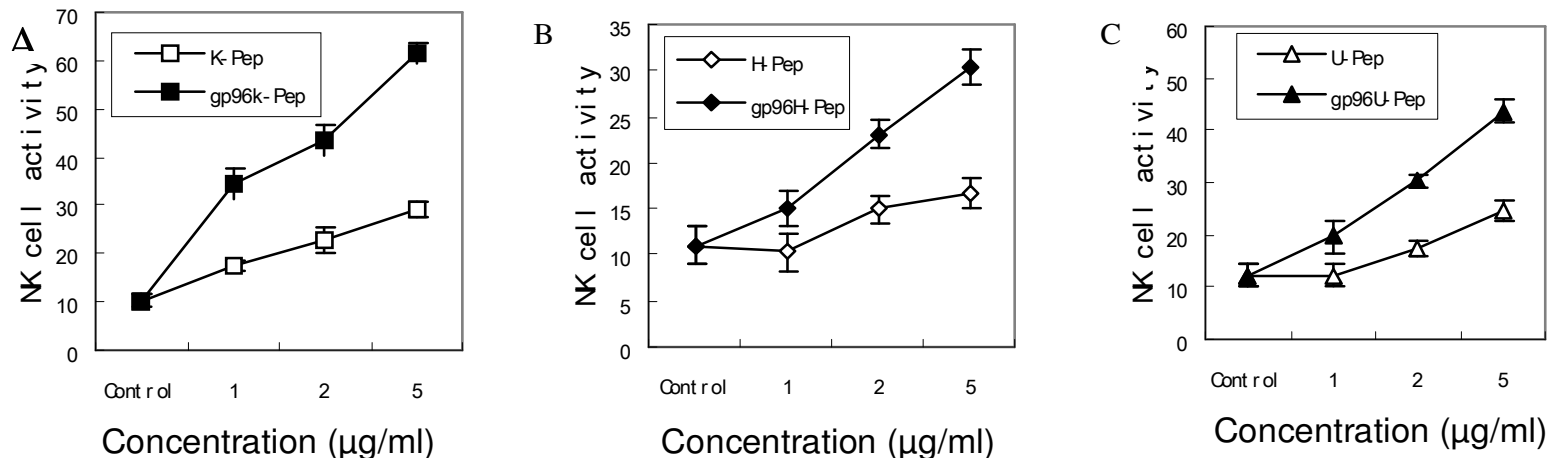


Figure 3. The activity of NK cells assayed in the presence of gp96-peptide complexes. At an effector to target cell ratio of 10:1, NK cells were incubated with three different leukemia cell strains in the presence of 2 µg/mL gp96-peptide complexes or gp96 purified from AD-gp96 expression. NK cell activity for K562 (A), HL60 (B), and U937 (C) cells incubated with gp96-peptide complexes (i.e. gp96K-Pep, gp96H-Pep, and gp96U-Pep) vs. gp96 purified from AD-gp96 (K-Pep, H-Pep, U-Pep) for each of the three cell lines, respectively.

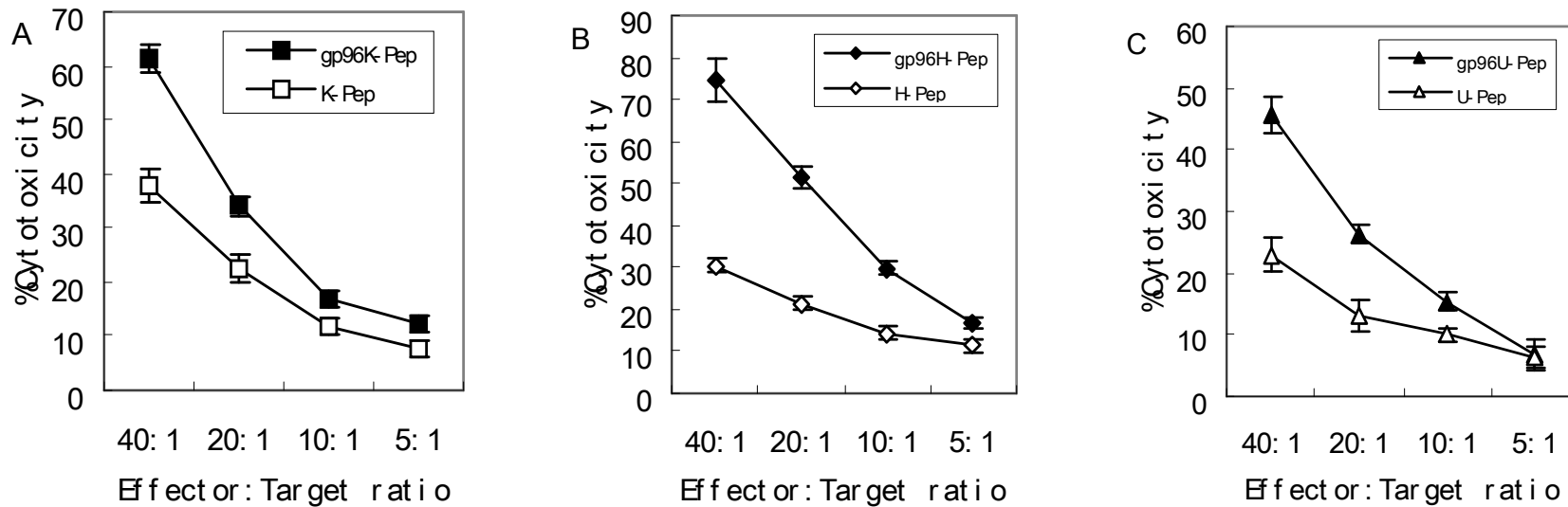


Figure 4. Cytotoxicity assays for T cells. CTLs were incubated with K562, HL60, and U937 cells in the presence of gp96-peptide complexes (gp96K-Pep, gp96H-Pep, and gp96U-Pep) vs. gp96 purified from AD-gp96 (K-Pep, H-Pep, U-Pep) at the target to effector cell ratios indicated.

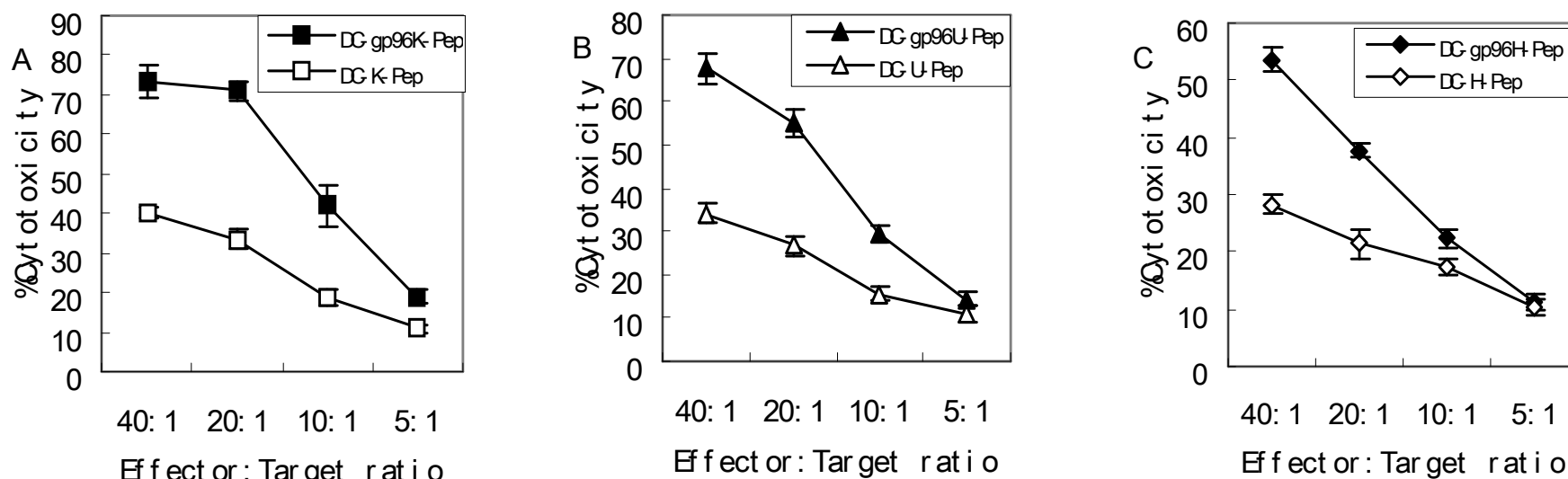


Figure 5. Cytotoxicity assays for DCs. DCs were incubated with K562, HL60, and U937 cells in the presence of gp96-peptide complexes (DC-gp96K-Pep, DC-gp96H-Pep, and DC-gp96U-Pep) vs. gp96 purified from AD-gp96 (DC-K-Pep, DC-H-Pep, DC-U-Pep) at the target to effector cell ratios indicated.

($p < 0.05$). K562, HL60 and U937 cells expressed PBMCs-gp96-peptide complexes VS K562 and HL60 and U937 cells expressed PBMCs ($p < 0.05$). K562, HL60 and U937 cells expressed DCs-gp96-peptide complexes VS K562 and HL60 and U937 cells expressed DCs ($P < 0.05$). Moreover, the secretion of IL-12(P70) and IL-10 from DCs was distinctly less than that for PBMCs. In contrast, the secretion of IFN- γ and TNF- α were opposite (Figure 6).

DISCUSSION

Similar to other types of tumors, leukemia is difficult to cure due to a residual number of cells that remain in a patient following treatment with chemotherapy or radiation. Even for patients who undergo complete remission following

chemotherapy and receive long-term consolidation chemotherapy, a chance for recurrence still exists even after allogeneic bone marrow transplantation. Therefore, in addition to the optimization of existing treatment methods, the identification of a leukemia antigen that can effectively stimulate CTLs would be of significant value. In previous studies of solid tumors (Belli et al., 2002; Mazzaferro et al., 2003; Wang et al., 2000) and other hematologic malignancies (Li, 1997; Oki and Younes, 2004), tumor-derived HSPs have been shown to generate tumor-specific immune responses.

Therefore, HSP-gp96 was studied to evaluate its capacity to stimulate a CTL response. Compared with AD-gp96, purified gp96-peptide complex was shown to remarkably improve the proliferation of allogeneic normal T lymphocytes and increase the activity of both NK cells and CTLs. As the

tumor-derived protein was from patient's tumor tissue, it avoids virosis' introduction and spread of transforming DNA (Wierecky, 2006). However, in the leukemia immunization therapy, what are the advantages of 96-peptide complex compared with other tumor-derived protein? As a protein that is able to associate with various antigen peptides and display a variety of T cell epitopes, the gp96-peptide complex has the capacity to activate many types of cytotoxic T cells. Accordingly, the immunogenicity of the gp96-peptide complex is more diverse and therefore, produces a stronger immune response than a single antigen. Furthermore, gp96 localizes to the surface of antigen presenting cells (APCs) and nanomolar levels of protein are sufficient to induce a CTL response. Correspondingly, the concentration of the immunization dose of gp96-peptide complexes would be low (Heike et al., 1999). It has also been shown

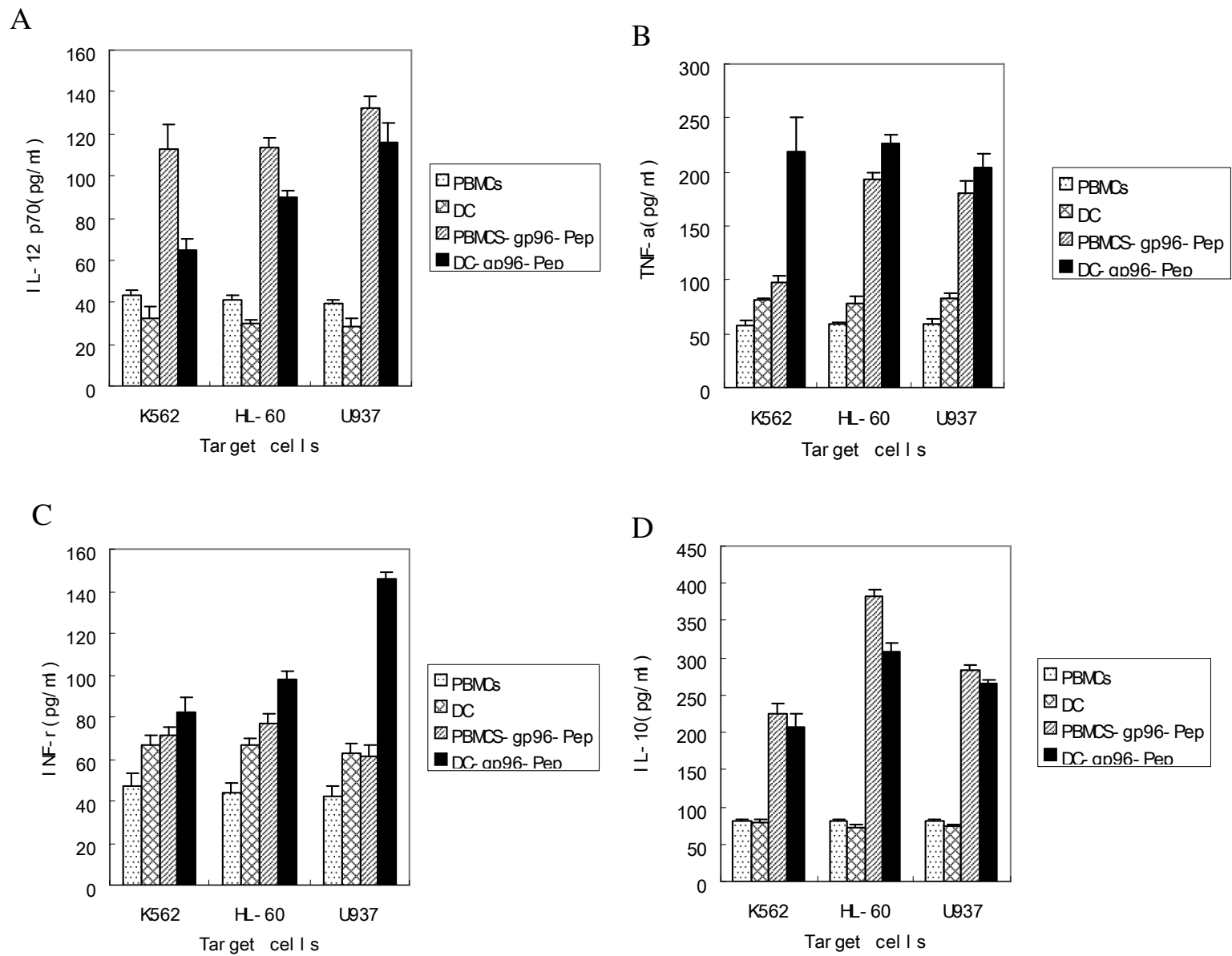


Figure 6. Cytokine-specific sandwich ELISAs. Cytokine expression by PBMCs and DCs in the presence or absence of gp96-peptide complexes purified from K562, HL60, and U937 cells. (A) IL-12 (P70); (B) TNF-α; (C) IFN-γ; (D) IL-10.

that, MHC class I restricted polypeptide epitopes can enhance an antigen-specific CTL response in the absence of adjuvant. However, the level of immunogenicity associated with this approach is low, while the amount of epitope needed is high. As a result, increased toxicity has been associated with this strategy, making it unacceptable for clinical application (Audibert, 2003). In contrast, gp96 represents a vaccine that would not require adjuvant, thereby eliminating any additional opportunities for toxicity (Heike et al., 1999; Caudill and Li, 2001).

DCs are well-characterized for being the most effective antigen presenting cell type in the immune system, although there is no evidence to indicate that DCs can directly target tumor cells (Gutether and Becher, 2007). However, the immune response mediated by T cells has been shown to play a key role in the anti-tumor defense of an organism. For example, DCs present antigen polypeptides for the sensitization, activation and amplification of T cells. DCs can also provide co-stimulatory signals to T cells. Therefore, overall DCs are a very useful component of the immune response, particularly as a stimulant of T cells (Srivastava et al., 1994; Levin et al., 1993). When DCs mature, they highly express MHC molecules and develop the capacity to provide co-stimulatory signals that result in an efficient antigen presenting ability. The differences among the cytokines secreted by Th cells can be divided into two cell subsets: Th1 and Th2. Th1 cells secrete IL-12, IFN- γ and TNF- α among others and mainly mediate an immune response. In contrast, Th2 cells secrete IL-4 and IL-10, as well as other cytokines to be involved in the humoral immunoresponse (HI) and mediate immune suppression. IL-12 is the cytokine of Th1, but P70 is a subunit functioning inhibition effect. In this study, the treatment of PBMCs and DCs with gp96-peptide complexes induced the secretion of cytokines. Compared with PBMCs, DCs were found to secrete higher levels of TNF- α and IFN- γ and lower levels of IL-12 (P70) and IL-10. These results suggest that, the gp96-peptide complex owns stronger molecular chaperones, to promote the maturity of DCs, thereby enhancing a greater Th1 response and inhibiting Th2. Accordingly, stronger cytotoxicity was generated.

Currently, a few clinical trials are investigating the use of gp96-peptide complexes as a treatment strategy for tumor patients. Previously, Janetzki et al. (2000) performed pre-clinical studies with gp96-peptide complexes in a melanoma model and DCs were found to markedly increased CTL activity compared with antigen peptides. These results suggest that, the gp96-peptide complex, in coordination with DCs, can enhance the antigen presenting role of DCs and significantly improve the ability of CTLs to target leukemia cells. In addition, the short half-life of gp96-peptide complexes in combination with longer-lived DCs has the potential to capitalize on the favorable features of each component to generate a stronger anti-tumor immune response.

In summary, the results of this study demonstrate that leukemia-derived gp96-peptide complexes play an important role in activating lymphocytes and increasing the presentation of tumor antigens by activated CTLs. Therefore, in combination with an increased secretion of cytokines, DCs stimulated with gp96-peptide complexes produced an overall stronger immune response towards leukemia cells. These results provide further support for the use of gp96 as an immunotherapy for the treatment of residual leukemia cells, where gp96-peptide complexes could be isolated from the patient's own tumor to avoid the need for virus-mediated therapies and the transforming DNA involved (Wierecky et al., 2006).

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