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**Gastric-cancer–derived heat shock protein gp96–peptide complex enhances dendritic cell activation**

Lu WW *et al.* HSP gp96 of gastric cancer

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**Abstract**

***AIM***

To investigate the role of heat shock protein (HSP)-glycoprotein (gp)96 to dendritic cells (DCs) and lymphocytes induction in gastric cancer.

***METHODS***

Human gastric cancer (GC) cell lines KATOIII, MKN-28 and SGC-7901 were adopted, and infected with adenovirus gp96 at a multiplicity of infection of 100. gp96–GC antigen peptide complexes were purified through \*\* procedure/maneuver? MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, lactate dehydrogenase (LDH) release assay and ELISA were used to determine allo-reactive T cell stimulation, natural killer (NK) cell activity and expression of cytokines (such as interleukin (IL)-10, IL-12, interferon (IFN)-γ and tumor necrosis factor (TNF)-α), respectively. Effect of cytotoxic T lymphocyte (CTL) on DCs incubated with HSP-gp96was also evaluated by LDH release. All assays were performed in triplicate and the average values were reported. Comparison between groups was conducted using Student’s *t* test.

***RESULTS***

T cells incubated with HSP-gp96 exhibited a marked increase in proliferation in a dose-dependent manner (*P* < 0.05). NK cell activity after gp96–GC peptide complex treatment was significantly higher than that after antigen peptide treatment (*P* < 0.05). The activity of CTLs incubated with DCs from three GC cells lines was obviously higher than that stimulated by GC antigen at ratios of 50: 1, 25: 1, 10: 1, and 5: 1 (*P* < 0.05). Furthermore, the secretion of TNF-α, IL-10, IL-12 (P70) and IFN-γ markedly increased after incubation with HSP-gp96 (*P* < 0.05).

***CONCLUSION***

HSP-gp96 promotes T cell response, enhances DC antigen presentation o and induces cytokine secretion, as well. HSP-gp96 has the potential to be immunotherapy for elimination of residual GC cells.

**Key words:** Gastric cancer; Heat shock protein gp96; T lymphocytes; Dendritic cells; Natural killer cells; Cytotoxic T lymphocytes; Immunization therapy

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**Core tip:** Heat shock protein (HSP)-gp96 was studied to evaluate its capacity to stimulate immune cells. Compared with gastric cancer (GC)-derived peptide, purified gp96–GC peptide complex markedly increased proliferation of allogeneic normal T lymphocytes, and activity of natural killer cells and cytotoxic T lymphocytes. Therefore, HSP-gp96 derived from GC cells has immunotherapeutic potential for elimination of residual GC cells.

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**INTRODUCTION**

Gastric cancer (GC) is the fourth most common type of cancer and its mortality rates rank second in the world[1]. Over 70% of newly developed GC patients and deaths occur in developing countries, especially in Eastern Asian, China, for instance[1]. One recent study indicated that 5-year overall survival of GC was 53% in Australian [2]. However, the 5-year survival rates for all stages are only 20%–25% in western countries, though with advanced interventions, the median survival time is only about 2 years[3].

Surgery and adjuvant chemoradiotherapy are traditional interventions of GC, however, prognosis is poor, which may due to complete removal of tumor cells. Therefore, how to eliminate residual GC cells has become the great focus of GC treatment worldwide. Immunotherapy is emerging as a new approach to control residual disease as an alternative to conventional chemotherapy. To explore more effective immunotherapeutic strategies for GC patients, the jobs to identify and use other potent GC antigens shared among GC patients are critically needed.

Heat shock proteins (HSPs), a group of highly conserved proteins, are induced by the perturbation or stress of various physical and chemical factors[4]. They participate in formation of various protein complexes, contributing to folding and extension as well as assembly. HSPs are defined as molecular chaperones regulating target protein transportation instead of protein synthesis[5]. HSPs are the members of the most conserved proteins known in phylogeny according to both structure and function[6,7]. Immunological significance of HSPs was realized from the observation that tumor-cell-derived HSPs could immunize against tumors, which is attributed to HSP-chaperoned peptides derived from tumor antigens[8–10]. HSP glycoprotein (gp)96 is namely from molecular weight of is 96 kDa and acts as a chaperone protein that localizes in the endoplasmic reticulum in eukaryotes[11-13]. Recent studies in solid tumors (such as melanoma and colon cancer) have shown that tumor-derived HSP-gp96 is immunogenic and able to stimulate tumor-specific cytotoxic T lymphocytes (CTLs) generation [14, 15]. It’s worth to notice that HSP-gp96 is an effective treatment for fibrosarcoma melanoma, colon cancer, lung cancer cancer, and spindle cell tumors induced by UV radiation in rat models[10,15,16]. In addition, a Phase III clinical trial of gp96-based vaccines treatment in melanoma and renal cell carcinoma is in progress[17,18]. So, whther HSP-gp96 has the capacity to stimulate cytotoxic T lymphocytes and facilitate complete clearance of GC is of great importance.

This study aimed to investigate whether GC-cell-derived HSP-gp96–peptide complexes could be used as GC-associated antigens in cancer immunological treatmtnt. The purified proteins were used to explore their ability to stimulate lymphocytes, facilitate transport of tumor antigen, enhance the transport of antigen and cytokine secretion in dendritic cells (DCs). Our results provide novel clues for HSP-gp96 as a potential immunotherapeutic target in GC and may provide hints for further studies in this concern.

**MATERIALS AND METHODS**

***Reagents***

KATOIII, MKN-28 and SGC-7901 GC cell lines were purchased from Shanghai Institute of Biochemistry and Cell Biology (China). Concanavalin A–Sepharose and ion exchange columns were purchased from Pharmacia (NS, United States). Markers for SDS-PAGE and bovine serum albumin were purchased from Zhongshan Biological Technology (Beijing, China). Phytohemagglutinin, ammonium persulfate, and a-pyran-1-methyl mannose were purchased from Fluka (MO, United States). Monoclonal antibody against HSP-gp96 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, United States). Glycine, acrylamide, and ammonium sulfate were purchased from Sigma (St. Louis, MO, USA). Cytokine kits for interleukin (IL)10, IL-12 (p70), interferon (IFN)-γ and tumor necrosis factor (TNF)-α were purchased from R&D Systems (Minneapolis, MN, United States). Flow cytometry reagents and antigens were purchased from BD Pharmingen (San Jose, CA, United States). Lactate dehydrogenase (LDH) release kit was purchased from Promega Biological Technology (Madison, WI, United States).

***Construction of HSP-gp96 recombinant adenovirus (Ad-gp96)***

Gene fragment of human HSP-gp96 was inserted into the pShuttle CMV shuttle vector, BJ5183-AD-1, which had been converted to facilitate homologous recombination in thallus (adenovirus, Ad) framework plasmid, pAdEasy-1, had already been pre-converted). Recombinant Ad plasmids containing target sequence were linearized for the transfection of AD293 cells. Virus produced in AD293 cells was concentrated using CsCl ultracentrifugation to produce high-titer recombinant Ad-gp96.

***Isolation and characterization of*** ***gp96-GC antigen peptide complex***

gp96–GC antigen peptide complexes were purified as previously described[19,20]. Human KATOIII, MKN-28 and SGC-7901 GC cell lines were infected with Ad-gp96 at a multiplicity of infection (MOI) of 100. One week later, cells were lysed and centrifuged for 30 min at 2000 × *g* at 4 °C. After the supernatants were aspirated, samples were centrifuged for an additional 15 min at 15 000 × *g* at 4°C. After the supernatants were aspirated, the samples were precipitated using 50%, then 70% saturated ammonium sulfate. The resulting precipitate was resuspended in 5 × its volume in equilibrium liquid (20 mmol/L Tris–HCl, pH7.4; 200 mmol/L NaCl; 2 mmol/L CaCl2, 2 mmol/L MgCl2), added to a concanavalin affinity column, and eluted using 10% a-pyran-1-methyl mannose. The eluant was further purified using a DEAE ion-exchange column and eluted using a gradient of 300–1000 mmol/L NaCl. Fractions containing gp96–GC antigen peptide complex were identified using 10% SDS-PAGE and Western blotting. Protein concentrations of the samples were determined using Bradford assays and the protein purity achieved was 84%.

***Preparation and identification of T cells and DCs***

DC cells were cultured according to previous report[21-24]. Peripheral blood mononuclear cells (PBMCs) were obtained from healthy volunteers and suspended in 5-mL culture flasks with RPMI 1640 containing 10% fetal calf serum. After cell concentration adjusted to 106 cells/mL, cells were incubated at 37 °C with 5% CO2 for 2 h. Cells that did not adhere were collected as T cells, and the adherent cells were treated with granulocyte–macrophage colony-stimulating factor (500 U/mL) and IL-4 (500 U/mL) before being divided into two separate DC populations. Both cells were cultured for 7 d, with half of the growth medium changed every 4 days. Cells were divided into two groups, one treated with purified gp96–GC peptide complex (1 μg/mL) and the other not, both groups were treated with 50 μg/mL TNF-α. On day 10, suspension cells were collected. Direct immunofluorescence labeling of the cells was performed to characterize the cell populations phenotypically. Cells were stained with CD3, CD14, CD19, CD83, CD1a, CD40, CD54, CD80, CD86, HLA-DR and HLA-ABC to identify DCs[25,26].

***MTS assays***

T cells (2 × 105/100 mL) were inoculated into 96-well U-bottom tissue culture plates prior to addition of purified gp96–peptide complex at three different concentrations (1, 2 and 5 μg/mL) in triplicate. After 5 days, the proportion of live cells was measured using a CellTiter 96 AQueous One Solution Cell Proliferation Assay kit (Promega). After 4 h incubation at 37 °C, the absorbance at 490 nm was measured using a Model 680 microplate reader.

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***Detection of NK cell activity***

NK cells were cultured as previously described[27]. Target cells included KATOIII, MKN-28 and SGC-7901 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 μg/mL with untreated NK cells as controls. LDH release was detected by colorimetry.

***Detection of CTL activity***

Conditions for the culture of CTLs have previously been described[27]. CTL activity was assayed for T cells and DCs incubated with purified gp96–GC peptide complex or tumor antigen peptide complex at 5 μg/mL for 7 d. KATOIII, MKN-28 and SGC-7901 cells were used at target: effector cell ratios of 50: 1, 25: 1, 10: 1, 5: 1 and 1: 1. LDH release was detected by colorimetry.

***Cytokine detection***

A sandwich ELISA method was adopted to detect expression levels of IL-10, IL-12 p70, IFN-γ and TNF-α in cell supernatants.

***Statistical analysis***

All assays were performed in triplicate and the average values were reported. Data are presented as average ± SD. SPSS version 10.0 was used for all analyses, with comparisons between groups made using Student’s *t* test. *P* < 0.05 was considered significant.

**RESULTS**

***gp96–GC peptide complexisolation and its effect on allogeneic normal T lymphocytes***

gp96–GC antigen peptide complex was purified from Ad-gp96-infected (MOI = 100) human GC cell lines, including KATOIII, MKN-28 and SGC-7901. The size and purity of isolated HSP-gp96–GC antigen peptide complex was confirmed by Western blot (Figure 1). To investigate gp96–GC peptide complex function, T cells (2 × 105/100 mL) were incubated with purified gp96–peptide complex at three different gradient (1, 2 and 5 μg/mL), or with antigen peptide. After 5 days, MTS assay was applied to evaluate T cells proliferation. T cells incubated with gp96–peptide complexes exhibited a significant and concentration-dependent increase in proliferation than that with antigen peptide, and greatest difference in proliferation was observed at the 5 µg/mL concentration (*P* < 0.05; Figure 2).

***Effect of gp96-GC peptide complex on NK cells***

To assess the effects of the gp96–GC peptide complex on NK cells, KATOIII, MKN-28 and SGC-7901 cells were incubated with NK cells at different ratios, 20: 1, 10: 1 and 5: 1, respectively. Co-cultured cells were stimulated with 1, 2 or 5 μg/mL purified gp96–GC peptide complex. NK cell activity after gp96–GC peptide complex treatment from three GC cells at different concentrations and target: effector ratios was significantly higher than that after antigen peptide treatment (*P* < 0.05; Figure 3).

***Effect of gp96–GC peptide complex on CTL activity***

On day 7, DCs expressed high levels of specific markers, including CD11c, CD80, CD86 and HLA-DR (data not shown). Cytotoxicity was evaluated in T cells and DCs incubated with purified gp96–GC peptide complex or tumor antigen peptide complex as control, at concentration of 5 μg/mL for 7 d. KATOIII, MKN-28 and SGC-7901 cells were used as target cells in co-culture, at ratios of 50: 1, 25: 1, 10: 1, 5: 1 and 1:1 to effector cells. Compared with tumor antigen peptide, cells treated with gp96-GC peptide complex from SGC-7901 cells and tumor antigen did not show significant difference in cytotoxicity when the target: effector ratio was 5:1 (*P* > 0.05). The CTL activity was highly increased by the gp96–peptide complex at target: effector ratios of 50: 1, 25: 1, 10: 1 and 5: 1 (*P* < 0.05) (Figure 4). The CTL activity after incubation with DCs stimulated by gp96–GC peptide complexes at target: effector ratios of 50: 1, 25: 1, 10: 1 and 5: 1 was significantly higher than tumor antigen group(*P* < 0.05) (Figure 5).

***Cytokine expression by PBMCs and DCs after gp96-GC peptide complex treatment***

Sandwich ELISA was used to detect IL-10, IL-12 p70, IFN-γ and TNF-α levels in supernatants of PBMCs and DCs, afterco-cultur with KATOIII, MKN-28 and SGC-7901 cells expressing gp96-GC peptide complexes, or without as control. Compared with untreated PBMCs and DCs, secretion of TNF-α, IL-10, IL-12 p70 and IFN-γ was markedly improved in the presence of 5 μg/mL gp96–GC peptide complex stimulation (*P* < 0.05, Figure 6) in both PBMCs and DCs.

**DISCUSSION**

Similar to other types of cancer, full recovery from GC is difficult due to residual cells that survive after aggressive postoperative chemotherapy or radiotherapy. Although immunotherapy combined with high-dose chemotherapy holds great promise for GC treatment, clinical studies have not yet achieved expected results. Other than optimizing immunotherapy, there is urgent need for novel GC antigen development that can be used as universal antigens to stimulate anti-GC CTL responses in most patients. In present study, we chose HSP-gp96 as our target in GC cells, because previous studies in solid tumors[28,29] have shown that tumor-derived HSP-gp96 had high efficacy in generating tumor-specific immune responses. However, there are no reports in GC yet. Considering the heterogenity of different tumors, preclinical studies are needed to explore the feasibility of GC-derived HSP-gp96as immunotherapy agent in GC.

Thus, HSP-gp96 was adopted for its capacity in stimulating immune cells. Compared with GC-derived peptide, purified gp96–GC peptide complex markedly improved allogeneic normal T lymphocytes proliferation, and increased the activity of NK cells and CTLs. HSP-based vaccines are personalized vaccines carrying the fingerprint of a given tumor, which can circumven the need to identify tumor antigens for each patient. It is still unknown, but verylikely that HSP chaperone peptides originate from endogenously degraded proteins, consisting of normal cell proteins and tumor-associated antigens[30,31]. Therefore, the CTLs were not reactive to normal cells, such as blood cells, including DCs, B cells and PBMCs[32]. CTLs can recognize antigens localized in the cytoplasm of target cells through peptide complexes formation with MHC class I molecules[33]. HSP-gp96 localizes at the surface of antigen presenting cells (APCs), and protein level though in nanomolar , still sufficient to induce a CTL response. Correspondingly, immunizing doses of gp96–peptide complexes are low[34]. HSP-gp96 is a vaccine that does not require adjuvant, thereby eliminating additional chances for toxicity[34,35].

Recent studies have shown that APCs, such as DCs and monocytes, can internalize HSPs spontaneously through receptor (such as CD91)-mediated endocytosis and then direct chaperoned proteins/peptides into the intracellular pathway for MHC-class-I-restricted presentation to CD8+ T cells, concomitant with induction of DC maturation and cytokine secretion[36,37]. Our results also showed that HSP-gp96 alone was able to activate immature DCs (data not shown). However, most mature or activated DCs were obtained after gp96 treatment and further maturation induced byTNF-α[32]. Here in our study, these DCs were used to generate gp96-specific CTLs.

The ability of HSPs in facilitating cross-presentation of MHC-class-I-restricted epitopes and priming CD8+ cell effector responses is well established[31]. Recent studies have also revealed that HSP–peptide complexes ciuld lead to antigen presentation on MHC class II molecules, and then activated CD4+ T cells[38,39]. In line with these findings, we might have induced a CD4+ T-cell response specific for MHC class II–restricted peptides that were chaperoned by gp96 in our experiment. This is further supported by the fact that IL-10, IL-12, IFN-γ and TNF-α were increased after PBMCs were stimulated by gp96–peptide complex. It is possible that HSP-gp96–peptide complexes uptake led to antigen presentation of both MHC class I and II molecules on mature DCs, thus activating CD8+ CTLsand CD4+ T cells as well. However, the evidence in support of this claim was not comprehensive. Hence, further studies are still in need, especially concerning role and mechanism of MHC class II molecules and peptides in activating specific CD4+ T cells.

In conclusion, our study shows that GC-derived gp96–peptide complex plays an important role in activating lymphocytes and promoting tumor antigens presentation by activating CTLs. These CTLs might be promising effector cells in GC immunotherapy because they are potent and cancer cell specific, able to target and kill GC cells, rather than normal cells. Thus, our study provides strong and direct evidence supporting application of HSP-gp96-based immunotherapy targeting GC, especially for residual GC cells. Another advantage of HSP-gp96–peptide complex is that it can be isolated from the patient’s own tumor tissue, to avoid the need for virus-mediated therapies and transforming DNA as well, which needs further clinical exploration[40].

**COMMENTS**

***Background***

Gastric cancer (GC) is the fourth most common type of cancer worldwide and has poor prognosis. Immunotherapy is a new approach to provide an alternative treatment to conventional chemotherapy for patients with GC. Heat shock protein (HSP)-glycoprotein (gp)96 is immunogenic and potent in stimulating the generation of tumor-specific cytotoxic T lymphocytes (CTLs), and may offer immunotherapy for elimination of residual GC cells.

***Research frontiers***

Previous studies in solid tumors, such as lung cancer, colon adenocarcinoma, fibrosarcoma melanoma, and spindle cell tumors, have shown high efficacy of tumor-derived HSP-gp96 in generating tumor-specific immune responses. However, there are no such reports for GC.

***Innovations and breakthroughs***

In this study, purified gp96–GC peptide complex was shown to improve markedly the proliferation of allogeneic normal T lymphocytes, and increase the activity of both natural killer cells and CTLs.

***Applications***

The results from this study provide novel clues for further investigation of HSP-gp96 as a potential target in an immunotherapy approach for GC.

***Terminology***

MTS assay is a colorimetric assay for assessing cell metabolic activity. NAD(P)H-dependent cellular oxidoreductase enzymes may, under defined conditions, reflect the number of viable cells present. Other closely related tetrazolium dyes, including XTT, MTT and the WSTs, are used in conjunction with the intermediate electron acceptor, 1-methoxy phenazine methosulfate.

***Peer-review***

The fact that HSP can be used as vaccination tool in gastric cancer, opens the possibility to another immune-based approach in this disease. This study has established that GC-derived gp96-peptide complex played an important role in activating lymphocytes and promoted the presentation of tumor antigens by activated CTLs. We need more data of HSP-gp96 in animal tests.

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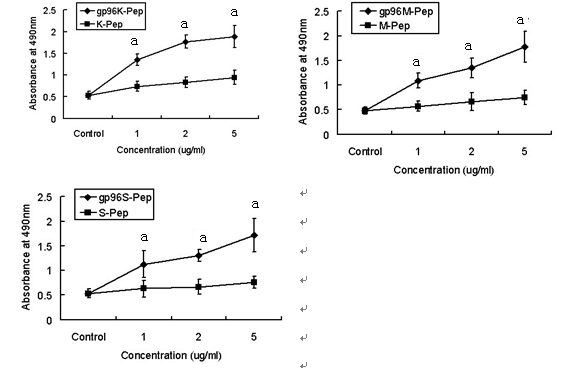
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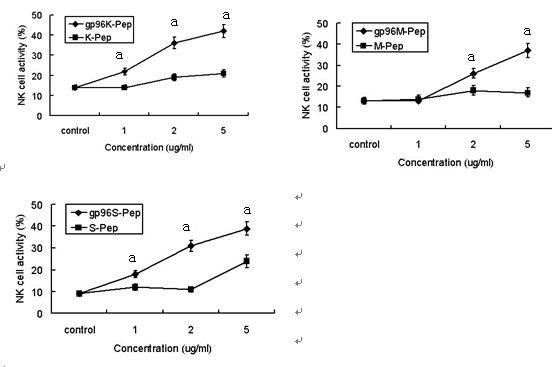
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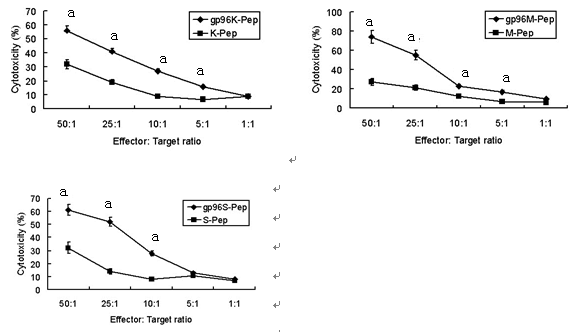
**Figure 1 Characterization of purified gp96–GC antigen peptide complexes.** A: Western blot of gp96–GC antigen peptide complexes from KATOIII cells; B: KATOIII GC cell proteins; C: Purified eluted fraction from KATOIII cells using DEAE-Sepharose anion exchange chromatography.



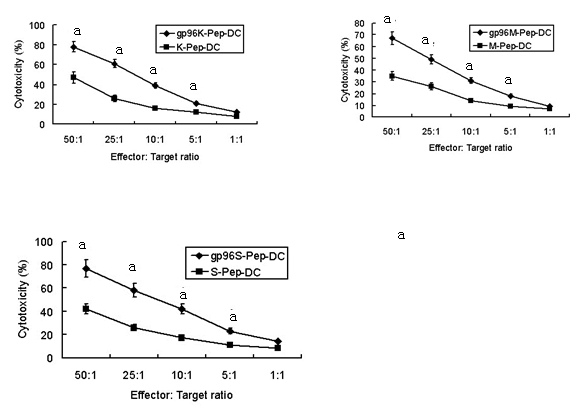
**Figure 2 Proliferation assays of gastric cancer cell strains treated with purified gp96–peptide complexes.** MTS assays were performed for: A: KATOIII source GP96–GC antigen peptide complexes (gp96K-Pep) *versus* KATOIII source antigen peptide (K-Pep); B: MKN-28 source GP96-GC antigen peptide complexes (gp96M-Pep) *vs* MKN-28 source antigen peptide (M-Pep); C: SGC-7901 source gp96–GC antigen peptide complexes (p96S-Pep) *vs* SGC-7901 source antigen peptide (S-Pep). a*P* < 0.05.



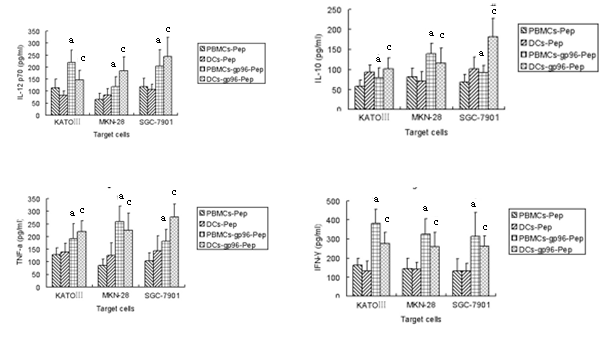
**Figure 3 Activity of NK cells assayed in the presence of gp96–peptide complexes.** At an effector to target cell ratio of 10: 1, NK cells were incubated with three different GC cell strains in the presence of 1, 2 and 5 µg/mL gp96–peptide complexes or gp96 purified from Ad-gp96 expression. NK cell activity for (A) KATOIII, (B) MKN-28 and (C) SGC-7901 cells incubated with gp96–peptide complexes (gp96K-Pep, gp96M-Pep and gp96S-Pep) *vs* GC cell source antigen peptide (K-Pep, M-Pep and S-Pep) for each of the three cell lines, respectively, a*P* < 0.05.



**Figure 4 Cytotoxicity assays for T cells.** Cytotoxic T lymphocyte (CTL) were incubated with KATOIII, MKN-28 or SGC-7901 cells in the presence of gp96–peptide complexes (gp96K-Pep, gp96M-Pep and gp96S-Pep) *vs* GC cell source antigen peptide (K-Pep, M-Pep and S-Pep) at the target to effector cell ratios indicated. a*P* < 0.05.



**Figure 5 Cytotoxicity assays for DCs.** DCs were incubated with KATOIII, MKN-28 and SGC-7901 cells in the presence of gp96–peptide complexes (gp96K-Pep-DC, gp96M-Pep-DC and gp96S-Pep-DC) *versus* GC cell source antigen peptide (K-Pep-DC, M-Pep-DC and S-Pep-DC) at the target to effector cell ratios indicated. a*P* < 0.05.



**Figure 6 Cytokine-specific sandwich** **ELISAs.** Cytokine expression by PBMCs and dendritic cells in the presence or absence of gp96–peptide complexes purified from KATOIII, MKN-28 and SGC-7901 cells. A: IL-12 p70; B: IL-10; C: TNF-α; D: IFN-γ. PBMCs cultured with gp96–GC peptide complexes *vs* PBMCs cultured with GC peptide, a*P* < 0.05; DCs cultured with gp96–GC peptide complexes *vs* DCs cultured with GC peptide, c*P* < 0.05.