Inhibitory Effect on Tumor Growth of Tumor-Associated Antigen Peptides Eluted from Murine Hepatocarcinoma H-22

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Abstract **Objective** The tumor-associated antigen peptides (TAP) are currently recognized as the vital target molecules for multi-peptides tumor vaccines. The study is to investigate the inhibitory effect on tumor growth of TAP extract (MW≤3000 Da). **Methods** TAP were prepared from H-22 cell membrane with mild acid elution method which was used to immunize the BALB/C mice by subcutaneous injection in backsides, and then to observe the effect of TAP on the development of hepatocarcinoma H-22 tumor in autotransplanted mouse model. **Results** After the treatment with TAP extract, the incidence of the transplanted tumor markedly reduced, the growth speed of the tumor decreased significantly, compared with control and adjuvant alone treated groups. Furthermore, cytokines products and the immune functions of thymocytes and splenocytes were greatly enhanced by the immunization with TAP, as detected in vitro assays. **Conclusion** The results indicate that the TAP extract has immunogenicity and could efficiently activate immune responses to inhibit tumor development in vivo. This study further confirms that immunization with TAP may have the potential application to the treatment of tumors via the enhancements of anti-tumor immunity.

Key Words Hepatocarcinoma/immunology; antigen, tumor; peptides; immunotherapy

T lymphocytes play an important role in mediating tumor immunity through recognizing the tumor antigenic peptide presented by major histocompatibility complex (MHC) molecules on the surface of tumor cells. With the progress of the study on the mechanisms of T cell activation and tumor antigen peptides processing and presenting, the tumor–associated antigen peptides (TAP), as efficacious target molecules of tumor cells identified by cytotoxic T lymphocyte (CTL), have been found to have an important effect in inhibiting tumor growth1. CD_8^+ CTL recognizes antigenic peptides expressed on the tumor cell surface in MHC class I–restricted fashion and cause lyses of the tumor cells[1–4]. Many extensive studies have demonstrated that these antigenic peptides are 8–12 amino acids long and are derived from the endogenous proteins, which were degraded of intracellular proteins via the antigen–processing pathway.

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Over the past decades, wide range of MHC class I-binding peptides that derived from tumor cells of mice and humans and recognized by CD_8^+ T cells have been identified[5–8]. Immunization with the tumor peptides could induce peptide-specific anti–tumor CTL activation in vitro and vivo. Similarly, experiments have indicated that Heat-shock Protein (HSP) extracted from tumor cells also has the anti-tumor effect [9]. It is thought that these molecules act as molecule chaperones during the assembly of MHC-peptide complexes and that the peptides, which is produced during antigen processing by HSP, have the vital role in tumor rejection. Therefore, it is essential to search for efficient immunogenetic components from tumor-associated multi-peptide pools in order to prepare for multi-peptides tumor vaccines, and it has become a novel strategy of tumor immune biotherapy.

Herein, we chose murine H-22 hepatocarcinoma to prepare TAP from the tumor cell membrane with mild acid elution method and then observed the effects of TAP on the development of autotransplanted tumor in the immunized mice. Our study demonstrated that the peptides derived from tumor cell membrane TAP as the target of antitumor immunity have the tumor inhibitory effects, inducing

specific cellular immune response. Furthermore, the results imply that the inhibitory effects of TAP on tumor could be achieved through the enhancement of immune functions.

MATERIALS AND METHODS

Animals and tumor cell line

6–8 weeks old Female BALB/c (H-2^d) mice were obtained from the division of animals of Norman Bethune University of Medical Sciences and maintained under germ-free conditions. Murine H-22 hepatocarcinoma cell line was kindly presented by Kitasato University, Japan.

Peptide isolation

Peptides were extracted from H-22 cell line as previously described 10. Briefly, 5×10^6 hepatocarcinoma H-22 cells were intraperitoneally inoculated in the abdominal cavity of mice. The mice were sacrificed 21 days after inoculation. The H-22 cells were taken out to prepare for single cell suspension; after three washes with PBS, the final wash of PBS was removed by pipet, 5ml of citrate–phosphate buffer (0.131M citric acid/0.066M Na_2HPO_4, pH=3.3) was added to the 10 ml tube, and incubated for 1 minute at room temperature, gently blew by Pasteur´s pipet, then centrifuged 1500 r/min for 5 min, the acid solution was harvested by pipet, then filtrated with 0.22 μm micropore filter to remove any cell debris. The resulting cell–free supernatant was harvested and stored at -80℃.

Purification of tumor associated peptide[11]

10 ml resulting H-22 tumor cell-free solution were loaded onto SepPak C18 (Waters Associates, America) devices. Peptides were eluted from those columns by 2 ml of 60% acetonitrile (in water) and subsequently lyophilized to remove the organic solvent. Samples were then reconstituted in 1 ml of PBS buffer and the resulting solution fractionated on the Centricon-3 (Amicon, Beverly, American) ultrafiltration devices, centrifuged at 6500 r/min for 120 min. The Centricon-3 flowthrough solvent consisted of peptides ≤3000 Mr (≈30 amino acids in length), then the eluent was reconstituted and stored at –20℃. The concentration of sample protein was 1.13 mg/ml, as determined with ultraviolet spectrophotometer.

Immunization with TAP

Experimental mice were randomly divided into 3 groups, including normal control group, adjuvant group and TAP immunized group. Using Freund´s Incomplete Adjuvant (FIA), TAP group´s animals were immunized of TAP peptides emulsified in an equal volume of FIA containing 20 μg/mouse by subcutaneous injection in backsides three times at weekly interval. Control group and adjuvant group were injected with the same volume of PBS or FIA, respectively. The mice were sacrificed 1 week after the last immunization. The spleens and thymuses were then taken out to be prepared into single cell suspension for immunological assays.

Tumor rejection in vivo

2×10^6 H-22 cells were inoculated in the hind limb of mice 1 week after the second immunization to observe the incidences of tumor, and the velocity of tumor growth.

CTL assay

^{3}H-TdR-release assay was used to assess CTL activity as reported by Higashi [12]. The amount of ^{3}H-TdR, which released from target cells, was counted by using a LKB-1214 liquid scintillation analyzer (ReckBeta, Sweden). The percentage of specific ^{3}H release was calculated using the following formula: CTL cytotoxic activity (%)=[Experimental release (cpm)-spontaneous release (cpm)]/[total release (cpm)-spontaneous release (cpm)]×100%.

Immunology index analysis

Thymocyte spontaneous incorporation analysis ^{3}H-TdR-incorporation assay was used. In brief, the thymocytes (1×10^6 cells per 200 ml per well, triple wells per sample) were added in a 96 well plant bottom plate (Costar, USA). Meanwhile, 18.5 kBq/25 μl ^{3}H-TdR were added per well, these cells were cultured in humidified atmosphere (5% CO_2, 37℃) for 4 h. The cells were harvested onto glass fibre filters using a Titertek cell harvester (ZT-II, China). The samples were counted using a liquid scintillation analyzer (LKB, Sweden). The results were denoted with ″cpm″, namely count per minute.

Proliferative reaction of splenocytes to Con A

Splenocytes were adjusted into 5×10^6 per ml, the concentration of mitogen Con A is 10 μg/ml. 100 μl of the cell suspension and Con A were added in a 96 well plane bottom plate (Costar, USA) respectively, triple wells of a sample were deter-

mined. these cells were cultured at 37℃ in humidified atmosphere at 5% CO_2 for 72 h, For the final 6 h of culture 18.5 kBq/25μl ^{3}H-TdR was added to each well. The cultures were harvested onto glass fiber filter disks, using a multiple cell harvester, and thymidine incorporation quantified using a liquid scintillation counter.

Analysis of T lymphocyte subsets by Flow Cytometry Freshly prepared thymocytes and splenocytes were suspended in PBS, with 0.1% BSA and 0.1% sodium azide. 1×10^6 cells were incubated on ice for 45 min with appropriated staining reagents. Then it was washed 2 times with PBS, T subsets were determined by flow cytometery (Becton Dickinson, American), and the monoclonal antibodies of CD4 and CD8 (labeled CD4-FTTC, CD8-PE) were purchased from PharMingen (CA, USA).

IL-2 activation assay of splenocytes Lymphoblast proliferation analysis method was used to determine the levels of IL-2 products in culture supernatant. The cell proliferation was detected using ^{3}H-TdR incorporations.

In vitro IFN-γ induction and IFN-γ assay The vesicular stomatitis virus (VSV) protection assay was used to assay IFN-γ levels. OD values were measured at 540 nm with Platereader (DG3022, China).

Statistics

Student´s t or χ^2 tests were used to evaluate statistics significant of the data. $P<0.05$ was recognized as statistic significance.

RESULTS

In vivo tumor rejection assessment

After the mice were immunized with TAP extract (TAP group) the velocities of the growth of transplanted tumor reduced compared with the control and adjuvant groups (Fig.1). The incidences of hepatocarcinoma on 21 days after transplanted of H-22 hepatocarcinoma lowered (50% in TAP group, $P<0.05$, Fig.2); the mean time of tumor development delayed for 4~11 days compared with control group (data not shown).

Thymocytes spontaneous incorporation analysis

Thymocytes spontaneous incorporation ^{3}H-TdR in TAP group was increased significantly as compared with that of the of Control and Adjuvant groups, ″ cpm″ value of TAP group is 1.35 fold than that of control group ($P<0.05$, Fig. 3).

Changes of T cell subset in thymus by FCM

As showed on the Table 1, the percentage of $CD4^+$ thymocytes has a decreased tendency as adjuvant group compared with that of the control group. The Ratio of $CD4^+/CD8^+$ was significantly decreased ($P<0.05$). TAP group compared with control group, and the percentages of $CD4^+$ and $CD8^+$ have an increasing tendency. Whereas, the percentage of CD4 CD8 double negative cells was signifi-

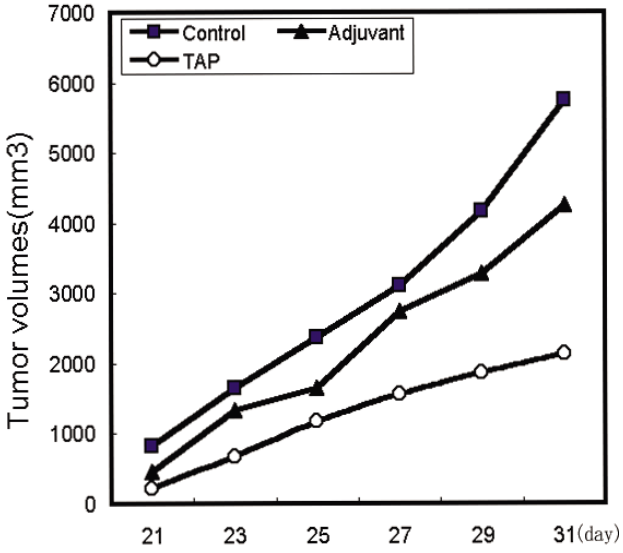

Fig.1 Effects of TAP extract on the development of transplanted hepatocarcinoma H-22 in mice

Ten mice each group were treated with TAP (20 ug per mouse), adjuvant or PBS (mock). One week later, the immunized mice were injected s.c. with 2×10^6 H-22 tumor cells and the tumor volume were then monitored every day. The data are the means of each group.

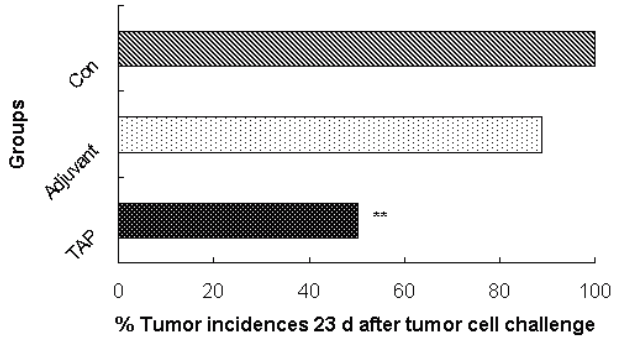

Fig. 2 Effects of TAP on transplanted H-22 hepatocarcinoma incidences in mice.

The tumor incidences (%) of each group was counted at 23 days after tumor transplanted H-22 tumor cells. n=10, ** $p<0.01$ vs control.

cantly increased (P<0.05). As TAP group compared with adjuvant group, the percentage of CD_4^+ significantly increased (P<0.05), but there were no significant changes in the ratio of CD_4^+/CD_8^+ T cells.

Changes in proliferative reaction of splenocytes to Con A

After TAP immunization, proliferative reaction of splenocytes to Con A was significantly increased as compared with that of control and adjuvant Groups (P<0.05), (Table 2).

Changes of cytokine activities and CTL kill rates in splenocytes

As showed in Table 3, the IFN-γ secreting activities of splenocytes was significantly increased (P <0.05); The secret activity of IL-2 has increased tendency as compared with control, but no significant changes 7 days after TAP immunization with TAP; The CTL kill ratio in spleen was 3.3 fold higher than that of control group, which was significantly increased compared with Control and Adjuvant groups (P<0.005 and P<0.05, respectively).

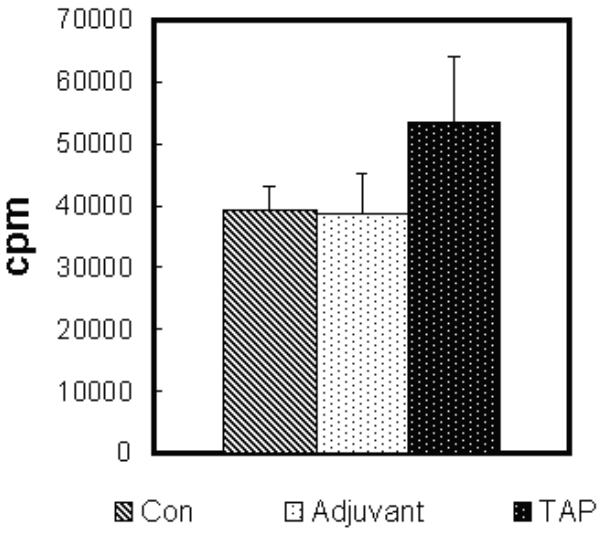

Fig.3 Changes in spontaneous incorporation of 3H-TdR into thymocytes 7 days after mouse immunization with TAP.

Thymocyte spontaneous incorporation of ^{3}H-TdR. The effect of TAP on thymocytes proliferation. The thymocyte spontaneous incorporation were collected and cocultured with ^{3}H-TdR (18.5kBq/well) to detect the thymocytes proliferation.

Table 1 Percentage changes of CD4 and CD8 SP cells in thymuses 7 days after mouse immunization with TAP extract

Groups	CD4 SP	CD8 SP	$CD4^+CD8^+$	$CD4^-CD8^-$
Control	14.06±3.67	6.66±2.40	77.5±6.22	1.772±0.58
Adjuvant	10.97±3.38	6.97±2.14	80.2±5.55	1.84±0.55
TAP	16.48±3.49▲	9.68±3.83	70.82±4.07▲	2.37±0.14*

Mean(SD, n=6; ▲ p<0.05 vs Adjuvant

Table 2 Changes in proliferative reaction of splenocytes to Con A at 7 days after mouse immunization with TAP extract

Groups	cpm
Control	20380(2338
Adjuvant	18559(3464
TAP	33519(4849 **

Mean (SD, n=6; ** p<0.01 vs Control and Adjuvant

Table 3 Changes of cytokine products and CTL function in splenocytes at 7 days after mouse immunization with TAP extract

Groups	IFN-γ (OD)	IL-2 (cpm)	CTL kill rates (E: T=50:1)
Control	0.89±0.07	6704±2577	7.57
Adjuvant	0.95±0.07	7893±3676	11.60
TAP	1.02±0.06*	8194±1967	25.16**▲

Mean(SD, n=6; * p<0.05 and ** p<0.005 vs Control, ▲ p<0.05 vs Adjuvant

DISCUSSION

T lymphocytes play an important role in mediating tumor immunity through identifying tumor cells efficaciously. In recent years, with the development of the study on tumor immunological mechanism, tumor-associated antigen peptides (TAP), as an efficacious target molecular of tumor cells identified by CTL, has been found to have an important effect in inhibiting tumor growth. Therefore, it is necessary to search for efficient components from tumor-associated multi--peptide pools in order to prepare for multi-peptides tumor vaccines, and this has become a novel strategy of tumor immune biotherapy.

In the present study, we chose murine H-22 hepatocarcinoma to prepare TAP from the tumor cell membrane with mild acid elution method and then observed the effects of TAP on the development of autotransplanted tumor in the immunized mice. The results showed that TAP reduced the incidence of the transplanted tumor, and decreased the growth speed of the tumor. These results indicate that TAP can inhibit tumor development.

The present experiment showed that the specific CTL kill rate in vitro induced in mice immunized with TAP was 3.3 fold higher than that of the control group and their reactive abilities to Con A enhanced. The secreting activities of interferon gamma (IFN-γ) and interleukin-2 (IL-2) of T cells in spleen increased significantly as compared with that of the control, the percentages of $CD4^+$ and $CD8^+$ went up and the expression of CD3 and CD69 molecules unregulated (data not shown). The spontaneous incorporation of ^{3}H-TdR into mouse thymocytes after the immunization with TAP increased as compared with that of the control, and the expression of CD3 and the percentages of CD4 and CD8 simple positive T cells increased. The results indicate that TAP can elicit the activation of T cells, especially for T helper (Th) cells, and induce specific CTL. Meantime, the increases of IL-2 and IFN-γ secreting activities and the reaction of the splenocytes to Con A can further enhance the toxic effects of CTL. In addition, with the enhancement of proliferation activity of the thymocytes and the accelerated progressions of the cell renewal, differentiation and maturity, the storage of the effector cells in thymus can be increased. In conclusion, our study demonstrated that the peptides derived from tumor cell membrane TAP have the tumor inhibitory effects, induce specific cellular immune response, at the same time, the results imply that the inhibitory effects of TAP on tumor could be achieved through the enhancement of immune functions.

Acknowledgments

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