Treatment Effect of Tuftsin and Antigen Peptide Combined with Immune Cells on Colorectal Cancer

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Background: The aim of this study was to investigate the effect of antigenic peptides on dendritic cell maturation and activation as well as the role of dendritic cell induced cell function. The tumor-specific cytotoxic T lymphocytes induced by activation of the dendritic cells were also evaluated.

Material/Methods: SW-480 cell lysate and peptide antigens were selected as adjuvants in dendritic cell sensitization, and tuftsin was used to induce the phagocytosis of dendritic cells. Immature dendritic cells were stimulated with the antigen and adjuvant as follows: group A was negative control; group B was SW-480 (20 μg/mL); group C was SW-480 (20 μg/mL)+tumor necrosis factor (TNF)-α (10 μg/mL); group D was SW-480 (20 μg/mL)+tuftsin (20 μg/mL); group E was antigen peptide (2 μg/mL); group F was antigen peptide (2 μg/mL)+TNF-α (10 μg/mL); group G was antigen peptide (2 μg/mL)+tuftsin (20 μg/mL). Cytotoxic T lymphocytes activation and in vitro anti-tumor effects were examined by detecting the maturation marks of dendritic cells as well as interleukin (IL)-10 and IL-12 levels secreted by dendritic cells. Cells with the strongest immunizing effects were injected into nude mice and tumor suppression status was evaluated.

Results: Group D (SW-480+tuftsin), group E (antigen peptides), group F (antigen peptide+TNF-α), and group G (antigen peptides+tuftsin) displayed significant differences compared to the control group (P<0.05). Group G (antigen peptides+tuftsin) could also promote the secretion of cytokines IL-12, as well as inhibit cytokine IL-10 secretion, compared to the other experimental groups (P<0.05). In the in vivo experiments of tumor inhibitions, antigenic polypeptide+tuftsin was the most effective (P<0.05).

Conclusions: Combination of cytotoxic T lymphocytes and T peptide therapy in treating human colorectal cancer might be used as a new treatment strategy based on adoptive cellular immunotherapy.

MeSH Keywords: Colorectal Neoplasms • Dendritic Cells • Immunotherapy, Adoptive • Tuftsin

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Background

Colorectal cancer is a common malignancy, its worldwide incidence rate increases every year [1]. This phenomenon may be ascribed to the changes in the human lifestyle and diet [2]. In China, colorectal cancer is currently among the first 3 fatal cancers, and it remains the most prevalent Chinese gastrointestinal disease. Despite years of research, conventional treatments still have not significantly improved patient survival rates. At the same time, adoptive cellular immunotherapy has achieved great progress during recent years, and has become one of the most important anti-tumor treatments following surgery, radiotherapy, and chemotherapy [3].

Dendritic cells are typically found in normal human peripheral blood with a low concentration. In healthy human peripheral blood lymphocytes, dendritic cells account for about only 1% to 5%, but they play an important role in immune regulation. Dendritic cells are currently known to be the most powerful cells in the human body responsible for activating resting T cells, also known as professional antigen-presenting cells, therefore, dendritic cells hold an irreplaceable position in the body’s anti-tumor immune system [4]. Immature dendritic cells have a strong antigen recognition function. With dendritic cell maturation, tumor antigen uptake, processing, handling, and antigen recognition capability gradually disappears in favor of secretion of various cytokines and adhesion molecules, which activate T cells to produce large amounts of specific cytokotic T lymphocytes (CTLs) to kill tumor tissue. However, due to tumor immune evasion mechanisms and various other factors, such as the fact that tumor cells cannot be efficiently identified by dendritic cells, which cannot be induced to produce a normal immune response, tolerance to tumor tissue could be induced. In recent years, many research studies have focused on the solution of this problem, such as the introduction method of antigen, to improve dendritic cell activity, etc., and while each method has its own advantages and made some achievements, the effect has not yet met scientists’ expectations.

Tuftsin is a 4-peptide substance (Thr-Lys-Pro-Arg) first discovered by scientists from Tufts University in 1970 [5]. Reports indicate that tuftsin has displayed significant anti-tumor effects, not by directly killing tumor cells, but by phagocytic cells. After binding receptors on polymorphonuclear cell surfaces, tuftsin activates the migration of these cells, causes them to engulf cancer cells and achieves the anti-tumor effect [6]. Existing research showed that tuftsin clearly increased phagocytosis and anti-tumor effects [7], primarily by enhancing macrophage phagocytic mobility and activity, while simultaneously secreting cytokines, such as interleukin (IL)-1, tumor necrosis factor (TNF), etc. However, the mode of action and the results of are still not clear.

Immunotherapy may be an effective and safe supplementary approach to cancer treatment and could improve the efficacy of chemotherapy and radiotherapy for colorectal cancer patients [8]. Many studies have indicated that dendritic cells may enhance the function of cytokine-induced killer cells by significantly improving proliferation and tumor-specific activity of cytokine-induced killer cells [9]. Indeed, the clinical benefits of dendritic cells and cytokine-induced killer have been reported in patients with solid tumors [10]. The key step in tumor vaccine screening is a good tumor antigen in the immunization. Tumor cells carry different types of antigens, such as DNA, RNA, and surface proteins. Tumor cell lysate is the most commonly used antigen in research due to its advantages in variety and immunogenicity. It was shown that polypeptide DNA-transfected dendritic cells enhanced MNC anti-tumor activity, increasing tumor cell death and the percentage of perforin positive lymphocytes. In addition, DNA-transfected dendritic cells elicited a cytotoxic response that was as efficient as that of tumor lysate-loaded dendritic cells [11]. However, there are a variety of factors associated with tumor escape, which, to varying degrees, affect the ultimate efficacy of immunotherapy.

Therefore, our current study focused on how to increase the specificity and cytotoxic activity of T cells. In our current study, a new antigen-antigenic polypeptide was obtained; compared with tumor cell lysate debris, it may not be compared in terms of the types of tumor antigens. However, after screening for antigens with some immunogenicity and removing unnecessary associated antigens, they may be recognized and removed by immunocompromised organism to decrease the tumor’s immune tolerance [12]. When combined with tuftsin as basic treatment, it may reestablish the immune surveillance system, thus significantly improve patient survival and quality of life. In our present study, we investigated the effect of antigenic peptide on dendritic cell maturation and activation as well as the role of dendritic cell-induced cell function. The tumor-specific CTLs induced by activation of the dendritic cells were also evaluated.

Material and Methods

Monocyte isolation and generation of dendritic cells

Human peripheral blood mononuclear cells (PBMCs) were first isolated by density gradient centrifugation. Monocytes were isolated by adherence on tissue culture plastic plates. Then the cells were seeded in 24-well plates at 1×10⁶ cells per well. Immature dendritic cells were prepared by culturing monocytes for 8 days with human-recombinant granulocyte-macrophage colony stimulating factor (GM-CSF) and human-recombinant IL-4 in Cellex901 medium in 24-well plates at 37°C in 5% CO₂. Also, the immature dendritic cells were stimulated with...
the antigen and adjuvant as follows. Group A was the negative control; group B was SW-480 (20 μg/mL); group C was SW-480 (20 μg/mL)+tumor necrosis factor (TNF)-α (10 μg/mL); group D was SW-480 (20 μg/mL)+tuftsin (20 μg/mL); group E was antigen peptide (2 μg/mL); group F was antigen peptide (2 μg/mL)+TNF-α (10 μg/mL); and group G was antigen peptide (2 μg/mL)+tuftsin (20 μg/mL). The cells were collected after 8 days of treatment.

The study was approved by the Ethics Committee of the General Hospital of the People’s Liberation Army (December 19, 2014).

Flow cytometry analysis of dendritic cells

To determine the stimulation and maturation of monocyte dendritic cells exposed to the antigen and tuftsin, flow cytometry was used to detect HLA-DR, CD86, CD80, and CD83.

Induction of CTLs

On the 8th day, the adherent PBMCs were mixed with non-adherent cells under cryopreservation after recovery, which were added to Celix601 serum-free medium and placed in an incubator at 37°C, cultured in 5% CO₂. The next day CD3 monoclonal antibody (1000 u/mL) and IL-2 (1000 u/mL) were added, with subsequent daily additions of IL-2 (1000 u/mL). On the 14th day, the CTLs was harvested.

Detection of cytokines

IL-10 and IL-12 secreted by dendritic cells and TNF-α secreted by CTLs were measured using an enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer’s instructions. Dendritic cells were cultured in the first 8 days, supernatants were obtained from each well and samples of approximately 2 mL were used to detect IL-12 and IL-10 content using the ELISA method.

Proliferation of CTLs detected by MTT

To collect the cells in the logarithmic growth phase and adjust the cell concentration to 1000–10 000 per well, the liquid was added to 100 uL in every well. The cells were placed in a 5% CO₂, 37°C incubator, waiting to fill the well at the end of the cell monolayer. Then the concentration gradient of the drug was added to each well at 0–10 uL. The cells were placed in 5% CO₂ in a 37°C incubator and incubated for 24 hours; a microscope was used to observe cells state. After 4 hours of incubation, 10 uL of MTT stain was added to each well. Then washed 3 times with PBS buffered medium containing MTT. Then 100 uL of formazan solution was added to each well and mixed by low speed oscillation for approximately 10 minutes until fully dissolved. Determination of absorbance 570 nm was done by ELISA.

Lactate dehydrogenase release assay

Lactate dehydrogenase release assay was used to detect the anti-tumor effect of CTLs induced by different antigen sensitizations and different activating agents in vitro. We used the lactate dehydrogenase method for the CTL cytotoxicity test, taking the 7 groups of cultures on the 14th day of CTLs, resuspended to a cell concentration of 1×10⁶ per ml; and also taking SW-480 knot colorectal cancer cells in logarithmic growth phase as the target cells. The resuspended concentration was adjusted to 1×10⁶ per mL and the effective target ratio was 5: 1 and 10: 1.

Tumor models and in vivo anti-tumor effect assay

Nude mice were randomly placed into groups. Human colorectal tumor cells (1×10⁶) were harvested and injected subcutaneously into the right abdominal flanks of the mice. Three days later, mice in each group had hard nodules in the right armpit. From the fifth day on, we began to observe the signs of tumor growth; only 1 or 2 mice per group exhibited no significant subcutaneous neoplasia. Seven days after vaccination, all 35 mice were found to have developed subcutaneous tumor formations; thus, the tumorigenic rate was 100%. Tumor size was measured prior to the start of treatment. After 7 days, the aforementioned groups were treated with a variety of treatment options, administered through intravenous injection. Two subsequent treatments were administered after 5 days and 10 days. Seven days after the final tail vein injection treatment, the nude mice were sacrificed. Then, the tumor tissue was carefully stripped for pathological examination. The spleen, liver, and other tissues were removed and observed for tumor metastasis. Each organ was weighed electronically and the inhibition rates and spleen indices of the nude mice in each group were calculated using the formula: tumor suppressor rate=control group tumor weight–experimental group tumor weight/control group tumor weight×100%; spleen index=spleen weight (mg)/body weight (g).

Statistical analysis

SPSS18.0 software was used to analyze the data. Quantitative data were presented as mean ± standard deviation. One-way ANOVA was used to compare multiple groups, and the least significant difference (LSD) test was applied for comparison between 2 groups. P<0.05 was defined as statistically significant.
The phenotype of dendritic cells was detected by flow cytometry. The maturity of dendritic cells in terms of HLA-DR, CD86, and CD80 was close to 100% in each group and there was no significant difference among groups. CD83 of each experimental group showed an increasing trend compared to the control group. Group D (SW-480+tuftsin), group E (antigen peptides), group F (antigen peptides+TNF-α), and group G (antigen peptides+tuftsin) showed a significant increase of CD83 compared to the control group (P<0.05). Moreover, group F (antigen peptides+TNF-α) and group G (antigen peptides+tuftsin) showed a significant difference; the CD83 was significantly increased compared with the positive control group (SW-480+TNF-α) (P<0.05) (Figure 1).

Results

Detection of the phenotype of dendritic cells

The phenotype of dendritic cells was detected by flow cytometry. The maturity of dendritic cells in terms of HLA-DR, CD86, and CD80 was close to 100% in each group and there was no significant difference among groups. CD83 of each experimental group showed an increasing trend compared to the control group. Group D (SW-480+tuftsin), group E (antigen peptides), group F (antigen peptides+TNF-α), and group G (antigen peptides+tuftsin) showed a significant increase of CD83 compared to the control group (P<0.05). Moreover, group F (antigen peptides+TNF-α) and group G (antigen peptides+tuftsin) showed a significant difference; the CD83 was significantly increased compared with the positive control group (SW-480+TNF-α) (P<0.05) (Figure 1).

Figure 1. (A–D) The phenotype of dendritic cells. HLA-DR, CD86, CD80, and CD83 were detected by flow cytometry on the 8th day.

Figure 2. (A, B) The cytokines IL-10 and IL-12 secreted by dendritic cells. IL-10 and IL-12 secreted by dendritic cells were measured using an enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer’s instructions.
Detection of cytokines IL-10 and IL-12 secreted by dendritic cells

In group D (SW-480+tuftsin) and group G (antigen peptides+tuftsin), cytokine IL-12 levels were 122.43±53.16 and 175.49±76.34 respectively, which were significantly higher than the rest of the groups (P<0.05). Regarding IL-10 levels, group D (SW-480+tuftsin) was 50.20±28.39 which was significantly higher than the rest of the groups (P<0.05). The groups sensitized by peptide antigens had lower levels, but the difference was not significant (Figure 2).

The proliferation of CTLs

For each experimental group, the CTL proliferation data showed a steady growth rate in the first 6 days of co-culture (P<0.05). From the 7th day, the CTLs of group F (antigen peptides+TNF-α) and group G (antigen peptides+tuftsin) showed a significantly increased proliferation, while the rest of the groups remained in the original proliferation trend. On the 14th day, in the dendritic cells and T cells mixed culture, the IFN-γ level in the supernatant was quantified for each group. The results showed that in group F (antigen peptides+TNF-α) and group G (antigen peptides+tuftsin), CTL supernatant IFN-γ levels were 202.58±8.71 and 239.94±14.45 respectively. These 2 groups displayed a significant increase compared to the rest of the experimental groups (especially the positive control group, SW-480+TNF-α) (P<0.05, Figure 3).

Anti-tumor effect of CTL

The “effective to target” ratio of each group’s mixed culture were 5: 1 for CTLs and 10: 1 for SW-480 human colon cancer cells. CTLs anti-tumor effects were measured every 1 to 2 hours, and co-culture experiments were terminated after 8 hours. It was found that each experimental group showed an increased anti-tumor effect trend compared to the control group. Group F (antigen peptides+TNF-α) and group G (antigen peptides+tuftsin) were more effective at the target ratios of 5: 1 and 10: 1 than the rest of the experimental groups (P<0.05). Moreover, group F and group G also showed a higher tumor cell killing rate than the positive control group (SW-480+TNF-α) (P<0.05). Group G (antigen peptides+tuftsin) had the best killing effects at the target ratios of 5: 1 and 10: 1 with the killing rate of 46.10±3.50 and 79.65±2.92, respectively. When SW-480 was used as the experimental antigen sensitizer, the killing activity of experimental group showed an increase trend compared with control group, which was irrelevant with the combination with TNF-α or tuftsin, but the difference was not significant (P>0.05). When using peptide antigen alone to sensitize without TNF-α or tuftsin, the killing effect was not satisfactory; the killing effect showed no statistical difference compared to the control group (Figure 4).

Figure 3. Cytotoxic T lymphocytes proliferation trend graph. Cytotoxic T lymphocytes proliferation trend graph drawn to illustrate number of days vs. actual measurement results.

Figure 4. (A, B) Anti-tumor effect of cytotoxic T lymphocytes induced by different antigen sensitized and different activating agents in vitro. With effective to target ratio 5: 1 and 10: 1, the experimental antigen peptide+tuftsin group shows the best killing effect in vitro.
Discussion

Currently, the gold standard used to validly judge the maturation and activation of dendritic cells is to measure costimulatory molecule expression on dendritic cell surface and secretion of cytokines [13]. However, a variety of experimental results have confirmed no effective way to activate dendritic cells. Therefore, researchers have been trying to find a sufficiently effective way to stimulate dendritic cell maturation. The most popular method for in vitro cultivation of dendritic cells has been to use GM-CSF and IL-4 added monocyte cells in a specific medium that induces immature dendritic cells. Therefore, in our current study, these cells were selected at the right time according to the specific state of antigens and activators for the activation of dendritic cells. Finally, mature dendritic cells with tumor-specific antigens as expected, which can effectively be the signal to T cells to induce a series of immune response, were harvested.

The cell phenotypes of dendritic cells used to measure and analyze maturity and activity were primarily CD80, CD86, CD83, and HLA-DR. Previous research has pointed out that CD11c (a dendritic cell surface molecule) is also a meaningful marker, but considering that the CD11c molecules play an important role only in the differentiation between dendritic cells and other subtypes [14], and it is less correlated with maturity, CD11c was not a focus of observation in this experiment. In this study, it was found that in each experimental group CD80, CD86, and HLA-DR phenotypes were close to 100%, suggesting that the method currently applied to dendritic cells culture was relatively mature. The SW-480+TNF-α group and the polypeptide antigen sensitized dendritic cell groups showed increased maturity compared to the control group, indicating that these groups would likely present antigens to T cells more effectively. In addition, the antigenic polypeptide+TNF-α group and antigenic polypeptide+tuftsin group both showed a significant increase of CD83 expression compared to the SW-480+TNF-α group, which had been shown to effectively activate dendritic cells. This may indicate that antigenic polypeptides can be a better antigen than SW-480. However, whether mature dendritic cells have a better cell function cannot be judged by cell phenotype.
alone, thus our determinations regarding dendritic cell supernatant factor levels are less certain.

IL-12 was mainly secreted by dendritic cells, macrophages, and other antigen-presenting cells able to promote the proliferation and differentiation of T helper cells, and enhanced natural killer cell and CTL killing activity, thereby killing tumor cells. IL-10 might inhibit the proliferation of T helper cells, which also indirectly inhibits the proliferation of CTLs. A large number of studies have confirmed the expression of IL-10 can downregulate co-stimulation molecules on the dendritic cell surface to interfere with the normal function of dendritic cells. This is a negative regulatory factor which could induce tumor immune escape [15], thus, we used the ELISA method to measure IL-12 and IL-10 quantitatively. Our data indicate that tuftsin can act as a potent cofactor to make dendritic cells capture antigens, thus facilitating dendritic cell maturation, secrete more of the positive regulatory factor IL-12, and therefore activate the body’s immune cells to produce anti-tumor effects. The most traditional loaded antigen is found in cell lysate debris, but in our study, it was not difficult to find, although the SW-480+tuftsin group was shown to secrete more IL-12, and IL-10 secretion was elevated as well. This may be associated with surface antigen-induced immune escape mechanisms activating the relevant signaling and causing the cells to secrete large amounts of IL-10, which affects dendritic cell function. As the roles of dendritic cell activators still required further confirmation, T lymphocyte and mature dendritic cells were mixed under specific conditions and cultured in our study. We found that the T cell counts between the groups were related to proliferation trends, moreover, T cell proliferation status were correlated with IL-12 secretion and IL-10 played the opposite role.

In this study, we found that the antigenic peptides+TNF-α group and antigen peptide+tuftsin group had the highest CD8+ expression rates, which meant that these 2 sets of dendritic cell activated T cells harvested more CTLs and theoretically could produce a stronger anti-tumor effect. The cell expression of CD4+ and CD25+ has only recently been recognized to play an inhibitory role in immune regulation. It displays characteristics of immune suppression and immune incompetence [16], which play an important role in tumorigenesis. In the present study, the polypeptide antigen sensitized dendritic cell induced T cell expression ratio of CD25+ showed only a lower trend than the other groups.

Previous research has concluded that tumor immune escape and the microenvironment of tumors are relevant factors in tumor growth [17]. The question of how to defeat the immune tolerance of tumor cells is a key problem that researchers have been pursuing. In this study, the proliferation of T cells and the specific number of CTLs were not consistent in each experimental group. Further research is required to confirm whether we can successfully breakdown the immune tolerance of these tumor cells. By way of lactate dehydrogenase release assay, the antigenic polypeptide+TNF-α and antigenic peptides+tuftsin groups’ SW-480 in vitro tumor cell killing rate was distinctly more favorable compared with other experimental groups. In vivo, IFN-γ plays an important role in anti-viral, anti-tumor, and immunomodulatory effects [18]. Related reports have indicated that interferon added after antigen sensitization could generate significant immune enhancement effects [19]. In addition, studies have shown that, under appropriate conditions, IFN-γ can promote CD8 T cell differentiation, but cannot promote its proliferation. Therefore, IFN-γ and CTL interaction can improve cellular immune function and thereby enhance anti-tumor effects [20]. Prior to dendritic cell induction experiments, the antigen polypeptide+TNF-α group and antigen peptide+tuftsin group displayed greater dendritic cell maturity. In particular, in this study we found increased expression of costimulatory molecules; and these costimulatory molecules are related to T cell proliferation and differentiation, coupled with cytokine IL-12 and other synergies, so immune cell function was improved. The antigenic polypeptide+TNF-α group and antigenic peptide+tuftsin group showed excellent in vitro anti-tumor results when compared to the positive control group (SW480+TNF-α group), suggesting that these methods specifically inhibit tumor cell survival and therefore can prolong patient survival.

The application of antigenic peptides in combination with TNF-α or tuftsin significantly inhibited tumor growth. This result mainly ascribed to the following factors. The application of tuftsin increased phagocytosis of dendritic cells. After subsequent processing of the antigen peptide, dendritic cells can easily capture antigen phagocytosis through endocytosis, and then be processed again in the cell. After treatment, the effective tumor antigen presented to effector T cells. Dendritic cells were also significantly more mature at this time and the surface also expressed some sticky adhesion molecules and costimulatory molecules [21]. This can promote the secretion of immune cytokines TNF-α, IL-12, etc., which further promotes T cell activation, strengthening the cytotoxicity of CTLs. While TNF-α can effectively promote dendritic cell maturation, it only produces a stimulant effect and does not fundamentally solve the problem of tumor immune escape. While effective in nude mice, the effects of the antigenic polypeptide+tuftsin group require more experimentation and exploration to be fully confirmed. However, the potential of this therapy is enormous.

Conclusions

Using antigenic polypeptides to induce dendritic cells alone resulted in no obvious effect, but combined with TNF-α or tuftsin, the results were promising. Antigen peptides+tuftsin can also
promote the secretion of cytokines IL-12, as well as inhibit cytoke

The in vivo tumor inhibition of antigenic polypeptide+tuftsin was the most effective. Based on existing treatments for colon cancer, in this study we added adoptive immunotherapy to traditional methods and found a very significant improvement, which improved the characteristics of immune cell tumor killing activity. This might be a potential strategy to rebuild the body’s immune surveillance system to achieve effective treatment of malignant tumors and improve patient survival.

Conflict of interest
None.

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