Effects of CIK on hypoxia inducible factor-1α and T-cell subsets on colon 26 cancer xenograft mice

HONGZHEN ZHANG¹, MINGLI GUO², HONGYAO CHEN², YING ZHANG³ and LI YA HE¹

Departments of ¹Fifth Oncology, ²Otorhinolaryngology and ³Human Resources, Hebei People's Hospital, Shijiazhuang, Hebei 050051, P.R. China

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Abstract. Colorectal cancer is a common malignancy of the digestive tract with a high mortality rate. However, current treatment approaches such as radiotherapy and chemotherapy are ineffective. Thus, it is imperative to identify ways to treat recurrence and metastasis. The aim of the present study was to investigate the effects of cytokine-induced killer (CIK) cells on the hypoxia microenvironment and immune function in colon 26 cancer xenograft mice. A murine model of colon 26 carcinoma tumors were divided into CIK, normal saline (NS) and control groups. Hypoxia inducible factor-1α (HIF-1α) mRNA in tumor tissue and the small intestine of mice was detected by FQ-PCR. The percentage of the CD4⁺, CD8⁺ and CD4⁺/CD8⁺ ratio in the spleen of mice was detected by flow cytometry. The tumor volume in the CIK group was smaller than that in the NS group and the difference was not significant (P>0.05). HIF-1α gene expression was present in the tumor tissue and small intestine. HIF-1α gene expression in tumor tissue was significantly higher than that in the small intestine (P<0.05), and lower in tumor tissue of the CIK group compared to the NS group (P<0.05) of xenograft mice. However, no HIF-1α expression was observed in the small intestine of healthy control mice. CD4⁺ T-cell percentage and CD4⁺/CD8⁺ ratio in the spleen of xenograft mice was significantly lower than that of normal mice (P<0.05). Compared to the NS group, the CD4⁺ T-cell percentage was higher, whereas the CD8⁺ T-cell percentage was lower in the CIK group (P<0.05). In conclusion, HIF-1α gene expression was present in the tumor tissue and small intestine. The immune function of colon 26 transplanted in tumor mice was decreased. Additionally, CIK improved the microenvironment of tumor hypoxia and promoted immune reconstitution.

Introduction

Colorectal cancer is a common malignant tumor of the digestive tract. It is the third most common cancer diagnosed in men and the second most common in women. More cases of male mortality compared to female mortality have been reported (1). The age-standardized incidence is 44% higher in males (20.6 vs. 14.3 per 100,000 individuals) and that of the mortality rate is 45% higher in males (10.0 vs. 6.9 per 100,000) (1). Surgery, radiotherapy and chemotherapy are therefore ineffective against the recurrence and metastasis of colorectal cancer. Cytokine-induced killer (CIK) is the newest tumor adoptive immunotherapy method at present (2-4). However, few studies have been performed regarding the association of CIK and tumor hypoxia and T-cell subsets.

The aim of the present study was to investigate the effect of CIK on HIF-1α, hypoxia microenvironment and immune function in tumor-burdened mice.

Materials and methods

Experimental animals and tumor cell line. Healthy male Kunming mice (6- to 8-weeks old, weight 20-24 g; certificate no. 902078) were purchased from the Experimental Animal Center of Hebei Medical University. The mice were housed in an environment with a temperature of 18-22°C and humidity of 50-60%, and had access to food and water ad libitum. The colon 26 cancer cell strain was purchased from the Research Center of Hebei Medical University Fourth Hospital.

Key reagents. The reagents used in the present study were: generic recombination interleukin (IL)-1 (PeproTech, Rocky Hill, NJ, USA), IL-2, interferon-γ (IFN-γ) (Beijing Sihuan Bio-engineering Products Factory, Beijing, China), anti-mouse antibody PE-CD4 mAb and FITC-CD8 mAb (eBioscience, Inc., San Diego, CA, USA), HIF-1 kit (R&D Systems, Inc., Minneapolis, MN, USA) were used. Human lymphocyte separation medium (Tianjin Virtue Asm Pacific Technology Ltd., Tianjin, China), and mouse anti-human CD3Mab antibody (Laboratory of Immunology in Health Science Center of Peking University, Beijing, China) were also used.

Peripheral blood separation and the induction of CIK effect on cells (5). Peripheral blood (50 ml) was taken in vacutainers...
with heparin from healthy adults. Lymphocyte apheresis collections were performed in the blood sampling machine at 1,007 g/ml Ficoll and were separated using density gradient centrifugation at 2,500 rpm for 30 min and peripheral blood mononuclear cells (PBMC). The RPMI-1640 liquid transfer of PBMC was used at the starting density of 5x10^6/ml. IFN-γ (1x10^3 U/ml) was added and set at 37˚C with 5% CO₂ in a culture incubator. After 24 h 50 U/ml of CD3 monoclonal antibody and 500 U/ml of IL-2 were added. Subsequently, IL-2 at a concentration of 500 U/ml in fresh medium was added every 2-3 days. The cells were then cultured for 14 days as effector cells.

**Effector cells inhibit tumor growth in tumor-bearing mice.**

The second generation of cultured cells 26 colon cells in the logarithmic growth phase was used for the experiment. After 8 days of feeding, the mice were inoculated with tumor cells. An axillary subcutaneous injection of 1 ml cell suspension (containing ~2x10^6/ml tumor cells) was injected into the mice. The inoculation site appeared to be a hard nodule identified as a tumor. When the average volume of the transplanted tumor in mice was ~0.30 cm³, modeling was successful. Six mice were randomly selected as the health contrast group (group A). After the successful modeling, EXCEL software (Excel list v1.0.909 randomized tools) was used to divide mice with transplanted tumors into two groups of 6 mice each. The normal saline (NS) contrast group (group B) was infused intravenously in tail with 0.2 ml physiological saline on days 0 and 3. The colon cancer CIK treatment group (group C) was infused intravenously in tail with CIK cells (4x10^6/0.2 ml) on days 0 and 3. On day 12 of treatment intervention, the mice were sacrificed and transplanted subcutaneous tumor length was measured using vernier caliper. Tumor volume was calculated. Spleen and colon tumor tissue were removed, and stored in liquid nitrogen at −80°C below zero.

The tumor volume \( V = \frac{a \times b^2}{2} \) and relative tumor volume \( \frac{V_{10}}{V_{11}} \) the tumor volume measured on day 0 and V11 the tumor volume measured on day 11] were calculated as per the general standard formula.

**Flow cytometry (FCM) detection of CD4⁺ vs. CD8⁺ expression in mice.** The mice were sacrificed and the spleen was removed, sterilized, sheared, and pulverized in 200 mesh steel. The spleen was washed with incomplete RPMI-1640 culture solution, and suspended in ice. Spleen cell suspensions were gradually placed in the prepared lymphocyte separation liquid (accounting for 1,080 of the Ficoll-Hypaque) to divide and separate lymphocytes. Cell suspension was centrifuged at 2,000 rpm for 20 min, and the second layer of opaque low-density cells was extracted, washed (2X) in incomplete culture fluid and centrifuged at 1,000 rpm to obtain the cell number of 1x10^6/ml in cell suspension. The cell suspension (1 ml) was added into a tube. PE-labeled antimouse CD4 mAb and FITC-labeled CD8 mAb (1:2,000) were also added. After 30 min, 2 ml red cell lysate was added and incubated for 10 min. Phosphate-buffered saline (2 ml) was added to the cell suspension and centrifuged (2X) at 1,000 rpm for 5 min, and the supernatant was discarded. The ratio of each subpopulation of cells among 1x10^6 cells was detected by FCM (FC 500; Beckman Coulter, Miami, FL, USA).

**Determination of hypoxia inducible factor-1α (HIF-1α).** HIF-1α mRNA expression was evaluated by reverse transcription-quantitative polymerase chain in tumor body and small intestine of the tumor-transplanted group and the health contrast group of mice.

**Statistical analysis.** SPSS 16.0 (SPSS, Inc., Chicago, IL, USA) software was used to process the data. Quantitative data were presented as mean ± standard deviation. The t-test was used for comparison of the two groups. \( P<0.05 \) was considered to indicate a statistically significant difference.

**Results**

**HIF-1α gene expression and changes in splenic T lymphocytes.** HIF-1α gene was expressed in the transplanted tumor and small intestine of mice. HIF-1α gene expression in tumor tissues was significantly higher than that in the small intestine (\( P<0.05 \)). As shown in Tables I and II, the percentage of CD4⁺ T cells and the CD4⁺/CD8⁺ T ratio in spleen tissue of mice with transplanted tumor was significantly lower than that of healthy mice (\( P<0.05 \)).

**Effect of CIK on transplanted tumor volume.** Transplanted tumors gradually increased in size subcutaneously in mice. Local tumor nodular growth led to the tumor began to be touched, 4-5 days after subcutaneous inoculation of the cells. For days 1-7, tumor growth was essentially the same, but for days 8-17, tumor growth in the physiological saline group was significantly more rapid than the other groups. The CIK group had the most retarded tumor growth compared to the NS group. The tumor volume was reduced in the CIK group but was not statistically significant (\( P>0.05 \)) (Table III).

**Effect of CIK on the HIF-1α gene expression in mice tumor tissue.** Fluorescent quantitative analysis showed that compared to the saline group, HIF-1α gene expression was decreased in the tumor tissue of the CIK group, although with no statistical significance (\( P>0.05 \)) (Table III).

**Effect of CIK on transplanted spleen tumor T lymphocytes in mice.** Compared to the health contrast group, the percentage of CD4⁺ T cells in tumor-transplanted mice decreased, and for the CD4⁺/CD8⁺ inversion, the ratio of \( <1 \), was statistically significant (\( P<0.05 \)). Compared to the NS group, the percentage of CD4⁺ T cells and CD4⁺/CD8⁺ of the CIK group were increased, and the result was statistically significant (\( P<0.05 \)) (Table II).

**Discussion**

Colon cancer is a common malignant tumor of the digestive tract, and ranks third among gastric intestinal tumors. The incidence of colorectal cancer in China has been on the increase at a rate of 4% annually (6). Among patients with stage IV colorectal cancer, the 5-year survival rate is only 8.1%, with poor side-effects and adverse reaction of radiotherapy and chemotherapy. The immune system is also suppressed when killing tumor cells. Therefore, it is crucial to identify an effective treatment for advanced colorectal cancer.
Hypoxia is an important characteristic of solid tumor, and HIF-1α is a key factor of tumor hypoxia response. It is an upstream transcription regulating protein for the regulation of tumor angiogenesis, energy metabolism, cell proliferation, metastasis and invasion associated with tumor occurrence, development, recurrence, chemotherapy resistance as well as multidrug resistance (12). HIF-1α is overexpressed in most tumors, including colorectal cancer with an overexpression of >90%. The results of the current study show HIF-1α gene expression in tumor body and small intestine. In tumor body, the expression level was higher than that in the small intestine, although no expression was observed in the small intestine of healthy mice, which is consistent with earlier reports (12).

The tumor microenvironment is an environment where the tumor is located in the developmental process, including tumor cells, stromal cells, microvascular and tissue fluid and a small amount of infiltrating cells, such as dendritic cells and macrophages (13). The tumor microenvironment is also the execution site for antitumor immune effect stage (13). However, low O2, low pH, and low nutritional status of the tumor microenvironment is insufficient to provide the necessary O2 and energy.
for the cellular immune response, which is not conducive to the contact reaction of immune cells and attacking targets, resulting in tumor immune suppression (14). The tumor microenvironment is equivalent to a chronic inflammatory environment and hypoxic tumor cells and necrotic regional cell debris release high levels of chemokines with activation of the nuclear factor-κB pathway and recruitment of immune cells to hypoxia (15). In the hypoxic microenvironment, immune cells targeted as cancer cells cannot entirely activate their killing effect allowing the tumor to elude the immune response. Thus, the effective implementation of the antitumor function cannot be carried out. Tumor cells exposed to hypoxic environment are likely to develop into inferior genetic stability and more aggressive malignant cells, and become resistant to the treatment (16). Therefore, the downregulation of HIF-1α gene expression can prevent the hypoxia-induced reaction required for tumor growth, thereby inhibiting tumor growth. This finding confirmed that the HIF-1α gene expression in the tumor body of mice decreased following treatment with CIK. The tumor volume in mice of the CIK group was smaller than that in the saline group (17). Thus CIK cells as immunocompetent cells induced in vitro amplification resulting in, not only enhancement of the immune function, but also the interruption of the immune suppression state by improvement of the hypoxia tumor microenvironment to further enhance immune function and play an antitumor effect.

Based on the results, the breakthrough and hotspot of colorectal cancer treatment is identified by two aspects: the improvement of the tumor hypoxic microenvironment where malignant cells are located, and the enhancement of the body's own immune system. Thus, based on the two aspects, we concurrently detected T-cell subsets of spleen tissue of mice and employed the immune effect. Immune suppression and HIF-1α gene expression are cytokines associated with inflammation as well as immune suppression and tumor microenvironment, serving as a breakthrough point to discuss the effect of CIK on colon cancer transplantation mice. However, in the tumor hypoxia microenvironment, the mutual effect of HIF-1α and the immune function remain to be elucidated in future studies.

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