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Analysis of the mechanism regulating the killing effect of EBNA1 on EBV-associated B-cell tumors

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ABSTRACT

In order to analysis of the mechanisms regulating EBNA1 killing of EBV associated B cell tumors, preparations were first made for EBV-associated B cells, and the cells were subsequently transformed. The killing effect of ebna1-28 T cells on EBV-positive B cell lymphoid tumor cells was detected using the FACS method. SF rats were also selected to analyze the inhibitory effect of ebna1-28t on transplanted tumors in nude mice with EBV-positive B cell lymphoma. Results showed that compared with the untransfected group, the expression of EBNA1 was higher in the empty plasmid SFG group, the recombinant plasmid rv-ebna1/car group compared with the empty plasmid SFG group, and the expression of EBNA1 was higher in the untransfected group compared with the empty plasmid SFG group, which was statistically significant ($P < 0.05$); As shown in Figure 1, in vitro studies found that, compared to the untransfected group, the empty plasmid SFG group, the recombinant plasmid rv-ebna1/car group showed better killing efficacy on Raji cells, and the recombinant plasmid rv-ebna1/car group showed better killing efficacy on Raji cells compared to the empty plasmid SFG group; The tumor volumes of the rats in group C were larger compared with those in groups A and B, and the tumor volumes of the rats in group A were smaller compared with those in group B. The tumor volumes of the rats in group C were larger compared with those of the rats in the three groups ($P<0.05$). In group C, the cells were more severely invaded, and the nuclei were damaged. In group B, cell invasion in tissues was mild in the nucleus. The infection of cells in the tissues of rats in group A was better compared to groups B and C. In vitro experiments found that inhibition of EBNA1 was able to kill EBV-positive B cell lymphoid tumor cells effectively. Animal experiments found that ebna1-28t was able to shrink the volume as well as tumor weight of transplanted tumors in nude mice with EBV-positive B cell lymphoma and played a better inhibitory role.

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Introduction

EBV, a human lymphocytic double-stranded γ-DNA herpesvirus, has an important role in infectious mononucleosis, X-linked lymphoproliferative disorders, nasal cancer, and other diseases (1,2). It has been noted in studies that EBV plays a key role in the development of B-cell lymphoma. EBV is not apparent among initial infections, and a very small percentage of patients develop the self-limiting disease, which in turn establishes latent infection in most infected individuals (3,4). EBV is not only capable of activating certain immune mechanisms or interfering with the body's normal immune response to promote tumorigenesis, but it also has a large number of latent proteins that not only have a role in transforming cells but some have the function of assisting EBV to evade the body's immunity (5,6). EBNA-1 clinically maintains the EBV genome in an additive state in latently infected cells and thereby regulates the binding and segregation of viral genes to chromosomes in conjunction with intracellular factors, which are essential in maintaining latent EBV infection in vivo (7). Moreover, EBNA-1 contains glycine-alanine repeats that are essential for transformed cells, thereby inhibiting the processing and handling of antigens by antigen-presenting cells. Based on this, the mechanism of its regulatory action on EBV-associated B-cell tumors was analyzed in this paper, using EBNA-1 as a key target.

Materials and Methods

Materials

The Raji tumor cell line was purchased from Fenghui Bio, China.

In vitro experiments

Cell culture and passages

Lymphocyte strain culture conditions were IMDM + 10% FBS. Raji, EBV-transformed velvet monkey leukocytes B95-8, and LCL cells were treated with RPMI1640 + 10% FBS. The cells were grown to a density of 2-3 x10^6/ml, passaged, collected by centrifugation, resuspended in fresh complete medium, adjusted to a density of 0.5x10^6/ml, and inoculated into T25 cell culture flasks.

Detection of EBNA1 expression on the cell surface

The expression of EBNA1 on the cell surface was detected by flow cytometry, and the assay was performed in strict accordance with the kit instructions to avoid errors.

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Preparation of EB virus

The B95-8 cell line was first resuscitated with RPMI1640+10% FBS as the medium. The medium was replaced with a medium containing 2% low serum after the cells had grown to a certain number. Then, the cells were starved for 7 days, followed by centrifugation to collect the cell supernatant. The cells were repeatedly frozen and thawed 3 times using -70°C and 37°C water baths. After that, the cell debris was removed by centrifugation for 15 min and placed in -70°C refrigerator for freezing and storage.

Expression validation of Rv-EBNA1/CAR vector

The Rv-EBNA1/CAR vector was extracted using the endotoxin removal small volume plasmid extraction kit (OMEGA), and the concentration was determined using a UV spectrophotometer for transfection of cells. 293T cells were recovered and cultured in T25 cell culture flasks with DMEM+10% FBS complete medium. When the cell confluence reached 80% or more, the cells were passaged into 6-well culture plates. Then, the cells were transfected with plasmids when the cell confluence reached about 70%. The experiment was divided into 3 groups, untransfected group, empty plasmid SFG group, and recombinant plasmid Rv-EBNA1/CAR group. In the transfection group,1μg of plasmid was diluted to 150μl with Opti-MEM medium. In addition, 2μl of transfection reagent (293fectinTM Reagent) was diluted to 150μl. After incubation for 5min, the diluted transfection reagent was added dropwise to the plasmid solution for 30min at room temperature.

Assaying the efficiency of EBNA1-CAR for lentiviral infection of T cells

The lymphocytes were first cultured and then tested for lymphocyte amplification ploidy. Then, T cells cultured for 5 days were taken, washed with PBS, and $2X10^6$ cells were removed into flow assay tubes, 2 tubes in total. 50μl of PBS solution was left to resuspend the cells after centrifugation. One tube was filled with 5ul PerCP-CD3 antibody, 5ul PE-CD8 antibody, 5ul APC-CD4 antibody, and the other tube without antibody were used as a control tube. Finally, lentiviral infection of T cells was performed, and the efficiency of infection was tested.

Analysis of the killing effect of EBNA1/CAR on EBV-positive B-cell lymphoid tumor cells in vitro

The killing effect of EBNA1-28 T cells on EBV-positive B-cell lymphoid tumor cells was detected using the FACS method.

Animal experiments

Detection of the inhibitory effect of EBNA1-28 T on EBV-positive B-cell lymphoma nude mice transplanted with tumors

(I) EBV-positive Raji cells were digested with trypsin, the cell concentration was adjusted to $5Xl0^7$/ml, and 100μl of tumor cells were injected subcutaneously into the back of nude mice. (II) The plvxT cells and EBNA1-28T cells were prepared according to the previous steps. One batch was prepared every 10 days, for a total of 4 batches. (III) The long and short diameters of subcutaneous tumors in nude mice were measured at l0th d after inoculation of tumor cells. The size of subcutaneous tumors in nude mice was calculated according to the formula ($V=1/2\times$ long diameter $\times$ (short diameter)^2). (IV) Nude mice were divided into three groups according to the size of the tumors, ensuring that the mean and standard deviation of the tumor volumes in each group were similar without statistically significant differences in the tumors between the groups. Group A: EBNA1-28 T-cell group; Group B: plvxT-cell group; Group C: saline group (NS). (V) T cells were injected intratumorally at 10d,20d,30d, and 40d after inoculation of tumor cells, 5x106 cells per mouse, 100μl in total, at 2-3 points in the peritumor and intra-tumor. The long and short diameters of subcutaneous tumors in nude mice were measured with vernier calipers before each injection as well as at d 50 and d 60, to dynamically monitor the growth of subcutaneous tumors in each group.

Statistical methods

SPSS18.0 statistical software was used for analysis. The measurement data were shown as mean±standard deviation(x s), and t-test was used. The count data were expressed as rate [n (%)], and the $\chi 2$ test was applied. F-values were calculated between multiple groups. The difference was considered statistically significant at $P < 0.05$.

Results

Analysis of EBNA1 expression in each group

As shown in Table 1, EBNA1 expression was lower in the empty plasmid SFG group and the recombinant plasmid Rv-EBNA1/CAR group compared with the untransfected group. EBNA1 expression was higher in the untransfected group and lowered in the recombinant plasmid Rv-EBNA1/CAR group compared with the empty plasmid SFG group, and the three groups were statistically significant compared with each other ($P < 0.05$).

Analysis of the killing effect of regulated EBNA1 expression on Raji cells

As shown in Figure 1, in vitro studies revealed that the empty plasmid SFG group and the recombinant plasmid Rv-EBNA1/CAR group were more effective in killing Raji cells compared to the untransfected group. Moreover, the recombinant plasmid Rv-EBNA1/CAR group was more effective in killing Raji cells compared with the empty plasmid SFG group.

Analysis of tumor volume in each group of rats

As shown in Table 2, the tumor volumes of rats in group C were larger compared with those in groups A and

Table 1. Analysis of EBNA1 expression in each group.

Group	EBNA1
Untransfected group	2.94±0.21
Empty plasmid SFG group	2.43±0.31
Recombinant plasmid Rv-EBNA1/CAR group	1.03±0.16
F	5.912
P	0.001

Table 2. Analysis of tumor volume in each group of rats.

Group	Tumor volume (mm^3)
Group A	23.46±2.84
Group B	35.49±1.02
Group C	64.23±3.61
F	8.516
P	0.001

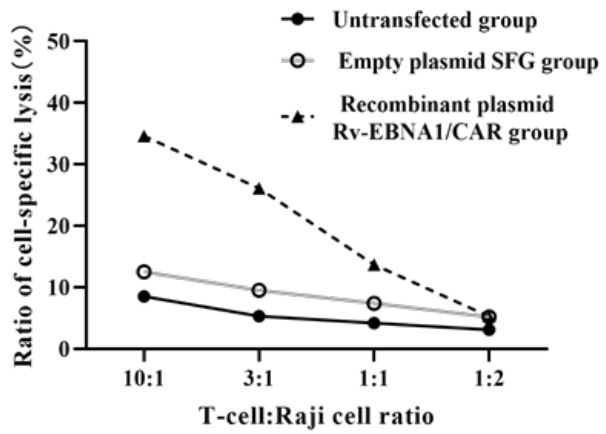

Figure 1. Analysis of the killing effect of regulated EBNA1 expression on Raji cells.

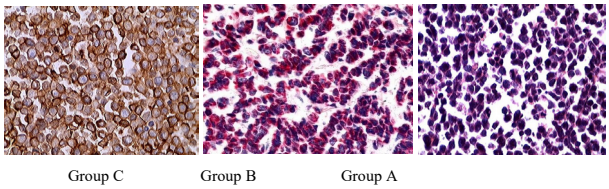

Figure 2. Tissue staining diagram of each group of rats.

B. The tumor volumes of rats in group A were smaller and those in group C were larger compared with those in group B. The tumor volumes of rats in the three groups were statistically different compared with each other ($P < 0.05$).

Tissue staining diagram of each group of rats

As shown in Figure 2, the cell infestation in group C rats was more serious and the nucleus was infiltrated. The cellular infestation in the tissues and nucleus of rats in group B was mild. Compared with groups B and C, the cellular infestation in the tissues of rats in group A was improved.

Discussion

When EBV infection is present in the body, the viral host is able to respond to the virus through intrinsic, humoral and cellular immunity, which in turn releases a series of cytokines and eventually produces specific antibodies and cytotoxic T cells that exert an antiviral effect (8,9). It can also interfere with the body's normal immune response and remain latent in the cells for a long time. In clinical studies, it has been found that the virus can effectively persist in circulation, quiescence, and memory B cells, which occurs because the cells described above are not able to express viral proteins with a normal metabolic mechanism and thus remain in vivo (10,11).

EBV, an important herpes virus, is closely associated with the development of many malignancies. Clinical investigations have revealed that most adults have been infected with EBV and will carry it for life (12,13). Among them, EBNA1, an EBV-encoded nuclear protein, plays an essential role in maintaining latent EBV infection by regulating the binding and segregation of viral genes to chromosomes (14,15). EBNA1 clinically has a unique glycine-alanine repeat sequence that inhibits the processing and handling of itself by antigen-presenting cells (16). Therefore, how the immune response is evaded clinically plays an important role in viral replication. In the present study, EBNA1 was found to be abnormally highly expressed in EBV-associated B-cell tumors, a finding that reaffirms the close association between EBNA1 and EBV. Therefore, it can be tested to effectively determine EBV infection. Moreover, the study in vitro in this paper found that the empty plasmid SFG group and the recombinant plasmid Rv-EBNA1/CAR group were more effective in killing Raji cells compared to the untransfected group, and the recombinant plasmid Rv-EBNA1/CAR group was more active in killing Raji cells compared to the empty plasmid SFG group. This result confirms that modulation of EBNA1 is effective in killing EBV-associated B-cell tumors.

EBV is transmitted mainly through the respiratory tract. After entering the respiratory tract, the envelope protein on the surface of the virus is able to bind to the CD21 molecule on the surface of B cells, which in turn mediates the entry of EBV into B lymphocytes (17,18). In previous reports on EBV infection of epithelial cells, it was found that infected cells were transfected (19). The interaction between the EBV envelope glycoprotein and the cell surface viral receptor is complex in its entry into epithelial cells, requiring the involvement of many glycoproteins. Each glycoprotein has a different function, with some of them mediating the binding of EBV to epithelial cells and others inducing the fusion of the viral envelope with the cell membrane, which in turn mediates EBV infection (20,21). The rats were selected for this study. T cells were injected intratumorally at 10 d, 20 d, 30 d and 40 d after tumor cell inoculation, and each mouse was injected with $5x10^6$ cells for a total of 100u1 in 2-3 points around and within the tumor. The long and short diameters of subcutaneous tumors in nude mice were measured by vernier calipers before each injection and at d50 and d60. The dynamic monitoring of subcutaneous tumor growth in each group revealed that the tumor volume was larger in group C rats compared with groups A and B. This result indicates that modulation of EBNA1 is effective in shrinking EBV-associated B-cell tumors and provides a better intervention effect.

In summary, in vitro experiments showed that EBNA1 inhibition was effective in killing EBV-positive B-cell lymphoma cells. Moreover, the animal experiments showed that EBNA1-28T could reduce the size and weight of EBV-positive B-cell lymphoma transplanted tumors in nude mice, which had a better inhibitory effect.

Acknowledgments
Not applicable.

Interest conflict
The authors declare that they have no conflict of interest.

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