

Full Length Research Paper

The immune-response enveloped-virus treatment for H₂₂ ascitic tumors in mice

Mintao Zhong, Min Huang*, Lei Liu, Xiaoli Wang, Xingyun Li, Anhong Ning and Jing Cao

Department of Medical Microbiology, Dalian Medical University, 9 Western Section, Lvshun South Road, Lvshunkou District, Dalian 116044, China.

Accepted 23 March, 2019

In addition to the standard therapies of chemotherapies and surgical interventions, viral therapy for tumor treatment has provided a new option for cancer patients. Whether the effects are safe, however, remains to be fully evaluated. Accordingly, we explored the immunological mechanisms of viral action against the H₂₂ ascitic tumor, using both the Newcastle disease virus (NDV) and the parainfluenza virus (PIV). We measured IL-2 levels, Natural killer (NK) cell activity, lymphocyte proliferation rates, and the CD4⁺/CD8⁺ cell numbers' ratio. All indicators were superior in the virus-treatment group compared with the control group. Tumor cells infected by either virus induced the expression of the tumor-associated antigen, which stimulated the host immune system.

Key words: Immunologic mechanism, tumor immunity, viral immunotherapy, tumor-associated antigen.

INTRODUCTION

Cancer is a major cause of death in developed countries. Standard therapies include surgery and chemotherapy to remove or shrink the tumor. However, the therapeutic efficacy of these methods is variable, and relapse and death rates remain high. Over the past hundred years, it has been observed in cancer patients that tumors regress after a viral infection. This observation has led to studies examining the ability of different viruses to kill tumor cells (Chou and Roizman, 1994; DeBiasi et al., 2004; Yun et al., 2005). During viral infection, the body produces an effective anti-tumor response through the stimulation of non-specific and specific immunity reactions. These responses can inhibit tumor growth or even kill tumor cells. In recent years, many viruses have been used for cancer therapy, including the reovirus (DeBiasi et al., 2004), herpes simplex virus (Chou and Roizman, 1994), and adenovirus (Yun et al., 2005). These viruses have achieved promising initial results. Because viruses can easily undergo mutation or genetic modification, however, their safety profiles still need to be evaluated (Alemany et al., 2000; Kay et al., 2001; Kirn et al., 2001; Kirn, 2000;

Qian et al., 2002; Somia and Verma, 2000; Vile et al., 2000). An optimum anti-tumor virus would stimulate an antigenic response against the tumor but at the same time show low mutagenic potential. In order to develop optimum viral therapies, we need to clarify the immune mechanisms that explain the anti-tumor effect. In this article, we focused on the use of NDV and PIV to treat H₂₂ ascitic tumors in mice in order to examine the immune response and provide a theoretical basis for viral anti-cancer treatment strategies.

MATERIALS AND METHODS

Experimental animals

Eighty healthy inbred 615-strain mice were selected. The (Spruce, Pine and Fir) SPF-grade mice were three months old and weighed 18 to 20 g. We used 40 male and 40 female mice, obtained from the Experimental Animal Center of Dalian Medical University. This study, approved by Dalian Medical University's Animal Ethical Review Committee, conforms to the guidelines for the use and care of animals that are published by the National Institute of Health.

Carcinoma cell lines

H₂₂ (mouse ascitic liver tumor) cell lines and YAC-1 (mouse lymphoma cells) were obtained from the Department of Microbiology in Dalian Medical University and were grown in RPMI-

*Corresponding author. E-mail: huangminchao@163.com. Tel: +86-411-86110007. Fax: +86-411-86110007.

1640 media (Gibco) with 10% fetal calf serum (China National Medicines Corporation, Ltd). The cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂.

Virus

The Newcastle disease virus (NDV) B1 strain and the parainfluenza virus (PIV) type α were obtained from the Institute of Virology at the Chinese Academy of Sciences, and cultured in chicken embryos by conventional methods (Paldurai et al., 2009). Virus titers were determined by the conventional hemagglutination test. Both of the virus titers were at least 1:1280, and were stored at -70°C until used.

Establishment of H₂₂ tumor-bearing mice

The H₂₂ ascitic tumor cell number and viability were determined, and the cell concentration was adjusted to 1×10^7 cells per ml with complete RPMI 1640 medium. The inbred 615-strain mice were inoculated with 1 ml tumor cells by intraperitoneal injection.

Animal groups and immunotherapy

We established H₂₂ ascitic tumor cells in mice (n=80) and divided the mice into four groups of 20 each: 1) normal saline (NS) control, 2) cyclophosphamide (CTX) group, 3) NDV-treatment group, and 4) PIV-treatment group. The four groups were injected intraperitoneally with NS, CTX, NDV, or PIV on days 1, 3, 5, and 7 after the tumors were established. At 12 days after inoculation, 10 mice in each group were taken for collection of blood, spleens, tumors, and ascites. Tissue was collected under anesthesia with ether. Blood was collected for serum by retro-orbital sampling. The enterocoelic cavities were opened, and the spleens were removed to sterile Hanks solution. The tumors were removed and placed in 10% formalin. The ascites were collected and transferred into sterile tubes.

Detection of IL-2 levels

IL-2 levels were measured in venous serum samples according to the manufacturer's instructions (China National Medicines Corporation, Ltd)(Corsi et al., 2004).

Preparation of mouse spleen cells

The spleens were minced in Hanks solution and passed over a 200-mesh strainer to remove erythrocytes. The remaining red cells were lysed, and the cell suspension was added to 5 ml Hanks solution and centrifuged at 12000 rpm for 10 min. The supernatant was discarded, and the cell pellet was resuspended in 2-3 ml RPMI 1640 and centrifuged at 1500 rpm for 5 min. After washing twice in RPMI 1640, the cells were again resuspended in RPMI 1640 with 10% fetal bovine serum (FBS). The cell survival rate was >95%, as determined by trypan blue staining. The cell concentration was adjusted to a final concentration of 5×10^6 cells per ml.

Preparation of target cells

The YAC-1 lymphoma cells were cultured in RPMI-1640 medium with 10% (100 ml/L) fetal bovine serum (FBS). The cell medium was changed and cultured for another 22-24 h before the experiment; the cells were then washed for 2-3 times in complete

media and centrifuged at 1500 rpm for 5 min. The cells were washed once with 5 mL Hanks solution, centrifuged at 1500 rpm for 5 min, and resuspended in RPMI media after adjusting the concentration to 2.5×10^5 cells per ml.

Determination of NK cell activity, lymphocyte transformation, and CD4⁺/CD8⁺ ratio

NK cell activity and lymphocyte transformation were measured as described previously (Kano et al., 2007; Mosmann, 1983). Fluorescently conjugated antibodies against CD4⁺ or CD8⁺ (Serotec) were added to the spleen cells (5×10^6 / ml), and the CD4⁺/CD8⁺ ratio was determined by flow cytometry.

The tumors were embedded in paraffin, sectioned at 5 μ m, stained with hematoxylin and eosin (HE), and examined under light microscopy.

The ascites were centrifuged, and the pellet was fixed in 2.5% glutaraldehyde for 2 h at 4°C. The ascites were further fixed in 1% osmium tetroxide, dehydrated by graded alcohol, and embedded with propylene oxide and epoxy resin. Ultrathin sections (50 \times 70 nm) were double-stained with acetate uranyl and lead citrate. The sections were viewed under transmission electron microscopy.

Other indices

The growth rates and survival times of tumor-bearing mice were determined. From these values, we calculated the viability of tumor-bearing mice that survived for a minimum of 60 days.

Statistical analyses

The data were analyzed by ANOVA using SPSS software. Results are reported as mean \pm standard deviation, and p < 0.05 was set as statistical significance.

RESULTS

Survival

The tumor visibly increased in size, and survival time decreased in the H₂₂ tumor-bearing mice. We observed peritoneal swelling in the NS-control group by the seventh day after inoculation, and mortality was 100%, with an average survival time of 16.3 days (Figure 1 and Table 1). In the CTX group, mortality was 100%, with an average survival time of 20.4 days (4.1 days longer than the control group; p < 0.05). In the (pelvic inflammatory disease) PIV-treatment group, mortality was 50%, with an average survival time of 33.3 days (17.0 days longer than the control group; p < 0.01). In the (Newcastle Disease Virus) NDV-treatment group, in contrast, mortality was only 60%, with an average survival time of 27.1 days (10.8 days longer than the control group; p < 0.01).

Serum IL-2 levels

As shown in Figure 2 and Table 2, IL-2 levels in the PIV- and the NDV-treatment groups were also significantly

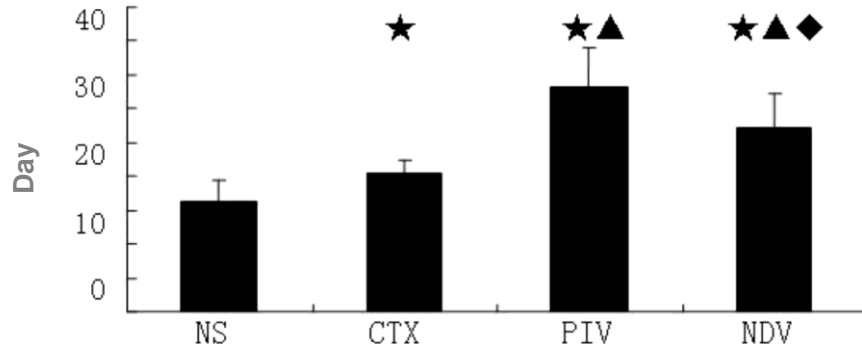


Figure 1. Comparison of survival times of H₂₂ tumor-bearing mice among the 4 treatment groups. ★ p<0.05 compared with the NS group; ▲ p<0.05 compared with the CTX group; ◆ p<0.05 compared with the PIV group.

Table 1. Comparison of survival times of H₂₂ tumor-bearing mice among the 4 treatment groups.

Group	Death/total	Average survival time	Survival rate (%)
NS group	10/10	16.3±3.1	0
CTX group	10/10	20.4±1.9★	0
PIV group	5/10	33.3±5.7★▲	50
NDV group	6/10	27.1±5.1★▲◆	40

★ p<0.05 compared with the NS group; ▲ p<0.05 compared with the CTX group; and ◆ p<0.05 compared with the PIV group.

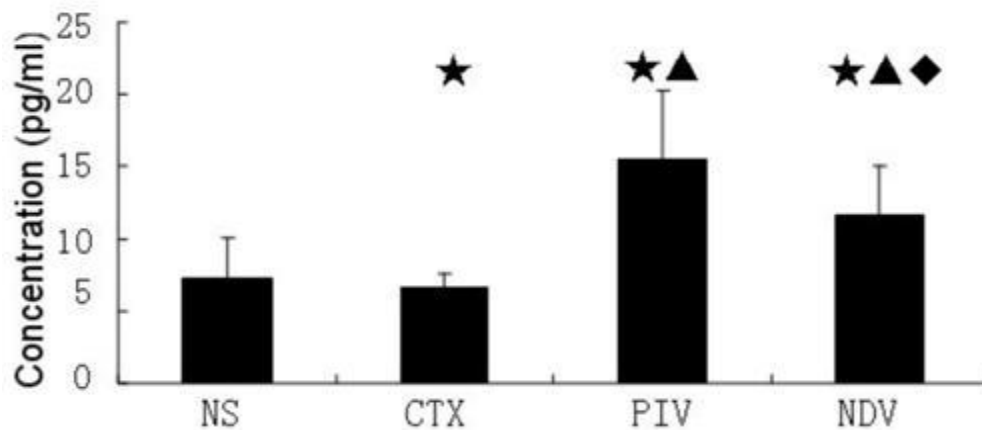


Figure 2. Comparison of serum IL-2 levels on H₂₂ tumor-bearing mice among 4 groups. ★ p<0.05 compared with the NS group; ▲ p<0.05 compared with the CTX group; and ◆ p<0.05 compared with the PIV group.

Table 2. Comparison of serum IL-2 levels on H₂₂ tumor-bearing mice among 4 groups.

Group	Animal	IL-2
NS group	10	7.25±2.83
CTX group	10	6.58±1.05★
PIV group	10	15.58±4.73★▲
NDV group	10	11.65±3.41★▲◆

★ p<0.05 compared with the NS group; ▲ p<0.05 compared with the CTX group; and ◆ p<0.05 compared with the PIV group.

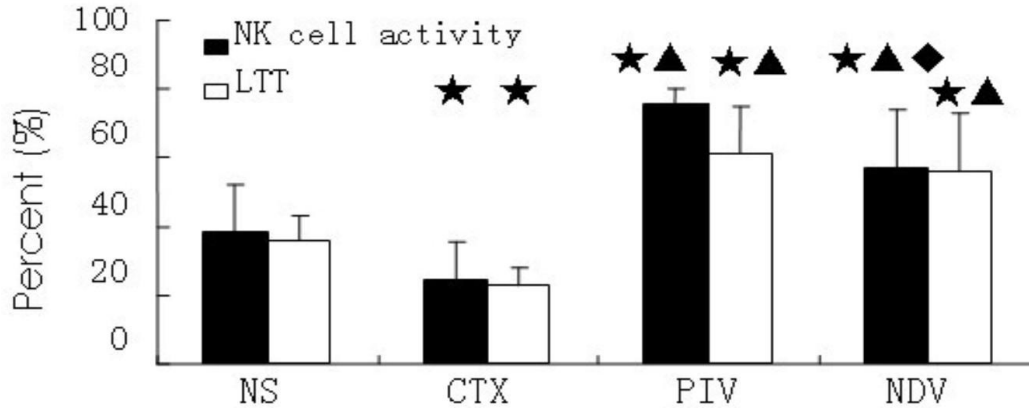


Figure 3. NK cell activity and lymphocyte transformation rates on H₂₂ tumor-bearing mice among the 4 groups. ★ p<0.05 compared with the NS group; ▲ p<0.05 compared with the CTX group; and ◆ p<0.05.

Table 3. NK cell activity and lymphocyte transformation rates on H₂₂ tumor-bearing mice among the 4 groups.

Group	Animal	NK cell activity	lymphocyte transformation rates
NS group	10	38.61±13.54	36.03±7.00
CTX group	10	24.61±10.99★	22.64±5.50★
PIV group	10	76.02±4.2★▲	61.09±13.96★▲
NDV group	10	57.06±16.76★▲◆	55.92±17.29★▲

★p<0.05 compared with the NS group; ▲p<0.05 compared with the CTX group; and ◆p<0.05 compared with the PIV group.

elevated over the NS and CTX groups (p<0.01 for all).

Lymphocyte transformation rate, NK cell activity, and CD4⁺/CD8⁺ ratios

As can be seen from Figure 3 and Table 3, NK-cell activity in the PIV- and NDV-treatment groups were significantly different from the NS and CTX groups (p<0.05 for all). NK-cell activity in the PIV-treatment group was higher than that in the NDV-treatment group (p<0.05). NK-cell activity in the CTX group was significantly lower than that in the NS-control group (p<0.05). Lymphocyte transformation rates in the PIV-treatment group and the NDV-treatment group were significantly higher than those in the CTX group and the control group (significant difference with p<0.05), but rates between the PIV group and the NDV group were not significantly different. The lymphocyte transformation rate was significantly lower in the CTX group than in the NS group (p<0.05). As shown in Figure 4 and Table 4, CD4⁺/CD8⁺ ratios in the PIV- and the NDV-treatment groups were both significantly elevated over the NS and CTX groups (p<0.05 for all).

Tumor cell morphology

1. Light microscopy evaluation of the tumor cells,

showing irregular, large nuclei, stained deeply and with more pathological mitosis and higher heteromorphism (Figure 5a).

2. Tumor cells with viral infection by light microscopy: the cell morphology and size are the similar, with large areas of cell necrosis (Figure 5b).

3. Observation of tumor cells with viral infection by electron microscopy: The tumor cells consisted of irregular, large nuclei, a large number of normal nuclear chromatin, and rare membrane protrusions (Figure 6a).

4. Observation of tumor cells with viral infection by electron microscopy: the endoplasmic reticulum has a high degree of expansion and comes in different sizes and shapes, with very irregular bubbles; mitochondrion begins swelling and degenerating; more protrusions with sticking a small amount of virus particles and budding body of virus particles are in the membrane (Figure 6b).

DISCUSSION

Each year, nearly 10 million new cases of malignant tumors appear all over the world; moreover, 7 million die of malignant tumors (Ebina, 2005). Surgery, radiotherapy, chemotherapy, and other methods induce substantial damage to the patient, particularly impacting the immune system. This damage to the immune system often leads to tumor-cell escape from immune surveillance, resulting

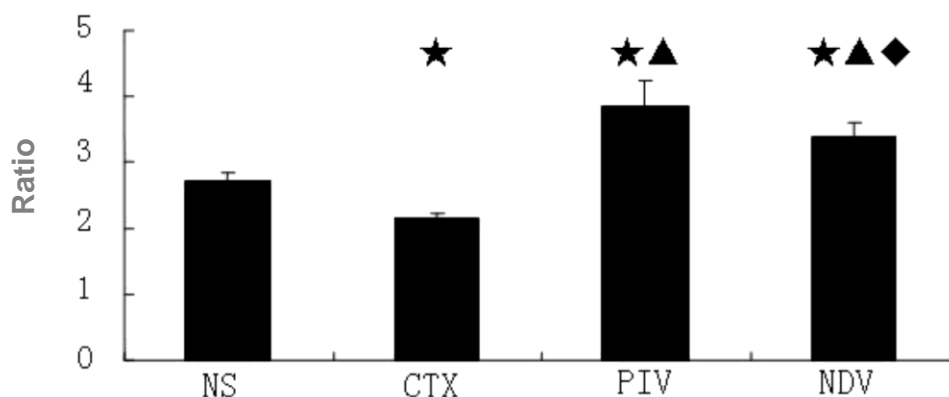


Figure 4. Comparison of CD4⁺/CD8⁺ ratios on H₂₂ tumor-bearing mice among 4 groups. ★ p<0.05 compared with the NS group; ▲ p<0.05 compared with the CTX group; and ◆ p<0.05 compared with the PIV group.

Table 4 Comparison of CD4⁺/CD8⁺ ratios on H₂₂ tumor-bearing mice among 4 groups.

Group	animal	CD4 ⁺ /CD8 ⁺ (%)
NS group	10	2.72±0.13
CTX group	10	2.15±0.07★
PIV group	10	3.85±0.38★▲
NDV group	10	3.39±0.21★▲◆

★p<0.05 compared with the NS group; ▲p<0.05 compared with the CTX group; and ◆p<0.05 compared with the PIV group.

in tumor spread or recurrence. Thus, it is very important to both kill cancer cells and enhance immune function. This study is the first to find that PIV and NDV treatment significantly prolongs survival in tumor-bearing mice.

NK cells play an important role in the non-specific immunity of the tumor. When NK cells kill sensitive cells without being restricted by MHC, the consequence is directly cytotoxic and results in the secretion of lymphokines (McFarlin et al., 2007). Lymphocytes could change into lymphogonia, differentiating and proliferating by concanavalin (ConA) stimulation. Once this occurs, the cells can release a variety of factors that improve overall anti-tumor levels (Zhang et al., 2010). While CD8⁺ T cells are the major component against tumor cells, CD4⁺ T cells play an important adjuvant role in tumor immunity. Therefore, the CD4⁺/CD8⁺ ratio directly reflects the overall cellular immunity function (Wang et al., 2004). IL-2 secreted by T cells is a primary component of cellular immune function (McFarlin et al., 2007).

Our experimental results show that NK cell activity and lymphocyte transformation rate, the CD4⁺/CD8⁺ ratio, and the IL-2 levels of PIV- and NDV-treatment groups were all higher than those in the NS group (p<0.05 for all). Furthermore, these values were also increased over those in the CTX group (p<0.05 for all). All of these

factors indicate an enhanced immune function, consistent with the anti-tumor effect of the virus through enhancing the immune function. The effect of the PIV-treatment group was superior to the effect of the NDV-treatment group.

Cyclophosphamide can inhibit immune function, so its anti-tumor effect is not achieved through positively modifying the immune system. PIV and NDV are enveloped viruses, which means that proliferation in the host cell is a virus-budding process. In this study, by observing the morphology of tumor cells, we found that within H₂₂ tumor cells with viral infection were large areas of necrosis, and viral particles and virus-budding bodies appeared on the cell membranes. Tumor cells without viral infection display no such phenotype, illustrating that the anti-tumor effect by enveloped viruses induces an enhanced antigenic response of the tumor cell. The immune mechanism involves the rapid adsorption of live enveloped viruses to infect tumor cells when the viruses enter the body. Viral biological synthesis in tumor cells induced enhanced antigen and a strong tumor-associated antigen (TAA), thereby stimulating the body's immune system to improve immune indicators for enhancing the immune response, and killing the tumor cells. This indicates that the virus has a direct anti-tumor effect while enhancing the immune function. Further research will

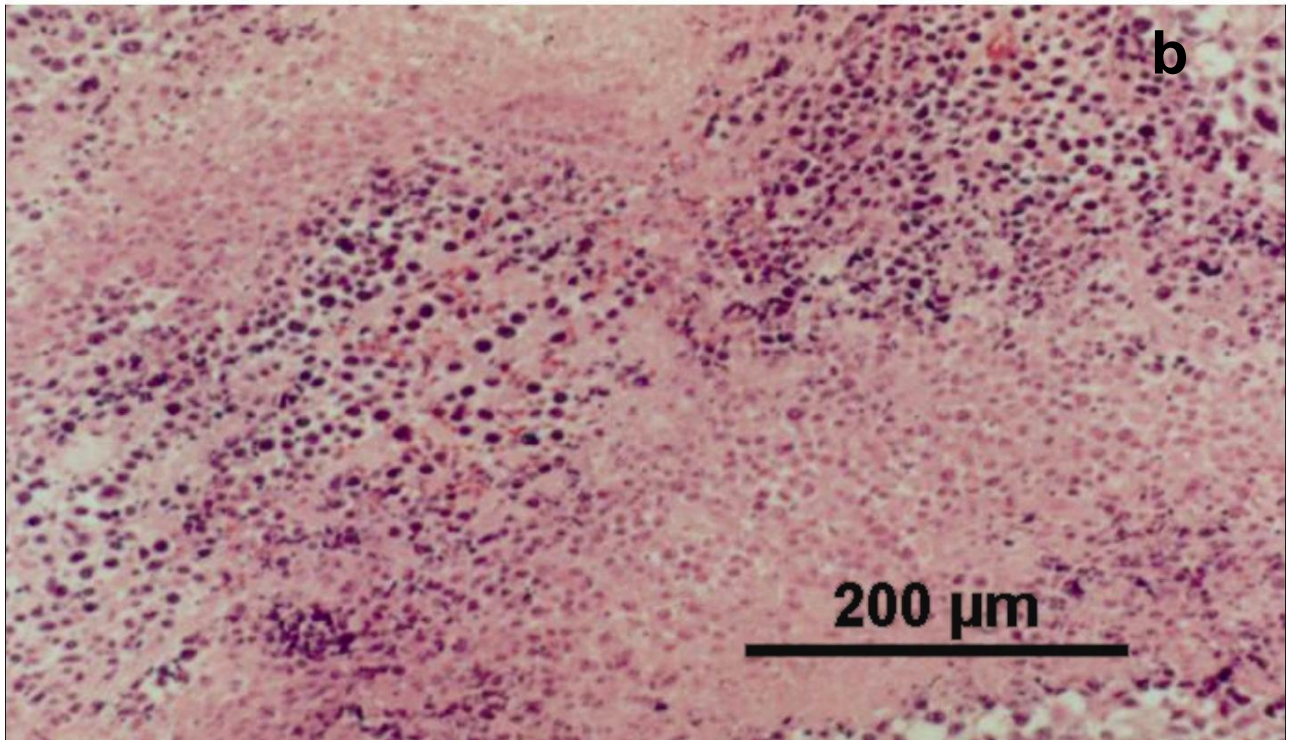
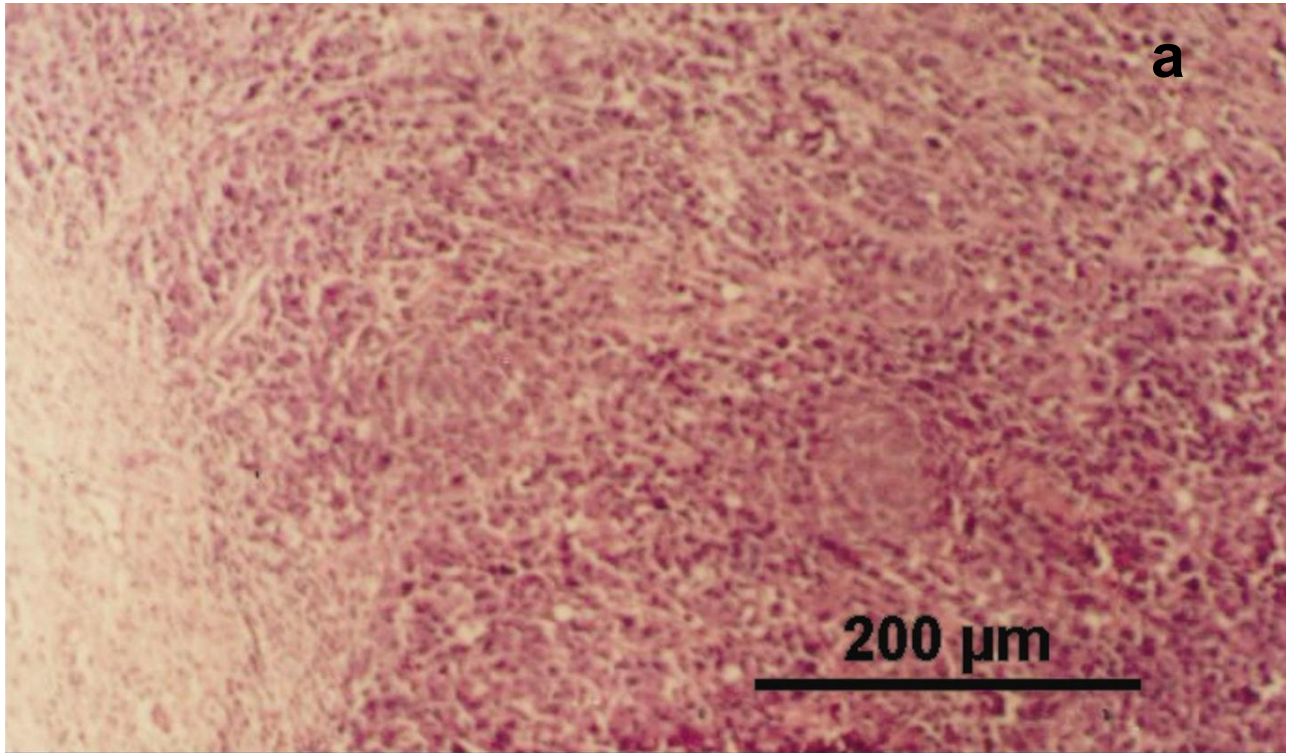


Figure 5. Top) Tumor cells without any kind of viral infection (×400 magnification). Bottom) Tumor cells infected with a virus demonstrating cell necrosis (×100 magnification).

focus on the structure and biochemical character of TAA. In conclusion, our study provides a new theoretical basis

for further study of the mechanism in viral immunotherapy tumors.

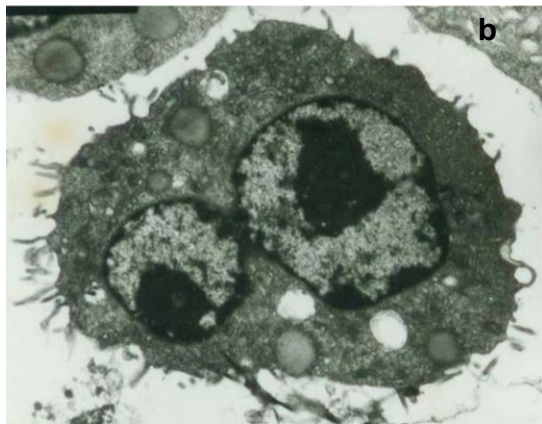
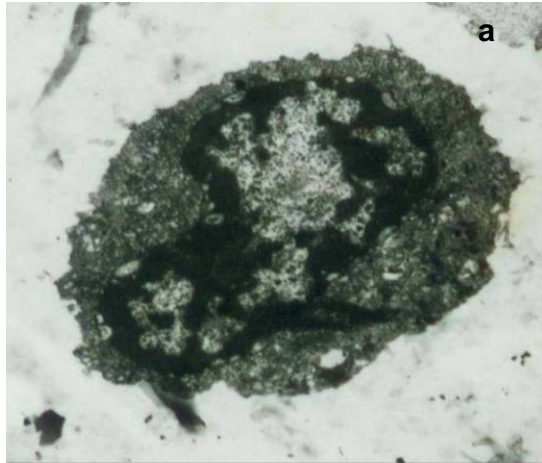


Figure 6. Top) Morphology of tumor cell without viral infection (x5000 magnification). Bottom) Morphology of tumor cell with viral infection (x5000 magnification).

REFERENCES

- Alemayn R, Balague C, Curiel DT (2000). Replicative adenoviruses for cancer therapy. *Nat Biotechnol.*, 18: 723-727.
- Chou J, Roizman B (1994). Herpes simplex virus 1 gamma (1)34.5 gene function, which blocks the host response to infection, maps in the homologous domain of the genes expressed during growth arrest and DNA damage. *Proc. Natl. Acad. Sci. USA*, 91: 5247-5251.
- Corsi MM, Ruscica M, Passoni D, Scarmozzino MG, Gulletta E (2004). High Th1-type cytokine serum levels in patients with infectious mononucleosis. *Acta Virol.*, 48: 263-266.
- DeBiasi RL, Robinson BA, Sherry B, Bouchard R, Brown RD, Rizeq M, Long C, Tyler KL (2004). Caspase inhibition protects against reovirus-induced myocardial injury *in vitro* and *in vivo*. *J. Virol.*, 78: 11040-11050.
- Ebina T (2005). Antitumor effects of intratumoral injection of Basidiomycetes preparations. *Gan To Kagaku Ryoho*, 32: 1654-1656.
- Kano Y, Hirahara K, Mitsuyama Y, Takahashi R, Shiohara T (2007). Utility of the lymphocyte transformation test in the diagnosis of drug sensitivity: Dependence on its timing and the type of drug eruption. *Allergy*, 62: 1439-1444.
- Kay MA, Glorioso JC, Naldini L (2001). Viral vectors for gene therapy: the art of turning infectious agents into vehicles of therapeutics. *Nat. Med.*, 7: 33-40.
- Kim D, Martuza RL, Zwiebel J (2001). Replication-selective virotherapy for cancer: Biological principles, risk management and future directions. *Nat. Med.*, 7: 781-787.
- Kim DH (2000). Replication-selective microbiological agents: fighting cancer with targeted germ warfare. *J. Clin. Invest.*, 105: 837-839.
- McFarlin BK, Flynn MG, Hampton T (2007). Carbohydrate consumption during cycling increases *in vitro* NK cell responses to IL-2 and IFN-gamma. *Brain Behav. Immun.*, 21: 202-208.
- Mosmann T (1983). Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J. Immunol Methods*, 65: 55-63.
- Paldurai A, Subbiah M, Kumar S, Collins PL, Samal SK (2009). Complete genome sequences of avian paramyxovirus type 8 strains goose/Delaware/1053/76 and pintail/Wakuya/20/78. *Virus Res.*, 142: 144-153.
- Qian Q, Sham J, Che X, Xu J, Xue H, Cui Z, Zhu B, Wu M (2002). Gene-viral vectors: a promising way to target tumor cells and express anticancer genes simultaneously. *Chin. Med. J. (Engl)*, 115: 1213-1217.
- Somia N, Verma IM (2000). Gene therapy: Trials and tribulations. *Nat Rev. Genet.*, 1: 91-99.
- Vile RG, Russell SJ, Lemoine NR (2000). Cancer gene therapy: hard lessons and new courses. *Gene Ther.*, 7: 2-8.
- Wang S, Zheng Z, Weng Y, Yu Y, Zhang D, Fan W, Dai R, Hu Z (2004). Angiogenesis and anti-angiogenesis activity of Chinese medicinal herbal extracts. *Life sciences*, 74: 2467-2478.
- Yun CO, Kim E, Koo T, Kim H, Lee YS, Kim JH (2005). ADP-overexpressing adenovirus elicits enhanced cytopathic effect by induction of apoptosis. *Cancer Gene Ther.*, 12: 61-71.
- Zhang Y, Yang H, Xiao B, Lu T (2010). Effect of RelB-silenced BMDC pulsed with Talpha146~162 on immunoreaction of T cells primed with TACHR. *Zhong Nan Da Xue Xue Bao Yi Xue Ban*, 35: 38-44.