RESEARCH COMMUNICATION

Anti-HER-2×anti-CD3 Bi-specific Antibodies Inhibit Growth of HCT-116 Colorectal Carcinoma Cells in Vitro and in Vivo

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Abstract

Objective: This study is conducted to evaluate the effects of anti-HER-2×anti-CD3 bi-specific antibodies(BsAb) on HER-2/neuover-expressing human colorectal carcinoma cells. Methods: Growth was assessed by MTT assays after exposure of HCT-116 cells to Herceptin, anti-CD3 and BsAb antibodies. Immunocytochemistry was applied to test the HER-2 level of HCT-116. In a nude mouse model, HER-2×CD3 BsAb was combined with effector cells (peripheral blood lymph cells from normal human being) for observations on in Vivo growth of tumors. Results: Compared with the control group, using effector cells combined with anti-CD3 McAb, Herceptin or HER2×CD3 BsAb, tumor cell growth in vitro and in vivo was significantly inhibited (P<0.05), most remarkably in the HER2×CD3 BsAb case. The growth of xenografts with HER2×CD3 BsAb combined with effector cells was also significantly inhibited when compared with the anti-CD3 McAb or Herceptin groups (P<0.05). Conclusion: HER-2/neu might be a useful target for immunotherapy in colorectal carcinoma, anti-HER2×anti-CD3 BsAb exerting clear anti-tumor effects.

Keywords: Anti-HER-2×anti-CD3 bispecific antibody - HER-2/neu - human colorectal carcinoma - nude mice

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Introduction

Colorectal carcinoma (CRC) is one of the most common malignancies in the world and the second most frequent cause of cancer-related death in the United States (Jemal et al., 2009). At present, traditional and combined therapeutic approaches (surgery, chemotherapy and radiation) for curing colorectal carcinoma does not show encouraging outcome. The five-year survival in the patients with stage III or IV disease is from 25 to 65 percent and from 5 to 7 percent, respectively. Recent years, anti-tumor T-cells and anti-tumor antigenic immunotherapy has become hot topics. Specifically, the role of proto-oncogene HER-2/neu in the diagnosis of cancers and the immunotherapy is progressing rapidly (Argyriou and Kalofonos 2009, Liu et al., 2012). It has been verified that Anti-HER-2 antibody has significant inhibition effect on the HER2-over-expression tumor (Morishita et al., 2010; Albarello et al., 2011; Schroeder et al., 2011; Liu et al., 2012; Somlo et al., 2012). As the time goes by, it is more and more concerned that the use of bispecific antibodies (BsAb) may be possible therapeutic agents for cancer treatment. Because of the important role in the process of antigen recognizing and activating, Anti-CD3 McAb is used as a member to construct double-functional antibody most frequently (Chatenoud, 2003). This article aims at evaluating the effect of HER-2×CD3 BsAb combined with peripheral blood lymph cells of normal human being on the growth of HER-2 expressing colorectal carcinoma. Our data lay a solid experimental foundation for further research on HER-2×CD3 BsAb anti-tumor mechanism in vivo and its clinical application.

Materials and Methods

Experimental material

Tumor cell line: human colorectal carcinoma cell line HCT-116 (given by the central lab of the Second Hospital of Jilin University). Animals: BALB/c-nu/nu nude mice (purchased from Animal Section, Tumor Research Institute of China Science Academy), half male and half female, at the age of 6 weeks, with average weight of 23.0g, raised in SPF condition. Effector cells: normal human peripheral blood lymph cells (from healthy volunteers). Major pharmaceutics: HER-2×CD3 BsAb (provided by Changchun Jinsai Pharmaceutical Co., Ltd); Herceptin (Roche Company, Switzerland); mice anti-human CD3 McAb (provided by Beijing Zhongshan Biological Technology Co., Ltd); mice anti-human HER-2 monoclonal antibody (provided by Immunity Research Office of Beijing Tumor Prevention Research Institute).

Cell culture

HCT-116 cells were cultivated with IMDM medium (provided by Gibco-BRL Company from USA), 37 °C, fully dissolved in 5% blood serum of fetal ox (provided

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by Hyclone Company from USA).

MTT colorimetric method

HCT-116 cells was digested and put into IMDM with 2% blood serum from newly born ox to make suspension liquid of cells with concentration of 2×10^5/ml; then lymph cells were separated and resuspended with IMDM medium to reach a concentration of 8×10^6/ml, 4×10^6/ml and 1×10^6/ml. The HER-2×CD3 BsAb, Herceptin and anti-CD3 McAb were diluted at the concentration of 400 ng/ml, 200 ng/ml and 40 ng/ml respectively. The 96-well plate was filled with 50 μl target cells, lymph cells and antibody respectively, and the final volume was 200 μl after being compensated with 2% IMDM. The plates were put into CO_2 hatching box and cultivated for 14 hours. The rate of effect/target was 40:1, 20:1 and 5:1 respectively. 10 μl/well of 5 mg/ml MTT was added 6 hours before the termination of the reaction, and cells were cultivated for additional 6 hours. The upper clear liquid was removed carefully by suction, 200 μl of DMSO was added into each hole, making it fully dissolved, OD490 was measured. Killing rate (%) = (OD490 in reference wel)/OD490 in experimental well)/OD490 in reference well ×100%

Immunocytochemistry

To exam HER-2-expressing level in human colorectal carcinoma cell line HCT-116, the slides were treated with 0.01% poly-lysine, and cells were digested with 0.25% pancreatin with 0.02% EDTA, and washed three times by PBS. The cell concentration was adjusted to 2×10^5/ml. The cells were then dropped on the slide and dried with cold air; fixed for 1 minute with fixative solution (methanol: acetone 1:1) at 4 °C, then washed twice with PBS (pH 7.4). 30 μl of peroxisome interceptive solution was added to each slide and incubated for 10min at room temperature (RT) and then washed three times with PBS. 30μl of non-immune animal blood serum (horse blood serum) was added and incubated for 5min at RT and then washed again with PBS; 30μl of mice anti-p185 monoclonal antibody was added and incubated for 60 min at RT, and then washed 3 times with PBS; 30 μl biotinylated sheep anti-mice IgG was added and incubated 10 minutes at RT, and washed 3 times with PBS; 30 μl streptoavidin-peroxisome solution was added and incubated for 10 min at RT, and then washed with PBS for 3 times. 100 μl newly compounded DBA-H2O2 solution was added, and washed with tap water to terminate the reaction. We sealed it with neutral resin, and observed the results under the microscope.

Animal experiments

Eight mice were inoculated with HCT-116 under the skin of the right back (1×10^7 cell/0.2 ml PBS per mouse), and passed it to the next generation after the tumor had formed. We choose a mouse whose tumor grew faster than others, and removed the tumor under sterile condition and cut it into a tumor block reaching a diameter of 2 mm. 48 nude mice were divided into 4 groups randomly, 12 mice per each group. After one week of plantation, we injected phosphate buffer, anti-CD3 monoclonal antibody (anti-CD3 McAb)+effector cells (CD3 McAb group), anti HER-2 monoclonal antibody Herceptin+effector cells (Herceptin group) and HER2×CD3 bi-specific antibody+effector cells (BsAb group), respectively from the abdominal cavity, twice a week for altogether 3 weeks. The animals were killed 8 weeks after the plantation. The tumor volume and inhibition rate were measured every week. The calculation equation was: tumor volume (mm^3)= $\pi/6 \times$ (smaller diameter)$^2 \times$ larger diameter.

Statistical treatment

The average of the volume (mean ± sd) of the tumors on the mice was indicated with standard deviation s, SPSS software was used to conduct analysis of variance of single factors.

Results

Cytotoxicity

The cytotoxicity of the cells intervened by HER-2×CD3 BsAb increased significantly. Comparing with the negative group, anti-CD3 group and Herceptin group, the difference was significant, $P<0.05$. The best effect-target ratio was 20:1; the optimal concentration of HER-2×CD3 BsAb was around 50 ng/ml; the killer activity was (92.6±3.7)% (Figure 1).

HER-2/neu expressing level of HCT-116 cells

The expressing level of HER-2/neu in HCT-116 cells was examed using immunocytochemical approach. The cell membrane of HCT-116 was stained with dark brown indicating expression of HER-2/neu (Figure 2).

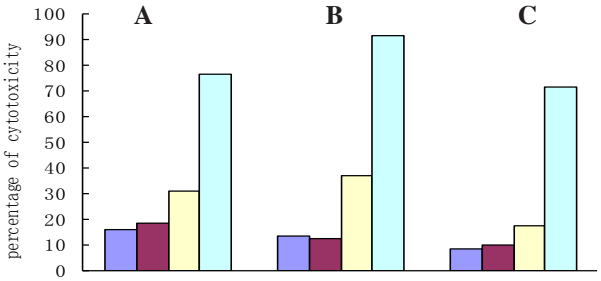

Figure 1. Cytotoxicity of the Cells Intervened by Antibody in Each Group. A, B and C represents ratio of Effect/Target (E:T) as 40:1, 20:1 and 5:1, respectively. a: negative reference group; b: Herceptin group (50 ng/ml); c: anti-CD3 McAb group (50 ng/ml). d:HER2×CD3 BsAb (50 ng/ml) group

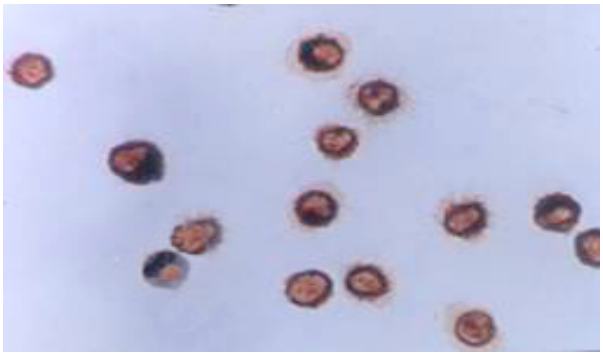

Figure 2. HER-2/neu Expression in Human Colorectal Carcinoma Cell Line HCT-116 [CONTROL IS MISSING]

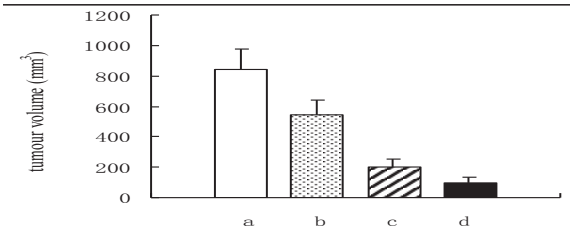

Figure 3. Effect of HER-2 ×CD3 BsAb on the Growth of Human Colorectal Carcinoma HCT-116 Transplanted Tumor in Nude Mice. a: PBS; b: Anti-CD3 McAb+effector cells; c: Herceptin+ effector cells; d: HER2/CD3 BsAb+effector cells

Establishment of nude tumor mice models using HCT-116 cells

We inoculated the HCT-116 cells in 8 nude mice, resulting in a tumor forming rate of 37.5% (3/8). Compared among the individuals, the difference was significant among the individuals in terms of the volume of the tumor.

Effect of HER-2×CD3 BsAb in the nude tumor mice model

The tumor size of nude tumor mice after treating with Abs was carefully analysized. The average tumor volumes of BsAb, Herceptin, Anti-CD3 and the reference group (PBS) were (0.10±0.02) cm^3, (0.22±0.05) cm^3, (0.56±0.03) cm^3 and (0.88±0.03) cm^3, respectively. The tumor inhibition rates were 79.1%, 58.0%, 47.2% and 0%, respectively. Compared to the reference group, the growth of transplanted tumors from Anti-CD3 McAb+effector cells, Herceptin+effector cells and HER-2×CD3BsAb+effector cells group was restrained ($P<0.05$). HER-2×CD3 BsAb+effector cell group turn out to be the outstanding candidate for further studies. Compared with Anti-CD3 McAb+effector cells and Herceptin+effector cells group, HER-2×CD3 BsAb+effector cell had a clear deference in restricting the growth of tumors ($P<0.05$) (Figure3).

Discussion

Cancer gene HER-2/neu (also called c-erbB-2, HER-2) encodes a transmembrane proteoglycan with a molecular weight of 185 kD, acting as epidermal growth factor receptor 2 (HER-2, erb-B2). HER-2/neu is a tumor associated antigen, and overepressed in many types of human cancers, which is closely associated to the invasion and growth of tumors. In 1998, the completely human McAb medicine Herceptin against solid tumors was put on sale, which was used to cure late mammary cancers (Baselga et al., 1999; Kiewe et al., 2006). Due to the large molecular weight of McAb, it's ability to penetrate tumor tissues is very limited, with negative impact on it's anti-cancer function.

In recent years, with the maturity of antibody engineering technology, the research for constructing in vitro small molecular antibodies has progressed rapidly, such as the construction of gene engineering BsAb. Anti-tumor BsAb is one of the strategies of combining T-cells with immune treatment based on antibodies, which has combined the above two advantages and overcome the limitations of each method to the large extent.

CD3 is a kind of cluster of differentiation on the T-lymph cells, which constitutes TCR-CD3 compound together with T-cell antigen receptor (TCR) and plays an important role during the process when T-cell recognizes the antigen and activates (Huang et al., 2012). The small-dose Anti-CD3 McAb can stimulate multi-clonal T-cells, induce the secretion of many kinds of cell factors, and produce the effector-cells with cytotoxicity and anti-tumor action. So in many cases, we selected CD3 molecular to construct double-functional antibodies to restrain tumors (Nishio et al., 2010).

In the above experiment, we have proved that HER2×CD3 BsAb can effectively connect HCT-116 with the effector cells. In order to further evaluate the biological reaction of the effector cells after they were connected to other effector cells, we adopted MTT assay to test the toxin activity of the cells. The results indicated that the cell toxin activity of HER2×CD3 BsAb group had been enhanced significantly, and the best effect/target ratio was 20:1, the best HER2×CD3 BsAb concentration was 50 ng/ml, and the killer activity was as high as (92.6±3.7)%. The difference is significant comparing with the negative reference group, Anti-CD3 antibody group and Herceptin group. This antibody can restrain the growth of HER-2/neu-expressing human colorectal carcinoma cells significantly in vivo and in vitro.

In this research, firstly we established models of tumor-carrying nude mice with HER-2-over expressing human colorectal carcinoma cell line HCT-116. The survival rate of the primary implantation in this experiment was 37.5% (3/8), and the difference in the tumor volume among the individuals was very large, which was in conformity with the reports of corresponding research (Xia et al., 2002). So we chose to conduct the in vivo experimental research on HER-2×CD3 BsAb after the tumors have been formed and passed to the next generation.

During the research on the Effect of HER-2×CD3 BsAb on growth of human colorectal carcinoma HCT-116 transplanted tumor in nude mice, we observed that HER-2×CD3 BsAb had more obvious inhibition action on the growth of HCT-116 xenogeneic transplanted tumors compared with the reference groups such as PBS, Herceptin and Anti-CD3 McAb. Notes: comparing with McAb, HER-2×CD3 BsAb has a more effective action in restraining cancers in vivo.

According to the report of Bang et al. (2010) and Cuello et al. (2001), HER-2/neu antibody played biological function through multiple organisms, including cutting the transmitting ways of cell signals, lowering HER-2/neu receptors, actuating apoptosis signals of tumor cells and collecting the immune cells to fight the killing tumors and so on. The above results laid a primary experimental foundation for further evaluation on the complicated organism of HER-2×CD3 BsAb for restraining the growth of tumors in vivo and for the clinical application.

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