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Research Article

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A New Detection Method for the Suppressing Effect of Enzyme Activity by Aminopeptidase N (APN/CD13) Inhibitor

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ABSTRACT

Aminopeptidase N, also known as CD13, is a transmembrance protease with many functions. CD13 is involved in inflammatory diseases and cancers. A convenient and reliable laboratory test method for detecting the suppressing effects of enzyme activity would be useful for the study of CD13 inhibitors. Traditionally, porcine CD13 (pCD13) is considered as the enzyme source. However, this method has significant practical disadvantages. The pCD13 is not human source, and the accuracy and reliability of experimental results are greatly reduced. In this paper, a modified detection method with K562-CD13 monoclonal cells, human-derived cell line, was established to detect the suppressing effects of enzyme activity by CD13 inhibitor. In this method, k562-CD13 monoclonal cells were used as enzyme source, and L-leucine p-nitroaniline hydrochloride as substrate. Using CD13 enzyme activity analyses, we found that the ability of the catalytic substrate was weaker in K562 cells than other cells and K562-CD13 cells expressed significantly higher levels of CD13 enzyme activity than the parental K562 cells. After the treatment of ubenimex, we detected the enzyme activity of CD13 with the new method. The enzyme activity of CD13 was significantly inhibited by ubenimex in a dose-dependent manner. In summary, the improved way for the inhibitory effect of CD13 inhibitor will be a sensitive, stable and objective laboratory method.

Keywords: Aminopeptidase N; CD13 inhibitors; Enzyme activity; K562-CD13 monoclonal cells; Ubenimex

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INTRODUCTION

Aminopeptidase N (APN/CD13) is a metallo-dependent integral membrane protease of the M1 family [1,2]. APN/CD13 is a Zn^{2+} dependent, amembrane-bound peptidase that cleaves the N-terminal peptide from small peptides [3-5]. In the past few years, many studies show that CD13 has many functions, and thus involved in signal transduction and immunological responses [6]. Recently, it was reported that CD13 participates in tumor metastasis, angiogenesis and virus infection [7-9]. Haraguchi et al. [10] have demonstrated that CD13 is a marker for human liver cancer. In mouse xenograft models, the tumor size was significantly reduced by combination of a CD13 inhibitor with a ROS-inducing chemo/radiation therapy.

Therefore, CD13 is a potential candidate for the development of targeted anticancer drugs and the synthesis of CD13 inhibitors will be of great significance. A convenient and reliable detection method for the suppressing effect of enzyme activity by CD13 inhibitor would be useful for the study of CD13 inhibitors. The traditional detection method is using porcine CD13 (pCD13) as the enzyme source which is expensive and lack of accuracy and reliability. It was reported that a new compound 16l was obtained which markedly inhibited the enzyme activity using the pCD13. However, 16l couldn't inhibit the enzyme activity when human and mouse tumor cells or isolated cell membrane were

used as enzyme source [11]. ES-2 cells are still used as the source of enzymes. The disadvantage of this detection method is that it cannot exclude the effects of other enzymes on the cell surface [12]. So far, there has no proper method for detecting the suppressing effect of enzyme activity by a homosapiens CD13 inhibitor.
This study aims to looking for a new improved method which is more accurate, sensitive, stable and cheap through the replacement of enzyme source.

EXPERIMENTAL SECTION

Cell Culture

Human K562 myeloid lymphoblastoma cell line, was cultured in RPMI 1640 supplemented with 10% fetal calf serum. These cells were incubated at 37℃ in a humidified atmosphere of 5% CO_2.

Western Blot

Cells were collected, and lysed with RIPA lysis buffer containing protease inhibitor. The protein concentration was quantified using BCA protein assay kit (Beijing Solarbio Science and Technology). Protein (30 μg) was separated by SDS-PAGE, and transferred onto PVDF membrane (Cat. IPVH00010, Millipore). The membrane was blocked with 5% skim milk in TBST and then incubated with goat anti-human CD13 antibody (Santa) at 4°C overnight. After washed with TBST, the membrane was incubated with the HRP-conjugated secondary antibodies (1:5,000; ZsBio, Beijing, China) for 1 h at room temperature. Then the membrane was visualized by enhanced chemiluminescence ECL (Cat. WBKLS0050, Millipore).

APN Activity Assay

K562 was infected with CD13 lentivirus and K562 cells were treated with different doses of ubenimex. After 30 minutes of treatment with ubenimex, the absorbance at 405 nm was measured with L-leucine p-nitroaniline hydrochloride as a substrate. The inhibitory rate of ubenimex on APN enzyme activity was calculated by the following formula.
Inhibitory rate=(the average OD value of control group - the average OD value of experimental group)/the average OD value of control group × 100% the average OD value of experimental group=the average OD value of experimental group with K562 cells overexpressing CD13 - the average OD value of experimental group with parental K562 cells the average OD value of control group=the average OD value of control group with K562 cells overexpressing CD13 - the average OD value of control group with parental K562 cell.

Flow Cytometry Assay

Cells were incubated with anti-CD13 antibody for 30 minutes at 4°C. Afer washed twice with PBS, the fluorescence intensity of the cells were measured using a FACS Calibur Cytometer. Flow cytometry data were analyzed using FlowJo 7.6 software.

Lentivirus Transfection

Myeloid lymphoblastoma cells K562 were plated in a 6-well plate overnight, and then infected with recombinant lentivirus vector to overexpress CD13. Lentiviruses were synthesized by Genechem (Shanghai, China). The lentivirus to overexpress CD13 was transfected into the K562 cells according to the manufacturer's instructions. The transfection efficiency was measured with fluorescence microscope. Cells were then collected and cultured to obtain stable cell lines.

Statistical Analyses

Data were expressed as mean ± standard deviation (SD). Statistical analyses were evaluated by the Student's t-test using SPSS 17.0 statistical software. In all of the experiments, $p<0.05$ was considered statistically significant difference.

RESULT AND DISCUSSION

K562 was Chosen to Overexpress CD13

Because there are still some enzymes that can catalyze substrates, leading to nonspecific reactions, so we need to screen a cell that have a weak ability to catalyze the substrate. L-leucine p-nitroaniline hydrochloride was used as a substrate for detecting APN enzyme activity. The APN enzyme activity in the K562 cells was obviously lower than

that of other cell lines such as PLC/PRF/5, MCF-7, A549, EA.hy926, BV2, RMC (Figure 1). Therefore, the K562 cells were selected for subsequent analyses.

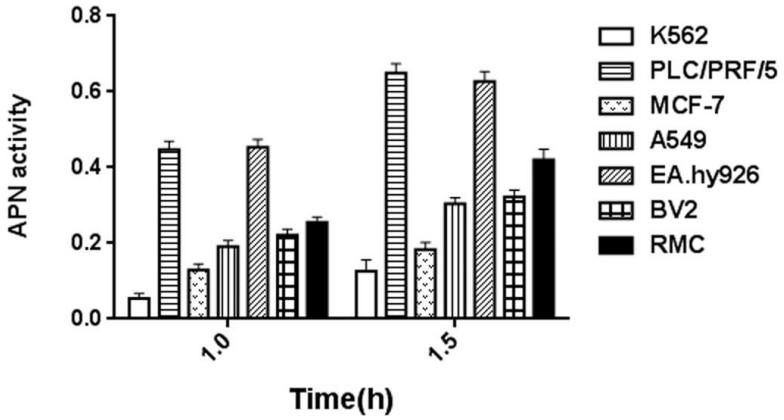

Figure 1: The APN enzyme activity of K562 cell line and other cell lines

All of the cells shown are without treatment. The enzyme activity was detected by measuring the absorbance at 405nm, as the substrate was L-leucine p-nitroanilide hydrochloride.

K562/CD13 Cells could be used to Detect the Suppressing Effect of Enzyme Activity by Aminopeptidase N Inhibitor

Myeloid lymphoblastoma cells K562 were infected with recombinant lentivirus vector to overexpress CD13. Then, the cells were selected to separate monoclonal cells. Six monoclonal cells were got and the enzyme activities were measured. The parental K562 cells were used as the control. It was showed that the APN enzyme activity of the K562 -CD13-1cells were higher than that of other monoclonal cells and parental cells. K562-CD13-1 cells were chosen to perform the next experiment (Figure 2A). And the CD13 enzyme activity of the monoclonal K562-CD13 cells was higher than that of the parental cells and polyclonal cells (Figure 2B). The green fluorescence representing the stable expression of CD13 can be observed (Figure 2C). Expressions of CD13 on these cells were analyzed by western blot and FACS. The expressions of CD13 in monoclonal K562-CD13 cells were significantly higher than that in the parental K562 cells (Figure 2D and 2E). As shown in Fig. 2F, after a 30 min treatment of ubenimex (an CD13 inhibitor commonly used in clinic), APN enzyme activity could be obviously inhibited by ubenimex in a dose-dependent manner.

APN/CD13 is a widely expressed plasma membrane extrapeptidase involved in pleiotropic functions. Recently, it is reported that CD13 plays an important role in tumor cell invasion, neovascularization and immune regulation. Therefore, CD13 is a potential candidate for the development of targeted anticancer drugs and CD13 inhibitors are

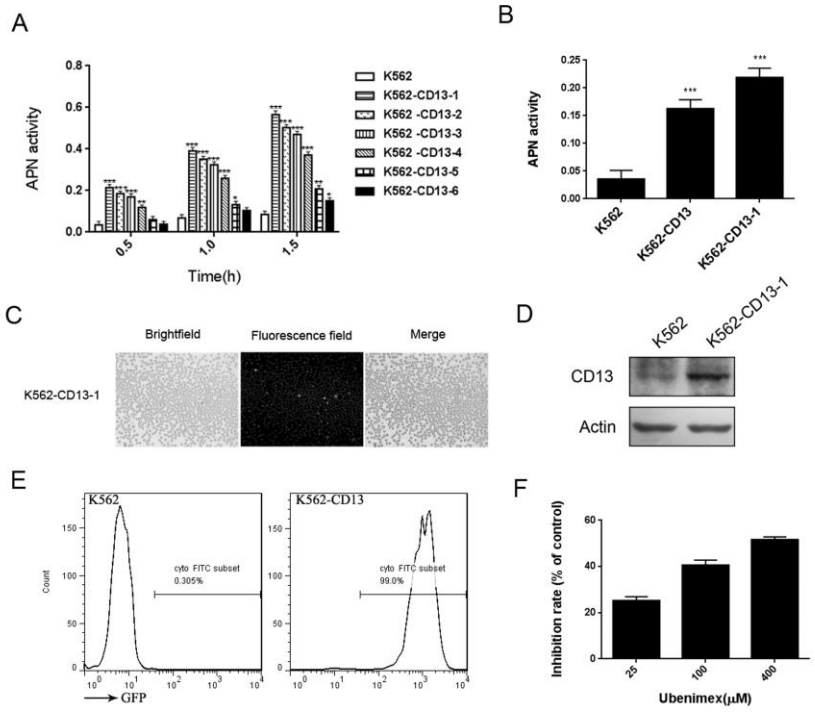

Figure 2: (A) Myeloid lymphoblastoma cells K562 were infected with recombinant lentivirus vector to overexpress CD13. The APN enzyme activity of some stable transfected monoclonal cell lines. (B) The APN enzyme activity of the monoclonal K562/CD13 cells was higher than that of the parental cells and polyclonal cells. (C) The green fluorescence representing the stable expression of CD13. (D, E) Western blot and flow cytometric analysis were performed with parental K562 and the monoclonal K562/CD13 cells. (F) Inhibition of cell surface aminopeptidase N activity by ubenimex. Aminopeptidase activity was measured after 30 minutes of treatment with different doses of ubenimex. Error bars represent means ± SD from triplicates or three independent experiments. *p<0.05, **p < 0.01

Being studied by more and more scholars. The detection method of enzyme activity was used to evaluate the suppressing effect of CD13 inhibitors. One of the two methods to detect enzyme activity is using pCD13 as the enzyme source. But there are differences in the amino acid sequence of the enzyme between porcine and homo sapiens (Figure 3). Therefore, the suppressing effect of CD13 inhibitors cannot be accurately reflected in this method. The other way is using the enzyme on the surface of ES-2 cells as a enzyme source, it cannot exclude the effects of other cell surface enzymes to catalyze the substrate. That is to say, they all may lead to a false positive test result which incorrectly indicates the suppressing effects of enzyme activity by CD13 inhibitors.

In this study, it was found that the CD13 enzyme activity of K562 cells was obviously lower than that of other cells. This result indicated that K562 cells can weakly catalyze the substrate. The weak reaction between the substrate and K562 cells can be used to avoid the the actions of other enzymes on the substrate. And K562 cells, which are derived from human, are suspension cells, not adherent cells, and need no digestion and centrifugation. Because of the less interference of other enzymes and convenient way to handle, K562 cells can be selected as the cell source to detect the suppressing effect of enzyme activity by CD13 inhibitors.

In this method, k562-CD13 cells, which were infected with CD13 vectors, were selected as enzyme source, and L-leucine p-nitroaniline hydrochloride as substrate. The K562-CD13 cells can integrate GFP and also carry the puromycin resistance. The infection efficiency can be evaluated by microscopic observation of green fluorescence, and the overexpressed cells can be screened and purified by puromycin resistance. So the K562-CD13 monoclonal cells can overexpress CD13 stably, thereby providing human amino peptidase N constantly, Human amino peptidase N is located on the surface of the cell membrane, and thus can directly catalyze the substrate, and is more suitable

for evaluating the inhibitory activity of the compound. In this paper, the CD13 inhibitor ubenimex was used as a positive control to evaluate the suppressing effect of LAP3 enzyme activity. We confirmed that APN enzyme activity could be obviously inhibited by ubenimex in a dose-dependent manner.

During the calculation of the inhibition rate on enzyme activity, the value of the overexpressed cells of the control group and the experimental group were subtracted the value of parental cells, through which it can exclude the influence of other enzymes and ensure the specificity for CD13 enzyme activity detection. This calculation can make up for the shortcomings of the detection method using ES-2 cells as an enzyme source.

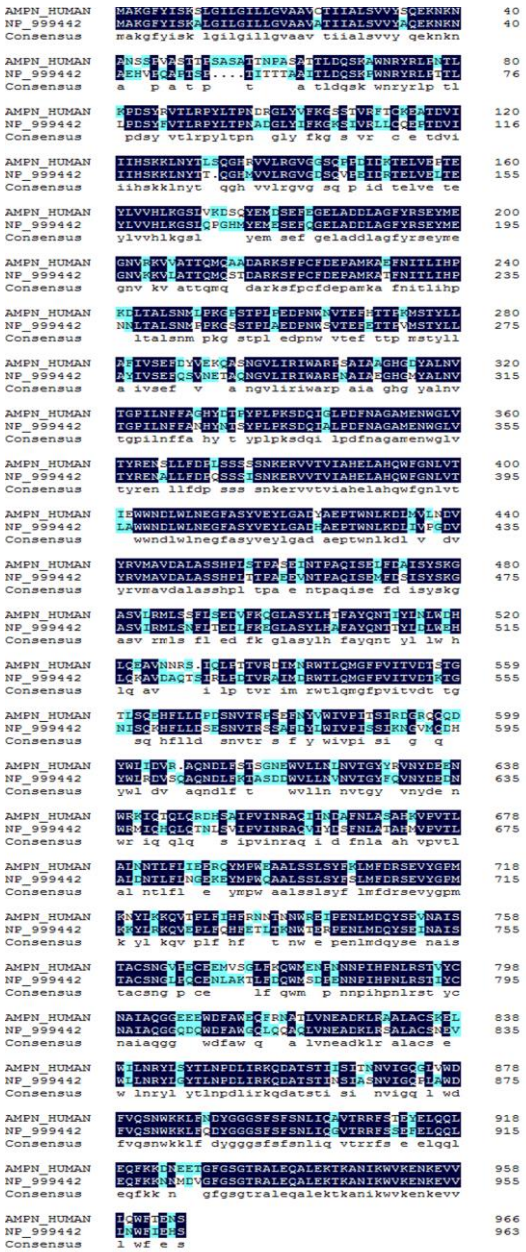

Figure 3: The sequence of the enzyme between porcine and homo sapiens

CONCLUSION

In conclusion, K562 cells with CD13 overexpression were used as enzyme source to detect the enzyme activity. And it solved many shortcomings of traditional methods. These studies thus offer a simple, sensitive, highly specific and

repeatable detection method for the suppressing effect of enzyme activity by amino peptidase N inhibitor, and this new method can be used for the evaluation of the activity of antitumor compounds.

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CONFLICT OF INTEREST

There are no conflicts of interest to disclose.

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