Effects of CD73 on human colorectal cancer cell growth in vivo and in vitro

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Abstract. The purpose of the present study was to explore the role and mechanism of extracellular ecto-5'-nucleotidase (CD73) in human colorectal cancer growth. Firstly, CD73 expression was detected in colorectal cancer cell lines both at the mRNA and protein levels. Secondly, recombinant CD73 interference and overexpression lentiviruses were used, respectively. Colony formation assay, CCK-8 assay and flow cytometry were used to investigate the impact of CD73 on colorectal cancer cell proliferation and cell cycle distribution. Then, adenosine and CD73 enzyme activity inhibitor (APCP) were used to study the effect of CD73 on Epidermal growth factor receptor (EGFR) and β-catenin/cyclin D1 signaling pathways. Finally, a human colorectal cancer transplantation nude mouse model was used to observe the effect of CD73 on tumor growth in vivo. As the results showed, CD73 was highly expressed in the colorectal cancer cell lines. CD73 promoted colorectal cancer cell proliferation both in vivo and in vitro. CD73 activated EGFR and the β-catenin/cyclin D1 signaling pathways through its enzyme and non-enzyme activities. All of the results confirmed that CD73 promotes the growth of human colorectal cancer cells through EGFR and the β-catenin/cyclin D1 signaling pathway. CD73 may be used as a valuable biomarker of colorectal cancer.

Introduction

Colorectal cancer is one of the most common cancers and is the leading cause of cancer-related deaths worldwide (1,2). Colorectal cancer is highly treatable when diagnosed and surgically removed at an early stage. Unfortunately, many patients are diagnosed with (distant) metastasis either during follow-up or at first presentation, 80-90% of which are unresectable (3,4). Despite considerable improvements in the treatment of colorectal cancer over the last few decades, the only option for unresectable metastatic CRC (mCRC) remains palliative systemic treatment (5,6). Recently, targeted and biological therapeutics have made substantial advances in mCRC treatment, and have prolonged overall survival and disease-free survival of patients, with fewer adverse effects than conventional chemotherapy (7,8). Since these therapeutics act on specific target proteins, they are restricted to certain individuals according to their molecular profiles. Therefore, more specific biomarkers, which determine the biological nature and behavior of colorectal cancer, is needed to benefit more patients (8,9).

Ecto-5'-nucleotidase (CD73), a glycosylphosphatidylinositol-linked 70-kDa cell-surface molecule, is expressed on many cell types, including subsets of lymphocytes, endothelial cells and epithelial cells (10). CD73 expression was found abnormally upregulated in many types of cancer tissues, such as colorectal, gastric, liver, ovarian and breast cancer (11-14). Studies have shown that CD73 on tumor cells is associated with tumorigenesis and tumor progression through inhibition of CD4+ T cells and NK cell proliferation, while increasing suppressive immune subsets such as regulatory T cells (Tregs), B cells, and myeloid-derived suppressor cells (15-18). Besides being originally defined as a lymphocyte differentiation antigen, CD73 has been identified as a co-signaling molecule on T cells and as an adhesion molecule required for lymphocyte binding to the endothelium (19,20).

More significantly, CD73 is a critical ectoenzyme in purine metabolism, which hydrolyzes extracellular AMP to adenosine (21). Thus, CD73 is a switch molecule of adenosine-related signaling pathways, which are important in a series of biological events, such as cell survival, proliferation and cell motility (22,23). In this study, we aimed to verify the hypothesis that CD73 promotes human colorectal cancer cell growth through its enzyme and non-enzyme activities, except for immune-related mechanisms.
Materials and methods

Cell culture. The colorectal cancer cell lines (RKO, SW480, HCT-15, LoVo and KM12) used in this study were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), and cultured in high glucose Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (both from Gibco, USA) at 37°C in 5% CO₂, under saturated humidity conditions.

Real-time RT-PCR and western blot analysis. Total RNA and proteins were routinely isolated from the cultured cells. Then, mRNA and protein expression levels were determined, respectively by real-time RT-PCR and western blot assays, where the housekeeping gene β-actin was used as control. Primary antibodies against β-actin, CD73, EGFR, β-catenin and cyclin D1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used at a dilution of 1:500 overnight at 4°C, respectively. HRP-labeled second antibodies (Santa Cruz Biotechnology) were used at a dilution of 1:1,000 for 1 h. To determine whether hydrolase activity of CD73 affects epidermal growth factor receptor (EGFR) expression, similar analyses were performed with cells pretreated with CD73 enzyme activity inhibitor APCP (10 µM) for 1 h or adenosine (20-200 µM) for 24 h.

Recombinant lentiviruses. CD73 interference and over-expressing recombinant lentiviruses were constructed using a ‘three plasmid system’ (Genechem, China). Interference-interference and overexpression sequences of CD73 were designed according to its gene sequence (gene ID: 4907). The multiplicity of infection (MOI) of the recombinant lentivirus in adherent monolayer colorectal cancer cells was 50. The effects of the recombinant lentivirus were observed at 1 week after infection.

Colony formation assay. Approximately 1x10³ colorectal cancer cells infected with the recombinant lentiviruses were plated in 60-mm culture dishes, fixed with ethanol and stained with 0.5% crystal violet 2 weeks later. The clone formation rate (CFR) = clone counts/seeded cell counts x 100%.

Cell Counting Kit-8 (CCK-8) assay. Adherent monolayer colorectal cancer cells in a 96-well plate were treated according to the manufacturer's instructions provided in the CCK-8 assay (Dojindo Laboratories, Gaithersburg, MD, USA). The cells were incubated in 10 µl CCK-8 agents for 1 h at 37°C. Absorbance of each well was quantified at 450 nm 3, 6, 12, 24 and 48 h later, respectively.

Cell cycle analysis. Adherent monolayer colorectal cancer cells were ethanol-fixed and stained with propidium iodide in buffer with RNase A. The DNA content was assessed using flow cytometer (Becton-Dickinson, San Jose, CA, USA). The proliferation index (PI) = [(G2/M + S)/(G0/G1 + G2/M + S)] x 100%.

Tumorigenicity assay. LoVo cells infected with CD73 interference and control lentiviruses were injected subcutaneously into 4-week nude mice (2x10⁶ cells/mouse), respectively. The tumors were measured every 3 days. The mean tumor volume was calculated as follows: 0.5 x a x b² (‘a’ is the largest
diameter, ‘b’ is the perpendicular diameter). Tumors were excised and weighed 3 weeks after injection.

Statistical analysis. All analysis of data was performed using SPSS 11.5 software. P<0.05 was considered to indicate a statistically significant result.

Results

CD73 expression in colorectal cancer cell lines. CD73 expression was examined in five human colorectal cancer cell lines (RKO, SW480, HCT-15, KM12 and LoVo) by real-time PCR and western blot analysis (Fig. 1A and B). CD73 expression was higher in the LoVo and KM12 cells with more malignant potential at both the mRNA and protein levels. On contrast, CD73 mRNA and protein expression was lower in the RKO, SW480 and hTC-15 cell lines with moderate malignant potential.

Effects of the recombinant lentivirus infection. Recombinant CD73 interference lentiviruses were infected into human colorectal cancer LoVo cells with a high background expression of CD73. Recombinant CD73 overexpression lentiviruses were infected into the RKO cells with a low background CD73 expression. In line with our expectation, both of the recombinant lentiviruses achieved a significant change in CD73 expression at the mRNA and protein levels (Fig. 1C and D).

CD73 promotes colorectal cancer cell growth and cell cycle in vitro. In the colony formation (Fig. 2A) and CCK-8 assays (Fig. 2B), colony formation ability and cell viability of RKO cells were increased after infection with the CD73 overexpression lentivirus. On the other hand, cell viability and colony formation ability of LoVo cells were decreased after infection with the CD73 interference lentivirus. More RKO cells were detected by flow cytometry to enter the S phase from the G0/G1 phase after infection with CD73 overexpression lentivirus, with an increased PI (Fig. 3A). On the contrary, more LoVo cells were found to enter the G0/G1 phase from the S phase after infection with the CD73 interference lentivirus, resulting in decreased PI (Fig. 3B).

CD73 increases EGFR and β-catenin/cyclin D1 expression. The important cell cycle protein cyclin D1, as well as its major transcription regulation molecule β-catenin, were found to be increased in the RKO cells infected with the CD73 overexpression lentivirus (Fig. 4A), while expression levels were decreased after infection of the CD73 interference lentivirus in the LoVo cells (Fig. 4B). EGFR, the critical cell growth
signaling pathway molecule and the major target of colorectal cancer biological therapeutics, was also found to be increased after CD73 was overexpressed (Fig. 4A), but decreased after CD73 was silenced (Fig. 4B).
The role of adenosine in the effects of CD73 on EGFR expression. Similar to the CD73 overexpression lentivirus, adenosine also increased the expression of EGFR in the RKO cells, but to a lower degree. APCP, a CD73 enzyme activity inhibitor, completely reversed the effects of adenosine and partially the effects of the CD73 overexpression virus (Fig. 5A). In contrast, APCP had a weaker effect than CD73 siRNA lentiviruses on EGFR expression in the LoVo cells (B). Similarly, adenosine reversed the effects of APCP (completely) and CD73 siRNA lentiviruses (partially) (n=3, *P<0.05).

CD73 promotes colorectal cancer cell growth in vivo. LoVo cells (2x10^6) infected with the CD73 interference or control lentivirus were injected subcutaneously into athymic nude mice. Tumor growth was obviously slower in the mice injected with cancer cells treated with CD73 interference lentiviruses when compared with these parameters in nude mice injected with cancer cells treated with the control lentiviruses (n=6, *P<0.05).
with the CD73-silenced LoVo cells than the control 20 days after injection (Fig. 6A and B). The weight of the tumors derived from the CD73-silenced LoVo cells was lower than the control (Fig. 6C). The results suggested that CD73 promoted the tumorigenicity of colorectal cancer cells in vivo.

Discussion

Metastatic colorectal cancer (mCRC) has a high incidence and mortality rate worldwide, and the effect of surgery remains limited (2,4). Molecular targeted therapy for mCRC has made progress in recent decades. For example, monoclonal antibody (mAb) drugs targeting EGFR (24), such as cetuximab (25) and panitumumab (26), have been recommended to treat mCRC singly or combined with systemic chemotherapy, for and panitumumab (26), have been recommended to treat (mAb) drugs targeting EGFR (24), such as cetuximab (25) limited (2,4). Molecular targeted therapy for mCRC has made in vivo control (Fig. 6C). The results suggested that CD73 promoted colorectal cancer cell growth both in vitro and in vivo. Flow cytometry showed that CD73 promoted colorectal cancer growth by regulating more cells into the S phase from the G0/G1 phase, resulting in an improved PI. These findings were consistent with the results of other research in other types of cancers (15,35,36).

Wnt/β-catenin/cyclin D1 is a classic signaling pathway for controlling embryonic growth and development, stem cell self-renewal, tissue structure maintenance and tumor progression (37,38). Cyclin D1 is a critical cyclin for regulating the colorectal cancer cell cycle (39). β-catenin is the major transcription regulator of cyclin D1, the abnormalities of which are commonly found in colorectal cancer (40,41). Our results showed that CD73 promoted both β-catenin and cyclin D1 expression, which well explained the effects of CD73 on colorectal cancer cell growth. EGFR is the critical molecule of many signaling pathways which control cell growth and is the major target of colorectal cancer biological therapeutics (24). Co-expression of CD73 and EGFR has been found in other types of cancers (34,36). Consistently, it was found in this study that CD73 promoted EGFR expression. The exact interactions among CD73, EGFR and β-catenin/cyclin D1 warrant further exploration.

In addition to immunoregulatory effects, CD73 plays a crucial role in adenosinergic signaling through catalyzing AMP into adenosine. Furthermore, CD73 also acts as an adhesion regulating molecule between cancer cells and extra-cellular matrix (ECM) components, which is not dependent on its enzymatic activity (19,20). In this study, we found that both enzymatic activity and non-enzymatic activity were involved in the regulation of CD73 in colorectal cancer cell growth, since adenosine and CD73 enzyme activity inhibitor ACPCP could only partly achieve the effects of the CD73 overexpression and interference virus, respectively. These results are supported by many studies in other cancers (42,43), suggesting that adenosine-related pathways and other signaling pathways warrant further study in colorectal cancer growth.

In conclusion, we found that CD73 promotes the growth of human colorectal cancer cells in vitro and in vivo. The effects of CD73 may be related to EGFR and β-catenin/cyclin D1 pathways. Our results suggest that CD73 may be a potential biomarker of colorectal cancer.

Acknowledgements

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References