Effects of ecto-5'-nucleotidase on human breast cancer cell growth *in vitro* and *in vivo*

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Abstract. Ecto-5'-nucleotidase (CD73) is an essential enzyme that generates adenosine, an essential molecule for cell growth. CD73 increases significantly in many breast cancers. In this study, α,ß-methylene adenosine-5'-diphosphate (APCP), a specific CD73 inhibitor was used to block the hydrolase's activity. Effects of CD73 were examined on human breast cancer cells MDA-MB-231 in culture for proliferation, cell cycle progression, and apoptosis before and after APCP treatment. The *in vivo* effect of CD73 was examined on MDA-MB-231 tumor xenograft growth in nude mice. Cell growth curve, cell cycle and apoptosis were observed with MTT assays and flow cytometry, respectively. Microvessel density (MVD) and lymph vessel density (LVD) of implanted tumor tissues was analyzed by immunohistochemistry for CD31 and VEGFR-3 staining respectively. Our results showed that APCP inhibited MDA-MB-231 viability in a dose-dependent manner. APCP (12 μM) increased the percentage of G_0/G_1 phase cells from 49.75 to 59.16% while it decreased S phase and G_2/M cells from 24.85 and 18.65% to 21.65 and 12.55%, respectively. The percentages of early and late apoptotic cells were also decreased after APCP treatment. However, APCP treatment did not affect the percentage of normal cells. Xenograft of MDA-MB-231 cells in the APCP treatment group had lower volume and weight than those of control group (2.70 ± 1.14 vs 1.41 ± 0.39 cm^3 and 2.7 ± 0.5 vs 1.3 ± 0.2 g), accompanied with less vessel formation with a MVD of 5 ± 1 compared to the control group's 10 ± 2 and an LVD of $4+1$ vs $7+2$. Our results suggest that CD73 may promote tumor growth and serve as a marker of breast cancer progression.

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Introduction

Cell proliferation and metastasis are major characteristics of malignant tumor. Cell proliferation depends on synthesis of a large amount of nucleotide. CD73, also known as ecto-5'-nucleotidase, is a membrane-bound glycoprotein (1). Its primary function is to hydrolyze extracellular nucleoside monophosphates into bioactive nucleoside intermediates (2) leading to the generation of extracellular adenosine. The functions of adenosine include vasodilation, stimulation of angiogenesis, cytoprotection, immunosuppression (3) and control of cell growth, maturation and differentiation. Adenosine has been shown as an important metabolite released by cancer cells that elicits physiological responses and promotes tumor progression (4). CD73 has also been described as an adhesion molecule that binds to other cells and extracellular matrix such as laminin and fibronectin (1,5,6). Given that progressive tumor growth depends on vasodilation, angiogenesis, cytoprotective and immunosuppressive activities, CD73 may promote cancer progression. CD73 expression in cancer cell lines is highly variable (5). CD73 expressing cancer cell lines are more invasive *in vitro* and tumorigenic in nude mice than CD73-negative cell lines (7-14). CD73 activity increased significantly in more aggressive breast cancer, gastric cancer, pancreas cancer and lymphoma (15). Spychala *et al* found that estradiol significantly down-regulates the expression of CD73 through the estrogen receptor (ER). Lack or loss of ER expression is an indicator of more advanced and aggressive breast cancer. Down-regulation of ER selectively increases CD73 expression and extracellular adenosine concentration. Increased generation of adenosine in ER-negative breast cancer has direct tumor-promoting consequences (4).

Although increased adenosine is consistent with faster proliferation of cancer cells (16), a number of studies have shown that activation of adenosine receptors may both stimulate and inhibit cell proliferation (5,16-23). Therefore, the role of CD73 on the growth of cancer cells remains uncertain. In order to further understand the function of CD73 on cancer growth, we investigated the effects of CD73 on human breast cancer cells MDA-MB-231 in culture for proliferation, cell cycle progression, and apoptosis for the first time. The *in vivo*

effect of CD73 was examined on MDA-MB-231 tumor xeno-graft growth in nude mice. We also examined microvessel density (MVD) and lymph vessel density (LVD) of implanted tumor tissues by immunohistochemistry for CD31 and VEGFR-3 staining.

Materials and methods

Animals and cells. Sixteen 6-week-old male BALB/C-nu/nu mice weighing 19-21 g were purchased from Shanghai Institute for Biological Sciences, Chinese Academy of Sciences, Shanghai, P.R. China. The mice were kept in a laminar-filtered airflow cabinet under pathogen-free conditions with a constant temperature of 25±3˚C, relative humidity of 40-60% and 12-h dark/light cycles. Human breast cancer cell line, MDA-MB-231 was purchased from the Institute of Biochemistry and Cell Biology (IBCB) of Chinese Academy of Sciences and cultured in Dulbecco's modified Eagle's medium (DMEM) supple-mented with 10% fetal bovine serum (FBS). DMEM, FBS and trypsin were purchased from Gibco (Langley, OK). α,ß-methylene adenosine-5'-diphosphate (APCP) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO). The Annexin V Apoptosis Detection kit was from Calbiochem (San Diego, CA).

Cell viability. Cell viability was determined using the MTT assay according to the manufacturer's instructions (Sigma-Aldrich). Only live cells reduce the yellow, water-soluble tetrazolium dye to a purple formazan product that is solubilized by DMSO and glycine. The purple color is proportional to the number of cells and the mitochondrial activity. MDA-MB-231 cells ($5x10^3$) in 200 μl DMEM were placed into each well of a flat bottom 96-well plate and incubated for 24 h. To evaluate the effect of CD73 on cell viability, the cells were then treated with different concentrations of CD73 inhibitor APCP (0, 1.5, 3, 6 and 12 μM) for 0, 12, 24, 36 and 48 h, respectively, prior to the addition of 20 μl of MTT solution (5 mg/ml in PBS). After being incubated for an additional 4 h at 37˚C, absorbance at 490 nm in both treated and untreated cells was measured with a microplate reader to determine cell viability.

Cell cycle. Cell cycle analyses were performed for cells with or without APCP treatment (12 μM APCP for 24 h) to deter-mine whether CD73 regulates the growth phase of breast cancer cells. Cells were prepared by trypsinization followed by centrifugation at 300 x g (1000 rpm) for 5 min. The cells were resuspended ($1x10^6$ cells/ml) and fixed with 70% ice-cold ethanol for 30 min. The cells were then centrifuged, washed and resuspended in 500 μl PBS with 10 μl of DNase-free RNase. After 30-min incubation, propidium iodide (0.05 mg/ml) was added to the solution. The cells were incubated for an additional 15 min in the dark and filtered through a nylon mesh to remove cell clusters. The fluorescence of propidium iodide of individual cells was measured using FACS Calibur Flow Cytometer (Becton-Dickinson, San Jose, CA). Cell subpopulations in G_0/G_1, S and G_2/M phases were calculated by gating analysis based on differences in DNA content. At least 20000 cells were analyzed per sample. Cell

proliferation index (PIa) was calculated as PIa = (S + G_2/M) x 100%/(G_0/G_1 + S + G_2/M).

Cell apoptosis. To determine if CD73 modulates programming death of breast cancer cells, the percentage of apoptotic cells and dead cells are measured by flow cytometry. Apoptotic cells are positive for Annexin V whereas cells that are propidium iodide (PI)-positive are those that have recently died. Briefly, cell staining was performed as follows. Cells were washed twice with cold PBS and resuspended in 300 μl Annexin V binding buffer (Becton-Dickinson). To the cell suspension, 5 μl fluorescein isothiocyanate-conjugated Annexin V and 5 μl 150 μM PI were added. The suspension was then vortexed and incubated at room temperature for 30 min. The samples were washed twice with the binding buffer, and then observed by flow cytometry (Becton-Dickinson). Data analysis was performed by CellQuest software (Becton-Dickinson).

Xenografts in nude mice and treatment. Sixteen mice received subcutaneous injection into the second right mammary fat pad with a 200-μl cell suspension containing $2x10^6$ MDA-MB-231 cells. Subcutaneous tumor node became palpable after a 7-day transplantation. After 10 days, when established tumors of 5-6 mm in diameter were detected, drug administration was started. From these 16 mice, 14 mice developed tumors, which were divided into two groups randomly for experiments. APCP (50 μl) (4 g/l in 9 g/l saline solution) was injected into the tumor every day in the treatment group and the same volume of 9 g/l saline solution was injected in the control group. After 8 days of treatment, 100 μl APCP (4 g/l in 9 g/l saline solution) was injected every day for 4 times. The mean tumor volume was measured and calculated according to the formula: V = a x (b)^2 x 0.5 (a, largest diameter; b, perpendicular diameter). Two weeks after the first APCP treatment, the two groups of mice were sacrificed and the tumors were removed. After the tumors were weighed, they were fixed with formalin and paraffin-embedded 4-μm sections were prepared for immunohistochemical analysis.

Immunohistochemical examination and assessment of MVD and LVD. CD31 and VEGFR-3 staining was used to identify the microvessel and lymph vessel density in the tumor tissues by immunohistochemical method. Briefly, after paraffin-embedded tissue sections were deparaffinized in xylene and rehydrated in alcohol, sections were incubated in 0.3% H_2O_2 to block endogeneous peroxidase activity. Each slide was incubated with normal goat serum for 20 min at room temper-ature, then incubated in the primary antibody, at 4˚C overnight. After incubation with the secondary antibody, biotinylated for 30 min at 37˚C, each slide was rinsed in phosphate-buffered saline and was incubated in the avidin-biotin peroxidase complex for 30 min at 37˚C. The peroxidase was visualized with 3-3'-diamino-benzidinetetrahydrochloride (DAB) solution and then counterstained with hematoxylin.

MVD was assessed according to the international consensus report (24). Immuno-stained slides were scanned at x100 magnification to identify the areas with the highest number of vessels (so called 'hot spot'). Counts were performed on 10 fields in the hot spot by two independent pathologists at x400 magnification and the mean was analyzed.
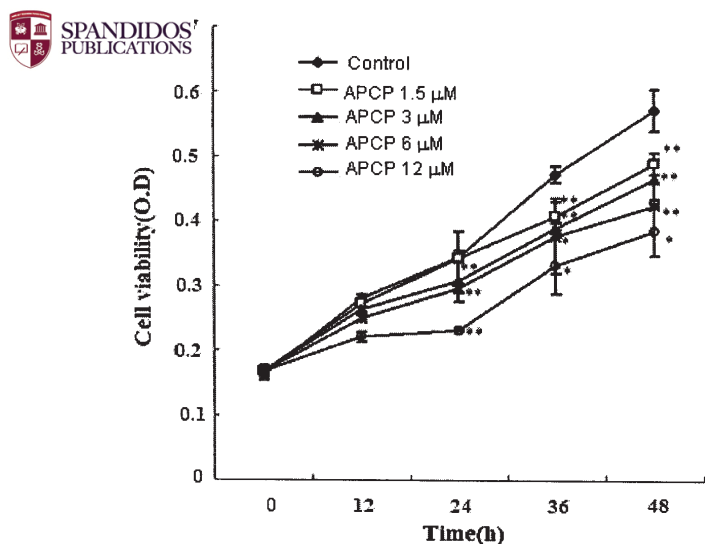
Figure 1. Effect of CD73 inhibitor on cell viability of the MDA-MB-231 cells. Cells were treated with different concentrations of CD73 specific inhibitor (APCP). Cell viability at 0, 12, 24, 36 and 48 h was detected using MTT degradation assay, n=3. *P<0.05, **P<0.01 vs control at the same time-points.

Statistical analysis. All data was expressed as mean ± standard error and analyzed using one-way ANOVA analysis. A P<0.05 was considered statistically significant.

Results

CD73 inhibitor decreased the viability of MDA-MB-231 cells. APCP is a specific inhibitor of CD73. To examine the effect of CD73 on cell viability, viability time curve of *MDA-MB-231* cells treated by different concentrations of APCP (0, 1.5, 3, 6 or 12 μM) was measured by MTT assay. The result showed that APCP inhibited cell viability significantly with a dose-dependent manner when compared to untreated control (Fig. 1).

Effect of CD73 inhibitor on cell cycle of MDA-MB-231 cells. To determine the effects of CD73 on cell cycle, DNA content in individual cells was measured by flow cytometry after being stained with fluorescent dye. APCP treatment (12 μM)
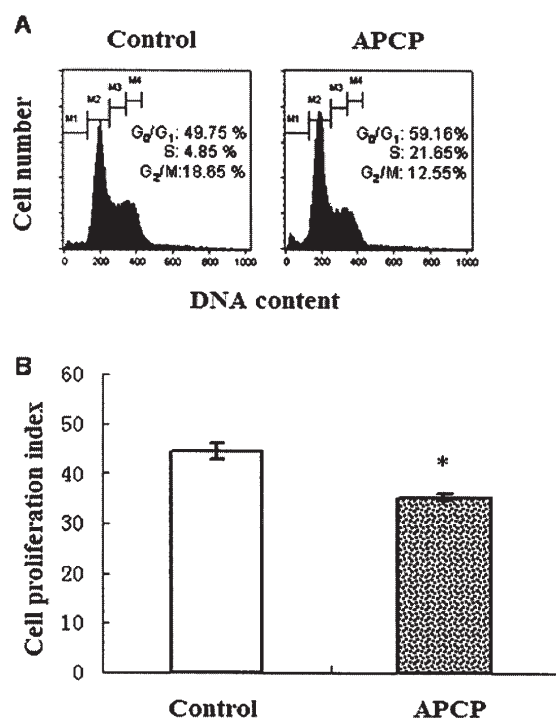

Figure 2. Effect of CD73 inhibitor on the cell cycle of the MDA-MB-231 cells. Cells were incubated with 12 μM APCP for 24 h, and then fixed and stained with PI as described in Materials and methods. The stained cells were analyzed by a flow cytometer. (A) Images from one representative experiment. M1, sub-G_1 phase; M2, G_0/G_1 phase; M3, S phase; M4, G_2/M phase. Percentage data were calculated from three independent experiments. (B) Cell proliferation index (PIa) of control and APCP treated cells. PIa = $(S + G_2/M)$ x $100\%/(G_0/G_1 + S + G_2/M)$. *P<0.01 vs control.

significantly increased G_0/G_1 phase cells from 49.75 to 59.16%, and decreased S and G_2/M phase cells from 24.85 and 18.65% to 21.65 and 12.55%, respectively (P<0.05, Fig. 2A). The proliferation index of APCP-treated cells decreased significantly when compared to untreated control (P<0.01, Fig. 2B).

Effect of CD73 inhibitor on apoptosis of MDA-MB-231 cells. Apoptosis was detected by double labeling method of Annexin
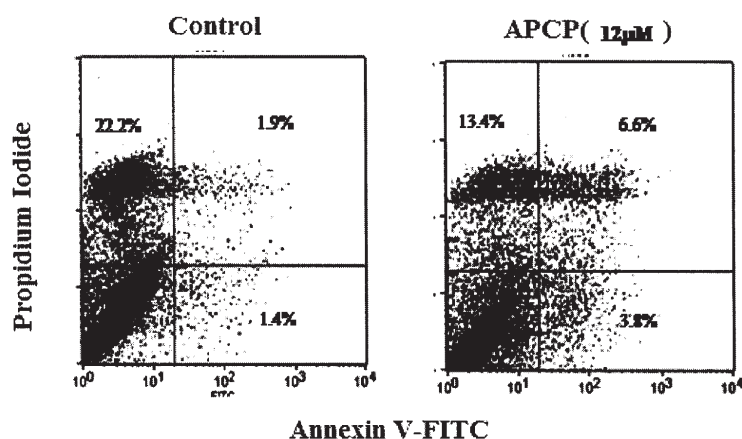

Figure 3. Effect of CD73 inhibitor on apoptosis of the MDA-MB-231 cells. Annexin V-PI staining was performed after the cells were exposed to 12 μM APCP for 24 h. The scatter plot of flow cytometry represented gated cells in different liability. Upper left, Annexin V$^-$/PI$^+$, completely dead cells; upper right, Annexin$^+$/PI$^+$, late apoptotic cells; lower right, Annexin V$^+$/PI$^-$, viable apoptotic cells; lower left, Annexin V$^-$/PI$^-$, normal cells.
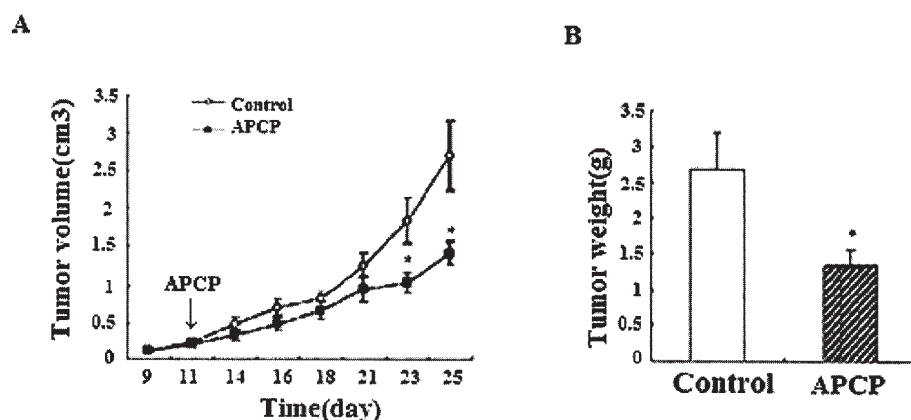
Figure 4. Effect of CD73 inhibitor on transplantation tumor volume and gravimetric of athymic nude mice bearing subcutaneously transplanted MDA-MB-231 human breast cancer cell line. (A) Effect of CD73 inhibitor on transplantation tumor volume. (B) Effect of CD73 inhibitor on transplantation tumor weight. Bars represent standard error (SE). n=7, *P<0.05, versus control.
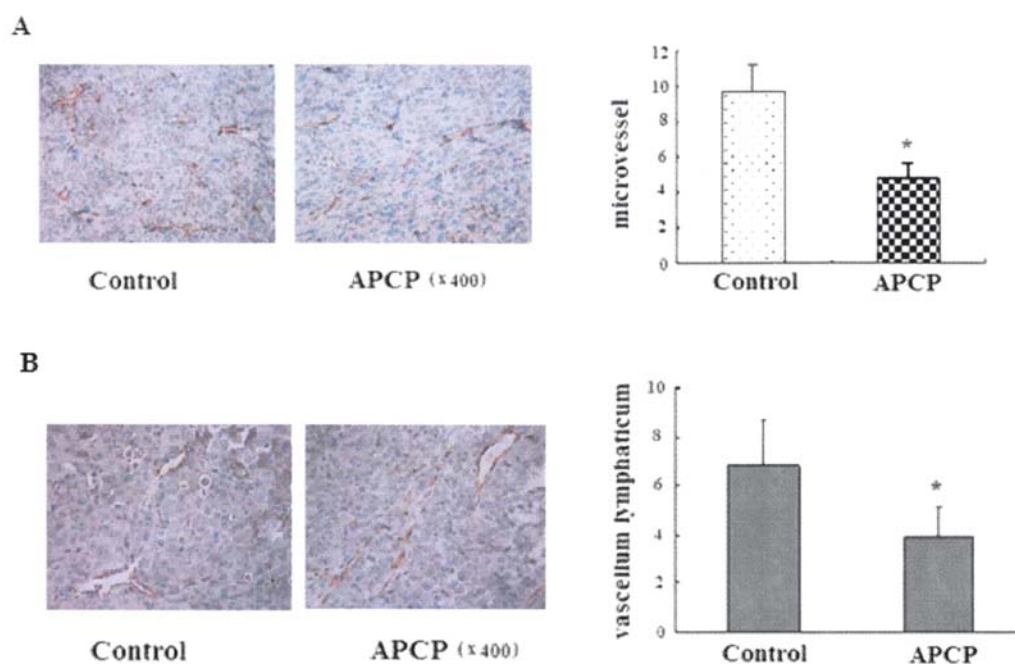

Figure 5. Effect of CD73 inhibitor on MVD and LVD in transplantation tumor. (A) CD31 staining. Athymic nude mice bearing subcutaneously transplanted MDA-MB-231 human breast cancer cell line had less tumor microvessel formation during treatment with the CD73 inhibitor APCP. Bars represent standard error (SE). (B) VEGFR-3 staining. Athymic nude mice bearing subcutaneously transplanted MDA-MB-231 human breast cancer cell line had less tumor lymph vessel formation during treatment with the CD73 inhibitor APCP. Bars represent standard error (SE). n=10, *P<0.01 compared with control group.

V-FITC and PI. After 24-h treatment with 12 μmol/l APCP, both early- (Annexin V-FITC positive only) and late-stage (both Annexin V-FITC and PI positive) apoptotic MDA-MB-231 cells increased significantly (P<0.05). However, the percentage of dead cells in the untreated group was significantly (P<0.05) higher than in the APCP-treated group. There was no significant difference in percentages of viable cells between the two groups (Fig. 3, P>0.05).

Effect of CD73 inhibitor on breast cancer xenograft growth. Xenograft model was employed to compare *MDA-MB-231* cell growth with and without APCP treatment. Finally, tumor grafts were collected and weighed two weeks after the first APCP treatment, and tumor tissues with APCP treatment showed significantly decreased volume and weight compared to those of the control group (2.70±1.14 vs 1.40±0.39 cm^3 and 2.7±0.5 g vs 1.3g±0.2 g; Fig. 4).

Influence of CD73 inhibitor on the tumor histology. To further elucidate whether reduced angiogenesis accounts for the suppressed *in vivo* growth of *MDA-MB-231* cells, MVD was assessed by immunohistochemistry. As shown in Fig. 5, microvessels were easily observed by CD31 staining. Statistics analysis showed a significantly less MVD was present in the APCP (100 mg/kg) treatment group (5±1) compared to 10±2 in the control group (P<0.01). We measured the LVD in tumor tissues by labeled VEGFR-3, which marked lymph vessel density. Statistics analysis showed a significantly less LVD

nt in the APCP treatment group (4±2) compared to control group (P<0.01).

Discussion

CD73 from tumors such as neurospongioma (25), colon adeno-carcinoma (26) and breast cancer (2) has higher activity than normal cells. We previously compared CD73 activity using high-pressure liquid chromatography and mRNA level by RT-PCR of different human breast cancer cell lines. The result showed that CD73 activity and mRNA level on MDA-MB-231, BT549 and MDA435 was higher than MCF-7 and T-47D (27). CD73 activity and metastatic potential is proportionally related (28). The mRNA transcriptional level of MDA-MB-231 human breast cancer cells was highest among the five human breast cancer cell lines tested. Therefore, the MDA-MB-231 cell line was selected to investigate the effect of CD73 on the viability, division and life cycle of human breast cancer cells.

Our study *in vitro* demonstrated that exposure of MDA-MB-231 cells to APCP, a specific CD73 inhibitor, resulted in a dose-dependent decrease in cell viability between the concentrations of 3 and 12 μM, which is consistent with previous study using glioma cells. In the same study, Wind *et al* found that besides the importance of the direct effects on cell proliferation and differentiation, CD73 overexpression enhances extracellular adenosine levels, which is an important proliferation stimulator (29). Adenosine stimulates bone marrow cell proliferation. Because the decreased cell viability caused by CD73 inhibition can be the result of either decreased cell proliferation or increased cell death, we observed the effects of APCP on cell cycle, apoptosis and death to clarify the mechanism.

Cell cycle analysis of B16-F10 melanoma cells treated with the A3 adenosine receptor agonist showed a significantly increased percentage of cells in the G_0/G_1 phase and decrease in the S and G_2 phases (30). Mujoomdar *et al* demonstrated that adenosine stimulates breast carcinoma cell proliferation, cell cycling and DNA synthesis by using MTT assay, flow cytometry and H^3-Tdr isotopic labeling (31). Because CD73 is one of the speed-limiting enzymes controlling the process of adenosine synthesis, we proposed that CD73 may have similar functions in regulating cell proliferation. In order to prove our hypothesis, cell cycling of MDA-MB-231 breast cancer cells was observed by flow cytometry after APCP treatment. The ratio of G_0/G_1 phase cells to S and G_2/M phase cells increased after exposure to APCP for 24 h. The result indicated that APCP may inhibit the progression of breast cancer cells by decreasing S and G_2/M phase cells and arresting cells in the G_0/G_1 phase of the cell cycle.

Further study was performed to determine the effect of CD73 inhibitor on cell death and apoptosis. Adenosine-induced apoptosis was reported in a number of studies (32-34). Adenosine triggers caspase-independent apoptosis of mouse thymoma cells through a non-classical receptor on the cell surface. However, the effects of CD73 on cell apoptosis and cell death have not been documented. Our data shows that when CD73 activity was inhibited, the percentages of early and late apoptotic cells increased significantly. Interestingly, the percentages of normal cells between the untreated and APCP-treated groups had no difference. The untreated group had more completely dead cells than the APCP-treated group. The result indicated that CD73 inhibition did not induce cell death.

Tumorigenicity assay showed that tumor xenografts of the APCP treatment group grew slower as their volume and weight were smaller than those from the control group. The development of a growing tumor requires an abundant blood supply. Angiogenesis is an important factor in the progression and enlargement of solid neoplasms and has a close relation to invasion and metastases (35). MVD assessment showed significantly decreased angiogenesis in tumors of the APCP treatment group, indicating that an inadequate blood supply might account for suppressed *in vivo* growth. To our best knowledge, this is the first direct evidence for the role of CD73 in breast cancer angiogenesis. The spread of tumor cells via the lymphatic system is one of the major causes of tumor metastasis. Because VEGFR-3 has been demonstrated to be expressed predominantly in the lymphatic endothelium, we measured LVD in tumor tissues by labeled VEGFR-3. Our results showed that decrease of the LVD in tumor tissue was related to treatment of APCP, suggesting that CD73 plays a crucial role in angiogenesis and lymphangiogenesis, and is closely related to invasion and metastases of tumors.

In summary, CD73 may accelerate the growth of breast cancer *in vitro* and *in vivo* by stimulating cell proliferation and tumor angiogenesis. Our results suggest that CD73 may serve as a marker of breast cancer progression.

Acknowledgements

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