

Original Article

PAR2 activates autocrine IL-8 signaling pathway to promote proliferation and migration of colorectal cancer cells

Dalei Jiang, Yuan Li, Huan Ma, Linhao Li, Shuhui Zhan, Yuqiang Gao

Department of Gastroenterology, Qingdao Municipal Hospital, Qingdao, PR China

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Abstract: Chronic inflammation is highly correlated with the development of colorectal cancer. Proteinase-activated receptor 2 (PAR2) plays pro-inflammatory roles in many type of cancers. By using trypsin or PAR2-activating peptide (AP2), we found PAR2 activation promotes the proliferation and migration of human colorectal cancer cells HT-29 and SW620. To clarify the downstream signaling of PAR2, we examined interleukin-8 (IL-8) secretion in human colorectal cancer cells. Trypsin and AP2 could up-regulate the expression and secretion of IL-8. Further, autocrine IL-8 activated ERK and AKT signaling pathways in cancer cells through CXCR1/2. The phosphorylation of ERK and AKT were suppressed by reparixin, an inhibitor of CXCR1/2. Together, PAR2 triggers autocrine IL-8 signaling pathway to promote proliferation and migration of colorectal cancer cells.

Keywords: PAR2, IL-8, CXCR1/2, colorectal cancer, proliferation, migration

Introduction

Colorectal cancer (CRC) is the third most common type of cancer and the fourth leading cause of cancer-related death worldwide [1, 2]. It is well established that chronic inflammation is highly correlated with the development of colorectal cancer [3, 4]. Infectious factors and their respond receptors on cell surface which lead to chronic inflammation may play roles in the initiation, promotion and progression of colorectal cancer [5].

Proteinase-activated receptor 2 (PAR2) is a cell surface receptor of G protein-coupled protease-activated receptor which responds to trypsin, mast cell tryptase, and coagulation factors VIIa and Xa [6]. PAR2 mediates a number of physiological and pathological pathways in a number of acute and chronic inflammatory diseases [7]. Activation of PAR2 by serine proteases is believed important player to the development of some cancers including human colon cancer [8, 9]. PAR2 activation can induce the secretion of pro-inflammatory cytokines and chemokines [10]. However, the underlying relationship between the function of PAR2 in

cancer development and secretion of cytokines and chemokines is still unclear.

Interleukin-8 (IL-8, also known as CXCL8) is a pro-inflammatory CXC chemokine. IL-8 activates a couple of intracellular signaling pathways downstream through binding to two G-protein-coupled receptors, CXCR1/2 [11-13]. Increased expression of IL-8 and/or its receptors are involved in the proliferation, invasion and metastasis of several cancers including colon cancer [14-16].

In this study, we showed activation of PAR2 could promote cell proliferation and migration in vitro. We demonstrated that expression and secretion of IL-8 increased after activation of PAR2 in colorectal cancer cell lines as revealed by ELISA. Further results indicated that autocrine IL-8 activated ERK and AKT signaling pathways in cancer cells. Blockade of IL-8 pathway by inhibition of CXCR1/2 caused suppression of phosphorylation of ERK and AKT, which resulted in a significant inhibition of cell proliferation and migration capacity induced by PAR2. So PAR2 could induce autocrine IL-8 signaling pathway to promote proliferation and migration of colorectal cancer cells.

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Materials and methods

Reagents

Trypsin and reparixin were purchased from Sigma, IL-8 recombinant protein was purchased from R&D systems. SLIGKV-NH₂ (AP2, PAR2 agonist) was purchased from abcam. Anti-phosphor-ERK1/2 (pERK1/2), anti-total-ERK1/2 (ERK1/2), anti-phosphor-AKT (pAKT), anti-total-AKT (AKT) and anti- β -actin antibodies were all from Cell Signaling.

Cell culture

Human colon cancer cell line HT-29 was maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% FBS (GIBCO). Human colon cancer cell line SW620 was cultured in Leibovitz's L-15 medium (Invitrogen) supplemented with 10% FBS. All the cells were cultured in a humidified atmosphere of 5% CO₂ at 37°C.

Before stimulation with agonists, cells were starved for at least 16 h.

Western blotting

After indicated treatment, cells were lysed in RIPA buffer (with proteinase cocktail (pierce)) and then boiled in SDS/ β -mercaptoethanol sample buffer, and 30 μ g samples were loaded into each lane of 4%-12% polyacrylamide gels and then transferred onto nitrocellulose membranes (BIORAD). The membrane was blocked with 3% BSA in PBS for 1 h at RT with shaking and then incubated with anti-phosphor-ERK1/2 (pERK1/2), anti-total-ERK1/2 (ERK1/2), anti-phosphor-AKT (pAKT) or anti-total-AKT (AKT) antibodies for 16 h at 4°C. Detection was performed using the ECL kit (Pierce). The β -actin signal was used as a loading control.

ELISA assay

Cells were seeded and grown for 24 h and then replaced with fresh serum-free medium 1 h before stimulation with trypsin (5 nM) or AP2 (100 μ M). Supernatant of the culture medium was collected after stimulation for 24 h. The amount of IL-8 secreted into the supernatant was quantified using a human IL-8 ELISA kit (R&D).

Cell proliferation assay

Cell Proliferation was determined by direct cell count. Cells were seeded in 24-well plates at low density (1×10^4), and allowed to attach overnight. Then cells were treated with indicated reagents and then cultured for 4 days. After 4 days culture, cells were detached by trypsin/EDTA and counted in a hemocytometer.

Cell migration assay

Cell migration assays were performed using modified Boyden chambers with 8 μ m-pore polycarbonate membrane (Corning). Cells (1.0×10^5 in 100 μ l) were seeded in the upper compartment of the migration chamber; trypsin or AP2 was added in both upper and lower compartments. Cells were allowed to migrate for 18 h at 37°C in 5% CO₂. Cells without any agonists or inhibitors in the assay were used as control. At the end of the assay, the upper surface of the membrane was wiped with a soft cotton swab to remove non-migratory cells. Cells that had migrated through pores to the lower side of membrane were fixed with 4% PFA, stained with 1% crystal violet and counted under bright field under high power field (100 \times). For each triplicate, the number of cells in ten random fields was determined, averaged counts were recorded.

Statistical analysis

All results were analyzed by SPSS Statistical Package version 16 and expressed as means S.E. Independent two group's analyses were used Student's t-test. $P < 0.05$ was considered statistically significant.

Results

PAR2 activation promotes proliferation and migration of colorectal cancer cells

To assess the function of PAR2 in the proliferation of colorectal cancer, we treated HT-29 and SW620 cells with trypsin or AP2, the results showed that both trypsin and AP2 could promote the proliferation of cancer cells (**Figure 1A** and **1B**). We then studied the role of PAR2 in controlling the migration of colorectal cancer cells. Using transwell assay we showed that activation of PAR2 resulted in a significant upregulation of migration capacity of both

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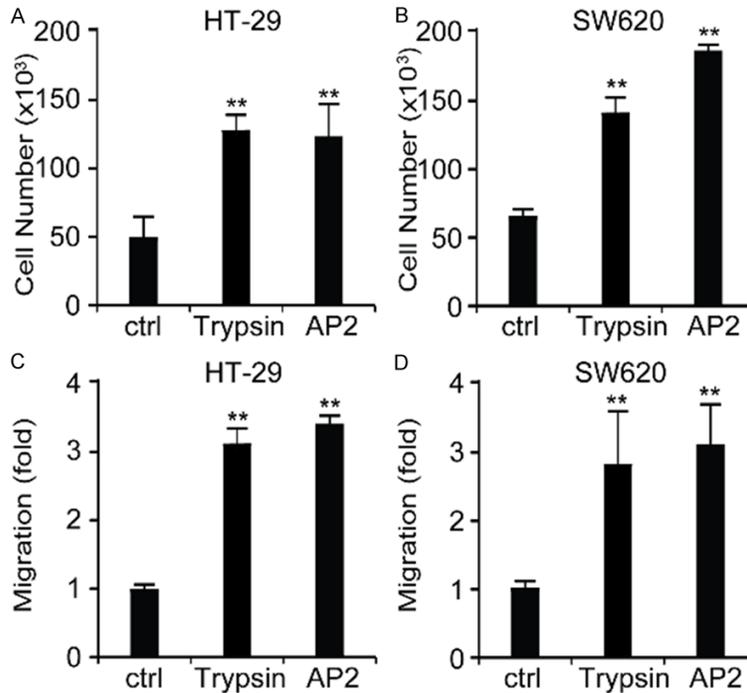


Figure 1. PAR2 activation promotes proliferation and migration of colorectal cancer cells. Cells were treated with trypsin (5 nM) or AP2 (100 μ M), then proliferation and migration assay were performed. Trypsin and AP2 can promote the proliferation of HT-29 (A) and SW620 (B). Trypsin and AP2 can also promote the migration of HT-29 (C) and SW620 (D). ** $P < 0.01$.

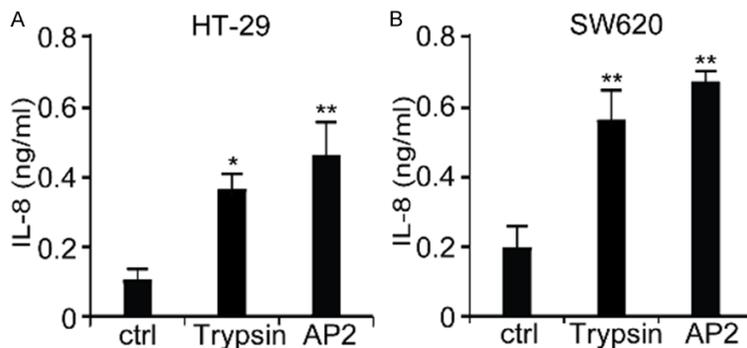


Figure 2. PAR2 activation upregulates expression and secretion of IL-8. Treatment of trypsin or AP2 increased the expression and secretion of IL-8 significantly in both HT29 (A) and SW620 (B) cells. * $P < 0.05$, ** $P < 0.01$.

HT29 and SW620 cells (Figure 1C and 1D). These results demonstrated that activation of PAR2 can promote proliferation and migration capacity of colorectal cancer cells.

PAR2 activation upregulates expression and secretion of IL-8

To gain further insight into the mechanism by which PAR2 promotes development of colorec-

tal cancer, expression and secretion of IL-8 under activation of PAR2 was detected. ELISA of IL-8 in the supernatant of medium showed treatment of trypsin or AP2 increased the expression and secretion of IL-8 significantly in both HT29 and SW620 cells (Figure 2A and 2B).

IL-8/CXCR1/2 signaling pathway is necessary to promote proliferation and migration of colorectal cancer cells

To further study whether IL-8 is involved in the development of colorectal cancer, we treated HT29 and SW620 cells with recombinant IL-8, the results showed IL-8 significantly promoted proliferation and migration capability of colorectal cancer cells (Figure 3A and 3B). Interestingly, we found that inhibition of CXCR1/2 by reparixin, an inhibitor of CXCR1/2, abolished the function of PAR2 (Figure 3C and 3D).

IL-8/CXCR1/2 activates ERK and AKT pathways to promote proliferation and migration of colorectal cancer cells

It has been reported that ERK and PI3K-AKT signaling pathways regulates cell proliferation and migration in colorectal cancer. However, it's still unclear how PAR2 activates ERK and PI3K-AKT signaling pathways.

To demonstrate the correlation of IL-8-CXCR1/2 and ERK and PI3K-AKT signaling pathways, we detect the phosphorylation of ERK and PI3K-AKT signaling in colorectal cancer cell lines. The results showed that AP2 upregulated the phosphorylation of ERK and AKT significantly. Interestingly, inhibition of CXCR1/2 by reparixin dramatically decreased the phosphorylation of ERK and AKT in PAR2-activating cancer cells by AP2 (Figure 4A and 4B).

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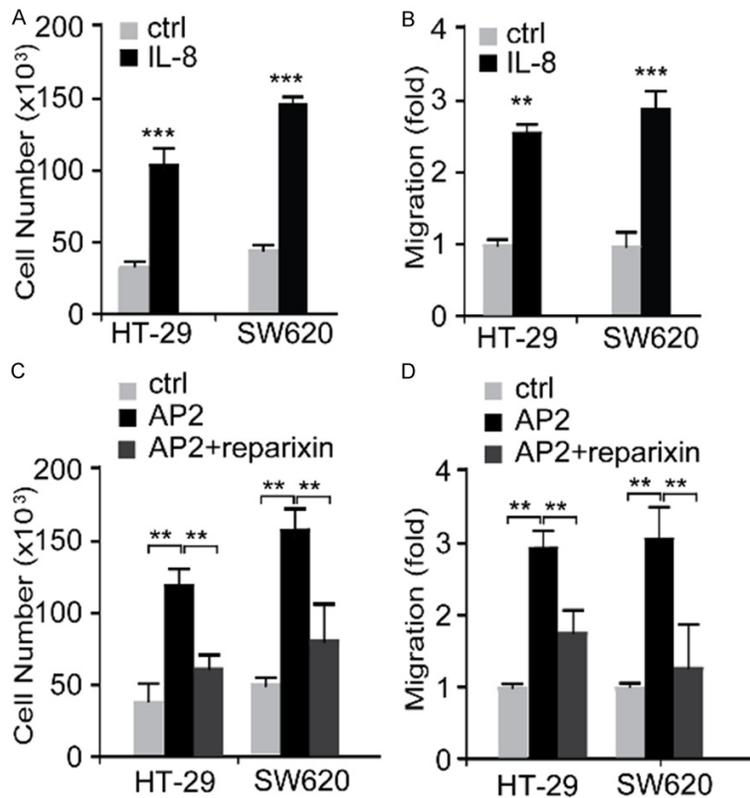


Figure 3. IL-8/CXCR1/2 signaling pathway is necessary to promote proliferation and migration of colorectal cancer cells. Treatment of IL-8 recombinant protein promoted proliferation (A) and migration (B) of both HT-29 and SW620 cells. Reparixin significantly abolished effects of AP2 on proliferation (C) and migration (D) of both HT29 and SW620 cells. ***P* < 0.01. ****P* < 0.001.

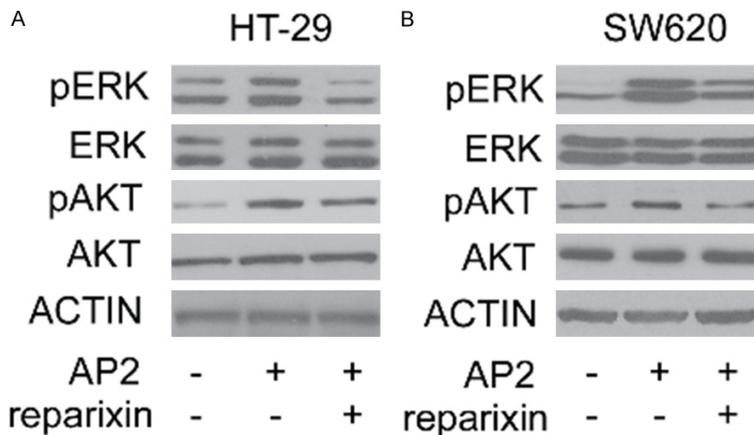


Figure 4. IL-8/CXCR1/2 activates ERK and AKT pathways in PAR2-activating cells. Treatment of AP2 increased phosphorylation of ERK and AKT significantly in both HT29 (A) and SW620 (B) cells. Reparixin dramatically blocks IL-8/CXCR1/2 signaling pathway by inhibition of phosphorylation of ERK and AKT in both HT29 (A) and SW620 (B) cells.

To summarize, our results demonstrated that PAR2-mediated progression of colorectal can-

cer is mediated by autocrine IL-8 signaling pathway.

Discussion

PAR2 is aberrantly expressed in a variety of malignant tumor cells and can be activated by tissue factor, trypsin and other protease produced by tumor cells and tumor-associated cells in the microenvironment. It's reported PAR2 is associated with cancer cell proliferation and migration and angiogenesis of tumor [17, 18]. In this study, we showed PAR2 promoted proliferation and migration of two colorectal cancer cell lines HT-29 and SW620 *in vitro* (Figure 1). PAR2 could stimulate pathways involved in acute and chronic inflammation which may play very important role in the progression of cancer [19]. Although these studies explained the correlation between PAR2 and colorectal cancer development, further studies are required to illuminate the deeper mechanisms.

Many studies have shown highly expression of IL-8 in tumor cells correlates with tumor progression and may be in response to environmental stresses such as hypoxia, which further show very significant potential in tumor progression and metastasis [20].

We examined that expression and secretion of IL-8 was highly up-regulated in colorectal cancer cell lines with PAR2 activation (Figure 2). Further we studied the biological function of IL-8 in the development of colorectal cancer.

Treatment of IL-8 recombinant protein significantly promoted proliferation and migration of colorectal cancer cells

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(**Figure 3A** and **3B**). It's reported that ERK and AKT signaling pathways are very important in the initiation and progression of colorectal cancer. In this study, we demonstrated that PAR2 significantly upregulated the phosphorylation of the ERK and AKT through IL-8-CXCR1/2 signaling pathway in human colorectal cancer cells.

Reparixin, an inhibitor of IL-8 receptor CXCR1/2 activation, has been shown to attenuate inflammatory responses in various injury models [21]. So we hypothesized reparixin could inhibit IL-8/CXCR1/2 signaling pathway and inhibit proliferation and migration of colorectal cancer cells. Indeed, inhibition of CXCR1/2 by reparixin resulted in significant inhibition of colorectal cancer cell proliferation and migration induced by PAR2 agonist (**Figure 3C** and **3D**). We then found dramatically decreased phosphorylation of ERK and AKT in PAR2-activating cancer cells (**Figure 4**), suggesting that PAR2 promoted colorectal cancer cell proliferation and migration via induction of autocrine IL-8-CXCR1/2 signaling pathway.

In conclusion, our data suggested IL-8 and its receptor CXCR1/2 is the main downstream signaling pathway of PAR2, which may be a new strategy and important therapeutic targets in colorectal cancers. Multiple small molecules such as reparixin showed highly potential that needs further extensive preclinical investigation.

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Disclosure of conflict of interest

None.

Address correspondence to: Yuqiang Gao, Department of Gastroenterology, Qingdao Municipal Hospital, Qingdao, PR China. E-mail: gyqgaoyuqiang@sina.com

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