Basic Research

Role of CCL21/CCR7 in invasion of colorectal carcinoma cell line SW480

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[Abstract] Background and Objective: Chemokine receptor CCR7 is up-regulated in gastrointestinal carcinomas and is significantly associated with lymphatic invasion and lymph node metastasis. This study was to investigate the role and mechanism of CCL21/CCR7 in invasion of colorectal carcinoma cell line SW480. Methods: The invasive capacity of SW480 cells was examined using Wound healing assay and Transwell assay. Expression of matrix metalloproteinase-9 (MMP-9) was measured by Western blot. SW480 cells were pre-incubated with CCL21 for 2 h before exposure to VP-16 (20 ng/mL). Cell proliferation was measured using MTT assay. Cell apoptosis was analyzed by flow cytometry and Hoechst33342 staining. Results: Compared to the control group, more cells in the CCL21 treatment group migrated into the gap at same time points: the count of SW480 cells penetrating through the membrane after the treatment of 100 ng/mL CCL21 increased significantly [(113±7) vs. (48±4)] (P<0.05); and the relative expression of MMP-9 in the CCL21 treatment group was enhanced evidently [(0.83±0.02) vs. (0.38±0.01)] (P<0.05). Although CCL21 alone did not promote proliferation of SW480 cells, pre-incubation of cells with 100 ng/mL CCL21 attenuated the inhibitory effect of VP-16 on proliferation of SW480 from 68.3% to 47.4%, and reduced the apoptotic rate from (65.2±5.2)% to (48.7±3.1)%. Conclusion: CCL21 enhances the invasive ability of SW480 cells, induces MMP-9 expression, and promotes the survival of SW480 cells under the suboptimal circumstance in vitro.

Key words: chemokine, CCL21, CCR7, invasion and metastasis, apoptosis

Colon carcinoma is one of the most common malignant tumors and its incidence is increasing in China recently. The main reason leading to the death of patients with colon carcinoma is the invasion and metastasis of tumor.1,2 It has been shown that the metastasis of malignant tumors is organ specific, which can not be simply explained by anatomic structures of the location of tumors or fixed blood and lymphatic drainage.3,5 Studies on the expression of chemokine receptors on the surface of tumor cells have provided a new view angle to investigate directional metastasis of cancer.6,7

In the past a few years, more and more attention has been paid
to the relationship between the expression of CCR7, a chemokine receptor in gastrointestinal cancer tissues, and the lymphovascular invasion and node metastases of tumors. In the present study, human colorectal carcinoma cell line SW480 with high-expression of CCR7 was selected according to the reference. Cells were stimulated by CCL21, the ligand of CCR7, to observe the change in invasiveness of SW480 cells and the effect of CCL21 on the proliferation and anti-apoptotic ability of SW480 cells.

Materials and Methods

Cell line and main reagents. CCL21 was purchased from PeproTech (UK). VP-16 was purchased from North China Pharmaceutical Co., Ltd. DMEM high glucose culture medium and fetal bovine serum (FBS) were purchased from Gibco. Transwell mini-well was purchased from Chemicon (USA). Cultrex Basement Membrane Extract (BME) was purchased from R&D (USA). Rabbit anti-human matrix metalloproteinase-9 (MMP-9) polyclonal antibody, peroxidase labeled goat anti-rabbit IgG antibody were purchased from Yide Biotechnology Co., Ltd (China). ECL chemiluminescence solution was purchased from Pierce (USA). Annexin-V flow cytometry apoptosis detection kit was purchased from Shenzhen Jingmei Biotechnology Co., Ltd. Hoechst 33258 was purchased from Beyotime Biotechnology Co., Ltd.

Methods. Cell culture. Human colorectal carcinoma cell line SW480 was purchased from Cell Culture Center of Wuhan University and maintained in the laboratory of General Surgery in our hospita. The cells were cultured in DMEM supplemented with 10% FBS, 100U/mL penicillin and 100μg/mL streptomycin at 37°C with 5%CO2 and saturated humidity. Cells at logarithmic growth phase were used for the experiment.

Cell proliferation experiment (MTT colorimetric method). SW480 cells were planted into a 96-well plate at 5 × 10⁴ cells/well. The following groups were included: negative control group in which serum-free DMEM was used, positive control group in which serum-free DMEM containing VP-16 (20ng/mL) was used, CCL21 treatment group in which serum-free DMEM containing CCL21 (10ng/mL, 100ng/mL) was used, and pretreatment group in which the cells were pretreated by CCL21 (10ng/mL, 100ng/mL) and then VP-16 (20ng/mL) was added. There were six parallel wells in each group. The cells were cultured for 48 h. Then 15μl of MTT (5mg/mL) was added and the cells were cultured for 4 h. The supernatant was removed by centrifugation and 150μl of DMSO was added to fully resolve the precipitate. Absorbance (A) was measured at 570nm on the microplate spectrophotometer. Cell proliferation inhibition rate was calculated by the following formula: cell proliferation inhibition rate = [A of control group-A of experiment group]/A of control group × 100%.

Flow cytometry. Apoptosis was detected by Annexin-V/PI flow cytometry. The following groups were included: positive control group in which serum-free DMEM containing VP-16 (20ng/mL) was used and pretreatment group in which the cells were pretreated by CCL21 (10ng/mL, 100ng/mL) for 2h and then VP-16 was added (20ng/mL). SW480 cells were harvested after incubation of 48 h and washed by PBS twice. The concentration of cell suspension was adjusted to 2×10⁵ cells/mL. Cell suspension (200μl) was incubated with 5μl of fluorescein isothiocyanate (FITC) labeled Annexin V and 10μl of propidium iodide at room temperature for 10 min. After adding 200μl of PBS, the cell suspension was detected by flow cytometry. The experiment was repeated for three times.

Hoechst 33258 staining. SW480 cells were planted into six-well plates with the cover glass at a concentration of 5 × 10⁴ cells/well. The following groups were included: positive control group in which serum-free DMEM containing VP-16 (20ng/mL) was used and pretreatment group in which the cells were pretreated by CCL21 (100ng/mL) for 2 h and then VP-16 was added (20ng/mL). The cells were cultured for 48 h and the cover glasses were washed with PBS for 5 min for twice. Then the cells were fixed with
4% paraformaldehyde for 10 min, followed by washing with distilled water twice. After drying at room temperature, Hoechst 33342 (5 μg/mL) staining solution was added and the cover glasses were placed at room temperature for 5 min. After being washed with distilled water for 5 min twice, the cover glasses were sealed and observed under a fluorescence microscope.

Cell scratch experiment. SW480 cells were planted into 24-well plates at a concentration of 2 × 10⁴ cells/well. When the cells reached full confluence, 10 μL transferpipetor tip was used to scratch on the cell monolayer in each well to establish a wound model in cultured cells. The cells were washed with PBS twice after scratching. The following groups were included: negative control group in which serum-free DMEM was used and CCL21 treatment group in which serum-free DMEM containing CCL21 (100ng/mL) was used. The cells were cultured and observed under an inverted microscope regularly for wound healing.

Transwell invasion experiment. The inner surface of the polycarbonate membrane (8 μm pore size) was coated with artificial matrigel BME (50μ L/well) to separate the upper and lower compartment. Cell suspension (100ML) at a concentration of 1 × 10⁶ cells/mL was added to the upper compartment, while 600μ L of serum-free DMEM supplemented with CCL21 (10ng/mL, 100ng/mL) was added to the lower compartment. After 48 h incubation, the filter membrane was taken out. Cells in the upper compartment were wiped out with a cotton bud and fixed with 4% parafomaldehyde for 5 min, followed by H&E staining. The number of cells that permeated the polycarbonate membrane was counted under a light microscope (× 200) and the invasive capacity of tumor cells was expressed with the relative number of invaded cells. The cell number in five randomly selected visual fields was counted and the mean was subjected to statistical analysis. Three samples were counted in each group.

Immunoblotting. SW480 cells were harvested after incubation of 48 h. The total cellular protein was isolated using 200μ L of lysis buffer and the protein concentration was determined by the BSA method. Protein sample (30μ g) was separated on 10% SDS-SAGE and then transferred to a nitrocellulose membrane. The membrane was incubated in TBST buffer solution containing 5% defatted milk powder at room temperature for 6-8 h, followed by incubation with rabbit anti-human MMP-6 monoclonal antibody (1:100) at 4°C overnight. Then the membrane was incubated with peroxide labeled goat anti-rabbit IgG antibody (1:3000) at room temperature for 2 h. The bands were visualized using ECL and analyzed by the scanning densitometer. The experiment was repeated three times. Mouse anti-human β -actin monoclonal antibody (1:300) was used as an internal reference.

Statistical analysis. All data were expressed as ± s. Students t-test was performed using SPSS 12.0 software, p<0.05 was considered of statistical significance.

Results

Effect of CCL21 on motility of SW480 cells. In the control group, SW480 cells on the edge of scratch spread and crept slowly to the center and the defect was repaired slowly. In the experiment group, the creeping speed of SW480 cells was significantly faster than that in control group. The cells spread quickly and there were obvious more cells crossing the edge of scratch (Fig.1).

Effect of CCL21 on invasiveness of SW480 cells. The results of transwell invasion experiment showed that the number of cells that permeated through member was (48± 4), (76± 6), and (113± 7) in the control group, 10ng/mL CCL21 treatment group and 100ng/mL CCL21 treatment group, respectively. The difference between treatment groups and the control group had statistical significance (t=12.16, p<0.05; t=18.84, p<0.05).

Effect of CCL21 on protein expression of MMP-9 in SW480 cells. The results of immunoblotting showed that relative protein expression of MMP-9 in SW480 cells after the stimulation of 10ng/mL and 100ng/mL CCL21 for 48 h was (0.59± 0.02) and (0.83± 0.02),
respectively. As shown in Fig.2, there was statistically significant difference (t=45.27, p<0.05; t=32.67, p<0.05) compared with control group (0.38± 0.01).

Effects of CCL21 on the cell proliferation of SW480 cells. The effect of CCL21 on the proliferation of SW480 cells in the in vitro culture system containing VP-16 was determined by the MTT method. There was no obvious pro-proliferation effect after the treatment with CCL21 alone for 48h (t=0.066, p>0.05; t=0.87, p>0.05). In the positive control group, VP-16 significantly inhibited the proliferation of SW480 cells with an inhibition rate of 68.3%. In the CCL21 treatment group, the proliferation of SW480 cells was also inhibited, however, at a weaker extent. The inhibition rate in the 10ng/mL and 100ng/mL group was 51.4% and 47.4%, respectively. There was statistical difference compared with the positive control group (t=4.74, p<0.05; t=10.19, p<0.05).

Effect of CCL21 on VP-16 induced apoptosis of SW480 cells. After being treated with VP-16 (20ng/mL) for 48h, the apoptosis rate of SW480 cells was (65.2± 5.2)%. In CCL21 pretreatment groups, the apoptotic rate of SW480 cells induced by VP-16 was decreased to (50.5± 3.2)% and 4 (8.7± 3.1)% in the 10ng/mL and 100ng/mL CCL21 group, respectively. As shown in Fig.3, there was statistical difference compared with the control group (t=12.78, p<0.05; t=13.61, p<0.05).

After being treated with VP-16 (20ng/mL) for 48h, significant morphological changes indicating apoptosis occurred in SW480 cells, as evidenced by condensed staining of nucleus or condensed staining of broken bits. The number of apoptotic cells was significantly fewer in the experiment group than that in positive control group (Fig.4).

Figure 1  Effect of CCL21 on motility of SW480 cells after 12 h treatment (x100)
A: control; B: treatment with CCL21 (100 ng/mL)

Figure 2  Effects of CCL21 on MMP-9 expression in SW480 cells
*P<0.05 , vs. control

Figure 3  Effects of CCL21 on VP-16 induced apoptosis in SW480 cells
1: VP-16 (20 ng/mL); 2: VP-16 (20 ng/mL)+CCL21 (10 ng/mL); 3: VP-16(20 ng/mL)+CCL21 (100 ng/mL)
*P<0.05 , vs. control
Discussion

Colon carcinoma is one of the most frequently seen malignant tumors. There are 1 million new cases each year worldwide. In China, its incidence is increasing over the year. The invasion and metastasis of tumor is one of the main reasons leading to death of patients with colon carcinoma. In recent years, the hypothesis of “soil and seed” for tumor metastasis has been re-emphasized again. With the discovery of chemokines and their receptors and the deepening of related functional study, the hypothesis of “call signal” was proposed to explain the metastasis of tumor. Common metastatic organs of tumors not only provide suitable microenvironment for the formation and growth of tumor metastasis, but also lead to selective invasion and metastasis of tumors that derived from specific tissues via “call signal”. Currently, it is considered that the interaction between chemokine receptors that are specially expressed in many malignant tumors and their ligands play an important role in organ specific metastasis of malignant tumors.

CCL21 is a CC chemokine that is mainly expressed in peripheral immune organs or tissues. Its specific receptor CCR7 is expressed on the surface of naïve T cells, B cells and dendritic cells (DCs). The interaction of CCL21/CCR7 mediates the homing of immune cells to secondary immune tissues or organs, which plays an important role in the inflammation and immunity of the human body. The expression of CCR7 was found up-regulated in many kinds of tumors, such as melanoma, lung cancer and head and neck cancer, which is related with lymphovascular invasion, lymph node metastasis and prognosis of tumor. Mashino et al. reported that after being stimulated by CCL21, F-actin was rearranged in gastric cancer NUG C3 cells expressing CCR7. In addition, cell morphology was changed and pseudopodium was formed, leading to a significant enhancement in mobility of the cells. In the present study, the effect of CCL21/CCR on in vitro invasiveness of SW480 cells was detected. The results of scratch experiment showed that within the same time period, SW480 cells that migrated into the gap of scratch in the experiment group were significantly more than those in the control group, and the healing of the scratch was significantly sped up. The results of transwell experiment showed that cells that permeated through the membrane in the experiment group were significantly more than those in the control group. CCL21 can promote the invasion of SW480 cells in a dose-dependent manner within the range of 10-100ng/mL. The above results suggest that CCR7 is functionally expressed in SW480 cells. The interaction of CCL21/CCR7 can enhance the migration of SW480 cells and
promote their invasion.

The invasion and metastasis of tumor is a multiple-factor and multiple-step process. Matrix metalloproteinase (MMPs) mediated degradation of the extracellular matrix and the basal membrane is a key step in the invasion and metastasis of tumors. The expression of MMP-9 is up-regulated in many kinds of malignant tumor tissues, cultured tumor cells and oncogene transformed cells. In vitro invasion experiment demonstrated that the high invasiveness of tumor cells is related with enhanced expression of MMP-9. The study of Brand et al. showed that chemokine CXCL12 can induce the secretion of MMP-9 in colorectal carcinoma HT-29 cells via acting on its specific receptor CXCR4. In order to investigate the promotion mechanism of CCL21 on in vitro invasion of colon carcinoma cells deeply, the effect of CCL21 on MMP-9 expression was also observed in the present study. We found that CCL21 enhanced the protein expression of MMP-9 in SW480 cells in a dose-dependent manner, suggesting that the interaction of CCL21/CCR7 can enhance the expression and secretion of MMP-9 in colon carcinoma cells, which would help degrade the extracellular matrix and the basal membrane and thus promote the invasion and metastasis of colon carcinoma.

Chemokines and their receptors not only play a role of chemotaxis and orientation in the process of cell migration, but also participate in the formation of local microenvironment, thereby providing necessary guarantee for the growth and proliferation of migrated cells in specific tissues. It was shown that the interaction of CCL21/CCR7 can enhance the anti-apoptotic ability of dendritic cells, T lymphocytes and mesangial cells and participate in the regulation of tissue microenvironment. Kim et al. found that the incidence of apoptosis of CD8 (+) CCR7 (+) T lymphocytes was lower than that of CD8 (+) CCR7 (-) T lymphocytes in the peripheral blood of patients with squamous cell carcinoma of the head and neck. Furthermore, the expression of CCR7 was positively related with the expression of Bcl-2 in CD8 (+) T lymphocytes, while negatively related with the expression of Bax and Fas. The expression of CCR7 can enhance the anti-apoptotic ability of T lymphocytes. In addition, CCL21/CCR7 can also promote the proliferation of chronic B lymphocytoma. In this study, apoptosis-inducing agent VP-16 was used as the positive control to observe the effect of pretreatment with CCL21 on proliferation of SW480 cells. The results of MTT cell proliferation experiment showed that CCL21 significantly enhanced the tolerance of SW480 cells in the toxic microenvironment of VP-16. The results of apoptosis experiment demonstrated that activation of CCL21/CCR7 enhanced the ability of SW480 cells against VP-16-induced apoptosis. Therefore, CCL21/CCR can enhance the survival ability of colon carcinoma cells, promote its growth and proliferation in metastatic site and then form cancer nests and metastatic tumors.

In summary, the interaction of CCL21/CCR7 can promote the invasiveness of human colon carcinoma cells and enhance the survival ability of tumor cells, which may be one of the important mechanisms for local infiltration and distant metastasis of colon carcinoma. Blocking agents aiming at interaction between chemokines and their receptors may become a new target for the treatment of malignant tumors.

References

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