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Review Article

Prospective Anticancer Therapy for CCL2–CCR2 Pathway Inhibition using Propagermanium

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ABSTRACT

Cancer stromal interactions have an essential role in the development and progression of most cancers. Cancer cells utilize the host's immune system for growth, invasion, survival, and metastasis. Because the CCL2 (chemokine (C-C-motif) ligand 2–CCR2 (C-C chemokine receptor type 2 pathway consists of a major inflammatory chemokine and receptor, various studies have been conducted to suppress cancer metastasis *via* this pathway. Many studies have indicated that the migration of tumor-associated macrophages (TAM) and monocytic bone marrow-derived suppressor cells (Mo-MDSCs) from the bone marrow into the microenvironment known as the "niche", which utilizes the CCL2–CCR2 pathway, promotes cancer metastasis. In line with this strategy, CCL2-neutralizing antibody CNTO888 (Carlumab), anti-CCR2 antibody (MLN1202, plozalizumab), and CCR2 antagonist (CCX872-B) proceeded to phase 1 and 2 clinical trials in cancer patients. However, no sufficient therapeutic effects have yet been proved. We focused on propagermanium (PG; 3-oxygermylpropionic acid polymer, Serocion®), which is an existing drug for hepatitis B virus (HBV) and has both CCR2 inhibition and natural killer (NK) cell activation properties. We conducted a single-arm clinical trial in refractory cancer patients. In both gastric and oral cancer patients, there was a tendency to prolong the survival period, and two out of eight patients with gastric cancer showed complete remission of liver and lung metastases. We considered that PG suppressed the growth of metastatic cancer by inhibiting the CCL2–CCR2 pathway and exhibited antitumor activity by activating NK cells. PG is a promising drug as a CCR2 inhibitor and an immune modulator activating NK cells. We review the recent progress in the development of CCL2–CCR2 inhibitors and the therapeutic potential of NK cell activation in cancer patients.

Keywords: CCL2; CCR2; FBXW7; TAM; NK cell; Metastasis; Cancer; Propagermanium

INTRODUCTION

The cancer microenvironment is composed of stromal cells, leukocytes, and extracellular matrix, which contribute to the progression of cancer [1,2]. Crosstalk between cancer cells and the microenvironment is affected by various soluble factors, including growth factors and cytokines such as chemokines. The expression patterns of chemokine receptors on cells that form the cancer microenvironment are diverse. Therefore, the function of chemokines in individual cell types also differs, for example, leukocyte migration, angiogenesis, tumor cell survival, adhesion, proliferation, vascular permeability, and immunosuppression. An essential function of inflammatory chemokines is to promote leukocyte migration and extravasation. CCL2 and CCL5 are major inflammatory chemokines [3,4] that recruit bone marrow-derived cells to the cancer niche. CCL2 and CCL5 signaling affects macrophages, vascular endothelial cells, and T cells, and ultimately cancer cell proliferation, invasion, and metastasis [5-7]. Recent experimental data indicates that cancer cell-derived CCL2 directly activated endothelial cells and promoted cancer cell extravasation in a CCL2- and CCL5-dependent manner [8,9]. These preclinical data strongly suggest that regulation of inflammatory chemokines and their receptors could be a novel and promising target for the suppression of cancer metastasis. PG inhibited CCR2 and decreased the growth of cancer metastatic lesions in a mouse metastasis model [10]. PG is also an existing therapeutic agent for hepatitis B, functioning as a multifunctional immunomodulator. It has been demonstrated that PG activates CTL and NK cells and promotes IFN-γ production to cause exclusion of HBV-infected cells. From 2015 to 2019, we administered PG to patients with refractory gastric cancer and oral cancer and obtained good results (UMIN-CRT 000017123) [11]. This review summarizes the latest findings in the development of CCL2–CCR2 inhibitors including PG and the therapeutic potential of NK cell activation

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for intractable cancers.

CHEMICAL AND PHARMACOLOGICAL PROPERTIES OF PROPAGERMANIUM

Organogermanium has various biological activities, and many compounds were developed in the 1970s. Since a double-blind phase 3 study in the 1980s demonstrated that PG treatment showed a reduction in HBV and improved liver function in chronic hepatitis patients compared with the placebo group [12], PG was approved in 1994 as a therapeutic agent for hepatitis B in Japan. It has been administered to a total of 140,000 patients in Japan to date, and is less toxic compared with cytotoxic chemotherapeutic drugs. In addition, there are reports of its therapeutic effects on atherosclerosis, renal fibrosis, and insulin resistance induced by a high-fat diet in PG monotherapy [13-15].

PG is insoluble in water at high concentrations but is easily dissolved in a protein solution to form a straight-chain polymer. This polymer interferes with glycosylphosphatidylinositol, which is an anchor protein of CCR2, and thus inhibits CCR2 function [16]. CCR2 is expressed on the surface of various cells such as neutrophils and monocytes. PG can suppress the chemotaxis of monocytes (macrophages) by inhibiting the CCL2–CCR2 signaling pathway.

PG (CAS No.1834584-83-6 or 126595-07-1; 3-oxygermylpropionicacid polymer; (C3H5GeO3.5)n) is the organogermanium compound used as a medicine in Japan. The structure of PG is a polymeric ladder-shaped structure consisting of a concatenated eight-membered ring [17]. However, the structure of repagermanium (Ge-132; CAS No.12758-40-6; poly-trans-[(2-carboxyethyl) germasesquioxane], (C18H30Ge6O21)n), which has the same basic formula as PG, is an infinite sheet structure with a twelve-membered ring[17]. Despite the apparent differences in the two structures, confusion has recently been observed in papers and chemical manufacturers' catalogs. When using PG in research, it is necessary to obtain and use a drug with a well-defined and guaranteed structure.

PROPAGERMANIUM-INDUCED NK CELL ACTIVATION

To understand the effect of PG on human immune systems, we conducted a clinical study (UMIN-CTR000030952) in healthy donors (HDs) [18]. Five HDs without cancer and any disease were administered PG 30 mg orally for 4 weeks (10 mg, three times a day [TID]). Peripheral blood mononuclear cells (PBMCs) were collected at pre-treatment and at 4 weeks and 8 weeks after the start of treatment. We prepared PBMCs and analyzed the surface antigen of immune cells (Th1, Th2, Th17, Treg, γδT, NKT, MDSC, B, and NK cells) using a multi-colored spectrum cell analyzer before oral administration after 4 weeks of oral administration and after 4 weeks of drug withdrawal (week 8). From the results of these comprehensive analysis, we focused on the changes in NK cell subsets. To assess NK cell activation, we classified NK cells (CD3−/CD19−lymphocytes) into four subsets according to their CD16 and CD56 staining levels: CD16−/CD56Bright (proliferative subset), CD16+/CD56Bright (intermediate subset), CD16+/CD56Dim (cytolytic subset), and CD16+/CD56− (CD56− subset). The CD16+/CD56Dim subset is the predominant subset in peripheral blood; it has abundant granzyme B, which induces apoptosis in abnormal cells. Among the five HDs, at week 4, CD16+/CD56Dim subsets tended to increase, and the CD16+/CD56 Bright subsets tended to decrease. After drug withdrawal, the CD16+/CD56 Bright subsets were significantly decreased to levels observed before PG administration at week 8. To evaluate the expression levels of granzyme B to determine the activation of NK cells, we performed a killing assay by apoptosis in HeLa cells using NK cells isolated from peripheral blood. As a result, it was confirmed that oral administration of PG promoted maturation of NK cells and enhanced cytotoxicity to HeLa cells. Our data revealed the possibility that oral administration of PG matures NK cells in the peripheral blood of HDs and activates the innate immune system. Therefore, PG activates innate immunity and can be expected to exclude circulating cancer cells or abnormal cells in peripheral blood.

ANTICANCER THERAPY BY PROPAGERMANIUM

A low concentration of FBXW7 (also known as Fbw7, Sel-10, hCdc4, or hAgo) in cancer cells or peripheral blood has a poor prognosis in breast cancer patients [19]. Nakayama and colleagues showed that FBXW7, an SCF receptor protein (SKP1–CUL1–FBOX) protein type ubiquitin ligase suppresses cancer metastasis in a CCL2-dependent manner [10]. They also showed that Fbxw7-deficiency in the bone marrow of a mouse model (Fbxw7bmΔ/Δ mice) induced a significantly higher metastatic rate of melanoma, lung cancer, and breast adenocarcinoma than in wild type mice. When FBXW7 expression levels are low or deficient, the concentration of CCL2 derived from metastatic tissue is high, and Ly6C+ Mo-MDSC and F4/80+ macrophages are increased in metastatic lesions, resulting in angiogenesis and metastatic tumor growth. It is thought that the immunosuppression occurs to form a niche suitable for the growth of metastatic lesions of cancer. Macrophages known as TAMs in cancer tissue supports the proliferation of cancer cells. If CCR2 inhibition can suppress the migration of monocytes to metastatic lesions (macrophages in tissues), cancer metastasis will be suppressed. Controlling the CCL2–CCR2 signaling pathway is an attractive therapeutic approach in cancer treatment. For this purpose, drug development and clinical research are being conducted.

Clinical studies using PG are being undertaken in several cancer types. In multiple myeloma patients, Tsutsumi et al. administered PG (10-40 mg/day) to 10 patients, resulting in two complete remissions, two partial remissions, four with stable disease, and two with progressive disease [20]. In a phase 1 study of breast cancer (UMIN-CTR041-001), 12 patients had no serious adverse events [21]. In response to this, we conducted a clinical trial (UMIN-CTR 000017123) in patients with refractory cancer (15 gastric cancer and eight oral cancer patients) [11]. All cases had refractory cancer with metastases, and all standard treatments resulted in failure or were intolerable. In patients with refractory gastric cancer, overall survival (OS) in the PG treatment group (PS 0-1, eight patients) was 172.0 days (61-800 days), and OS in the historical control group (39 people) was 66.0 days (8-386 days). Complete remission of multiple liver metastases (one patient) and multiple lung metastases (one patient) was observed. In patients with refractory oral cancer, OS in the PG treatment group (PS 0-1, eight cases) was 87.5 days (39-304 days), and OS in the historical control group at the same time was 36.0 days (23-126 days). Both gastric cancer and oral cancer tended to be prolonged in the PG treatment group. Treatment-related adverse events (AEs) were assessed using the Common Terminology Criteria for Adverse Events, version 4.0 (NCI-CTCAE ver.4.0). Anemia (grade 1-2) was reported in 10 patients (66.7%), while

aspartate transaminase elevation (40.7%), alkaline phosphatase elevation (53.0%), constipation (26.7%), γ-glutamyl transferase (40.0%), and creatinine elevation (26.7%) were also reported. Neutropenia and autoimmune reactions were not reported among treated patients. Anemia was frequent but was not exacerbated during PG treatment in 10 patients. One patient suspended PG treatment because of bleeding from their primary lesion of gastric cancer and received irradiation to manage the bleeding. This trial was a comparison with the historical controls at a single hospital, but no increase in AEs was observed with the combination of palliative and PG treatments. We considered that PG treatment is promising and is tolerable in advanced cancer patients.

CCL2-CCR2 AXIS INHIBITION FOR TREATMENT OF CANCER

CCL2 has been linked to cancer progression, especially metastasis. High expression of CCL2 in the blood of cancer patients is associated with a poor prognosis of breast, colon, pancreatic, prostate, and cervical cancer [5,22-27]. The CCL2–CCR2 pathway is considered as a major mechanism of angiogenesis because of the infiltration of TAMs in the cancer niche. Interestingly, immunohistochemical staining of CCL2 in cancer tissues indicates that high levels of CCL2 production are derived from the surrounding stromal cells as well as cancer cells. The CCL2–CCR2 axis has been suggested to be involved in cancer growth or survival in an endocrine and paracrine manners [28]. Regardless of cancer type, CCL2 expression is associated with increased infiltration of tumor-associated macrophages, tumor growth, and angiogenesis. CCL2 supports a potential antitumor role by tumor-entrained neutrophils, while CCL2 increases the recruitment of CD4+ T cells to the lung's premetastatic niche, stimulating tumor cell dissemination and proliferation [29]. CCL2 upregulates the expression of the immunosuppressive molecular MCP-1-induced protein (MCPIP1), and promotes macrophage-associated chemoresistance *via* the dual catalytic activities of MCPIP1 in multiple myeloma [30]. The function of inflammatory chemokines is complex and sometimes contradictory.

There are different approaches to control the CCL2-CCR2 axis in the treatment of cancer. Approaches to inhibit CCL2 or CCR2 using antibodies or CCR2 using small molecule compounds have been developed. As a clinical trial of CCL2 inhibitory antibodies, a phase 1 clinical trial (NCT00537368 and NCT01204996) using CNTO888 (Carlumab) was conducted, followed by a phase 2 clinical trial (NCT00992186) [31]. CCL2 concentration in the blood was temporarily decreased by antibody administration, but CCL2 concentration was increased after treatment. Some studies in mouse metastasis models have also indicated that anti-macrophage therapy targeting CCL2 may paradoxically promote metastasis [32,33].

A phase 2 clinical trial (NCT01015560) of anti-CCR2 antibody (MLN1202, plozalizumab) was conducted in 44 refractory cancers with bone metastases (various primary lesions) [34]. Urinary N-telopeptide was measured as a biomarker to evaluate bone metabolism. As a result, bone turnover was improved in 14% of treated cases, but adverse events of grade 3 or higher were also seen in 7%.

As a CCR2 antagonist small molecule compound, PF-04136309 has shown metastasis-suppressing effects in combination therapy with gemcitabine and FOLFIRINOX in a mouse pancreatic cancer metastasis model. CCX872-B showed some efficacy in a mouse breast cancer metastasis model. A phase 1 clinical trial (NCT01413022) of PF-04136309 and FOLFIRINOX and a phase 1b/2 clinical study (NCT02732938) using nab-paclitaxel and gemcitabine in combination with PF-04136309 were conducted in cases of metastatic pancreatic cancer [35,36]. However, PF-04136309 in combination with nab-paclitaxel plus gemcitabine had a safety profile that raised concerns for synergistic pulmonary toxicity and did not show an efficacy signal above nab-paclitaxel and gemcitabine. The subsequent development was canceled at the company's decision. At present, its efficacy in phase 2 or phase 3 clinical trials has not been fully demonstrated.

ANTITUMOR FUNCTION OF NK CELLS

NK cells are innate lymphocyte subsets and critical players in antitumor and antiviral responses. Human NK cells have been shown to be involved in development and maturation in the bone marrow and in secondary lymphoid organs such as lymph nodes. NK cells do not express polymorphic clonotypic receptors and utilize inhibitory receptors (killer immunoglobulin-like receptor and Ly49) to develop, mature, and recognize “self” from “non-self” [37]. NK cells recognize MHC-I-deficient or low-expressing cells as non-self and attack them to induce elimination. In order for NK cells to exert such an innate immune function, it is necessary for them to crosstalk with other immune cells to develop and mature [38]. Since the development and maturation pathways of NK cells are complicated and there are no suitable markers, they are classified through a combination of multiple CD antigens and their distribution in lymphoid tissue [39,40]. Mature NK cells have strong antitumor activity, and are CD16+ CD56Dim. In our analysis of PBMCs in five healthy donors, CD16+ CD56Dim mature NK cells were increased by oral administration of PG and decreased to normal levels after oral administration was discontinued. Although the mechanism of NK cell maturation has not been fully elucidated, oral administration of PG improves the ability of NK cells in peripheral blood to eliminate abnormal cells. It is speculated that this is one of the anticancer mechanisms of PG.

DISCUSSION

Cancer-stromal reactions, especially inflammation and immune reactions, have an essential role in the progression and development of cancer. With the recent advances in cancer immunotherapy, the prevention and treatment of metastases utilizing chemokines, chemokine receptors, and immune responses have been some of the most important scientific, clinical, and medical challenges in cancer research over the last decade. Immune checkpoint inhibitors such as nivolumab have revolutionized immunotherapy for refractory cancers. Many cancer patients receive immunotherapy, and the involvement of immune cells in the progression and metastasis of cancer is becoming clear. However, efficient suppression of metastatic cancer or the cancer niche has not yet been fully achieved, mainly because cancer metastasis is the result of a series of interrelated events that occur through complex interactions between different types of host cells and cancer cells. In other words, controlling cell-cell interactions leads to the efficient treatment of cancer metastases, and the modulation of chemokines and their receptors is attractive as a therapeutic target. However, certain chemokine-chemokine receptor-targeted cancer therapies may paradoxically promote cancer at certain stages of metastasis

formation. In 2020, drugs that are clinically used to block chemokine receptors include CCR4 inhibitor (mogamulizumab) for ATL, CCR5 inhibitors (Maraviroc) for HIV-1, and CXCR4 inhibitor (Plerixafor) for autologous stem cell transplantation in non-Hodgkin lymphoma and multiple myeloma [41- 45].

Low levels of FBXW7 expression assists cancer growth through the accumulation of Mo-MDSCs and TAMs in the cancer niche along with the Notch1 and CCL2–CCR2 pathways in cancer metastasis. Yumimoto et al. showed in a mouse metastasis model that PG inhibits cancer growth by inhibiting CCLR2 function [10]. In accordance with these data, we conducted a clinical study on refractory cancer aimed at suppressing metastatic lesions by PG. The findings revealed that there was a tendency of PG to prolong the survival period of both gastric and oral cancer patients, whereby two out of eight patients with gastric cancer showed complete remission of liver and lung metastases. To elucidate the mechanism of complete remission, we analyzed the PBMCs of healthy donors and found that PG treatment resulted in NK cell activation [11]. Although the relationship between CCR2 inhibition and NK cell maturation caused by PG treatment is still unclear, our clinical trial indicated that PG had a certain effect on refractory advanced cancer with metastasis. Considering the experimental results of this mouse metastasis model, PG therapy may be expected to suppress micro-metastasis. However, it seems that the efficacy of PG therapy is not sufficient for advanced or refractory cancer. Many clinical trials indicated that the combination of immunotherapy and anticancer therapy is effective in advanced cancer [46,47]. Besides the effective combination of anticancer agent and CCL2–CCR2 axis inhibitor [48,49], it has been also reported that the combination of sorafenib and natural CCR2 antagonist is effective for liver cancer [50]. These data suggest that therapies targeting chemokines and their receptors are more effective in combination with anticancer agents.

Recently, the transcription factor RUNX3 was reported to suppress the production of the inflammatory chemokine CCL5, enhance the antitumor activity of NK cells, and suppress melanoma lung metastasis in mice [51]. These data are interesting because the antitumor activity of NK cells is regulated by chemokines, and the elucidation of complex mechanisms such as immune cell crosstalk could lead to new therapeutic approaches.

CONCLUSION

CCL2–CCR2 axis-targeted therapy is an attractive treatment that affects the cancer niche and NK cells, but there are still many unresolved issues. Although CCL2–CCR2 axis inhibition is effective in suppressing micro-metastasis in mouse models, it is more promising to use it in combination with an anticancer agent for the treatment of human refractory cancer. Since there are some data suggesting that this approach has paradoxical effects on cancer growth, it is important to conduct clinical trials including appropriate monitoring.

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