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Blocking on the CXCR4/mTOR signalling pathway induces the anti-metastatic properties and autophagic cell death in peritoneal disseminated gastric cancer cells

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ABSTRACT

Patients with advanced gastric carcinoma, especially peritoneal dissemination, have a poor prognosis even after any treatment. Chemokines are now known to play an important role in cancer growth and metastasis. We recently reported that the chemokine CXCL12 plays an important role in the development of peritoneal carcinomatosis from gastric carcinoma. In this study, we investigated signalling pathway involved in the peritoneal carcinomatosis induced by chemokine CXCL12.

Akt was rapidly and strongly phosphorylated by chemokine CXCL12. CXCL12 also induced the activation of p70S6K (S6K) and eukaryotic initiation factor 4E binding protein 1 (4E-BP1) included in mammalian target of rapamycin (mTOR) pathways which are located downstream of Akt, resulting in enhancements of metastatic properties such as MMP production, cell migration and cell growth in peritoneal disseminated gastric cancer, NUGC4 cells. Furthermore, mTOR inhibitor rapamycin not only drastically inhibited migration and MMP production, but also induced type II programmed cell death, autophagic cell death.

In the present study, we have shown for the first time that the mTOR pathway plays a central role in the development of peritoneal carcinomatosis, and blocking this pathway induces autophagic cell death in disseminated gastric cancer.

Therefore, blocking on the CXCR4/mTOR signalling pathway may be useful for the future development of a more effective therapeutic strategy for gastric cancer involved in peritoneal dissemination.

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1. Introduction

Patients with advanced gastric carcinoma, especially serosa-invading tumours, have a poor prognosis even after curative resection. In these cases, peritoneal dissemination originating from free cancer cells seeded from primary gastric cancer often occurs after surgery and is the most common type of recurrence.[1–3] To date, various treatments have been used for peritoneal dissemination of gastric cancer, but there is no effective therapy for this condition. The 5-year survival rate of patients with peritoneal carcinomatosis is only 2% even if patients receive multidisciplinary treatment, including aggressive surgery[4–6] and intraabdominal or systemic chemotherapy.[7–12]

Chemokines are a family consisting of a large number of small cytokines and two cleavable transmembrane proteins that induce the direct migration of cells through interactions with a group of seven transmembrane G protein–coupled receptors.[13] It is now known that chemokines play important roles in the rapid recruitment of leukocytes in inflammatory responses as well as in homeostatic migration and tissue homing of lymphocytes.[13] Recently, it has been shown that various types of cancer cells express chemokine receptors and that chemokines may play a role in cancer progression and/or organ-selective metastasis.[14–16]

There have been no reports about the involvement of chemokines in peritoneal disseminated gastric cancer, although endothelial growth factor (EGF), hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF) and tumour necrosis factor-α (TNF-α) are well known as mediated cytokines of the disorder.[17–20]

Recently, we first reported that chemokine CXCL12 developed peritoneal carcinomatosis from gastric carcinoma, and furthermore, the administration of AMD3100 (a specific CXCR4 antagonist) was dramatically effective in peritoneal carcinomatosis of a gastric cancer model in mice.[15]

However, the molecular mechanisms of cell signalling pathways by which gastric carcinoma undergoes peritoneal carcinomatosis induced by chemokines remain to be clarified.

In this study, we have revealed that the mTOR signalling pathways activated by chemokine CXCL12 stimulation are involved in the development of peritoneal carcinomatosis in gastric cancer. We have further shown that an mTOR inhibitor, rapamycin, induced type II programmed cell death, autophagic cell death.

2. Materials and methods

2.1. Cell culture and reagents

The human gastric cancer cell line NUGC4 was kindly provided by Dr. Kazuo Yasumoto (Division of Experimental Therapeutics, Cancer Research Institute, Kanazawa University, Kanazawa, Japan). The cell line was maintained in RPMI 1640 supplemented with 10% foetal bovine serum (FBS), 2 mmol/l L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. NUGC4 disseminates, early after inoculation, into the abdominal cavity of nude mice and forms bloody ascitic fluid.[21] Recombinant human CXCL12 was purchased from R&D Systems (Minneapolis, MN, USA). Rapamycin was purchased from Carbiochem (San Diego, CA, USA).

2.2. Reverse transcription-PCR

This was performed as previously described.[16] In brief, total RNA was extracted using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's directions. First-strand cDNA was prepared from an RNA template (2 μg) using oligo(dT)18 primer and SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed at 42 °C for 50 min and then at 70 °C for 15 min. PCR amplification was performed by denaturation at 94 °C for 60 s, annealing at 60 °C for 60 s, and extension at 72 °C for 60 s for 28–35 cycles [28 cycles for glyceraldehyde-3-phosphate dehydrogenase (GAPDH)] using a TaKaRa Ex Taq HS PCR kit (Takara Bio, Shiga, Japan). All primers were verified to yield the expected products under the indicated conditions. PCR products were electrophoresed on 1.5% agarose gels and stained with ethidium bromide.

The sequences of the primers were as follows:

GAPDH sense 5′-TGAAGGTCGGAGTCAACGGATTTGGT-3′ and antisense 5′-CATGTGGGCCATGAGGTCCACCAC-3′,
MTI-MMP sense 5′-ATCAACACTGCCTACGAGAG-3′ and 5′-AAGACTTCATCGCTGCCCAT-3,
MMP-2 sense 5′-TGATGGTGTCTGCTGGAAAG-3′ and antisense 5′-GACACGTGAAAAGTGCCTTG-3′,
MMP-7 sense 5′-TCTTTGGCCTACCTATAACTGG-3′ and antisense 5′-CTAGACTGCTACCATCCGTC-3′,
MMP9 sense 5′-CATTTCGACGATGACGAGTTG-3′ and antisense 5′-AAGCCCCACTTCTTGTCGCT-3′.

2.3. Western blot analysis

Cells were cultured in RPMI 1640 supplemented with 0.1% FBS for 24 h. After the indicated treatments, cell lysates were prepared with sample buffer [25 mmol/l Tris-HCl (pH 6.8), 5% w/v glycerol, 1% w/v SDS, and 0.05% w/v bromophenol blue] and were subjected to SDS-PAGE and transferred to Immobilon-P membranes (Millipore, Bedford, MA, USA). Blots were probed by primary antibodies and then treated with horseradish peroxidase (HRP)–conjugated secondary antibodies (DAKO Japan, Kyoto, Japan) and an enhanced chemiluminescence (ECL) kit (Amersham, Piscataway, NJ, USA). Antibodies against phospho-Akt, p70S6k, phospho-p70S6k, 4E-BP1, phospho-4E-BP1 (Thr37-46) and anti-LC3 were purchased from Cell Signaling Technology (Beverly, MA, USA). Akt, β-actin, and PCNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.4. Chemotaxis assay

Migration assays were performed in 24-well Transwell plates (Costar, Cambridge, MA, USA) using inserts with 8 μm pore membranes as described.[22] Briefly, the lower surfaces of the membranes were precoated with 50 μl per filter fibronectin (Iwaki Glass, Tokyo, Japan) in PBS (25 μg/ml), allowed to dry

at room temperature, and washed in PBS. Gastric cancer cells were suspended at 1×10^6 cells/ml in chemotaxis buffer [RPMI 1640/0.1% bovine serum albumin (BSA)] and placed in the upper chambers of the membrane with recombinant CXCL12 (100 ng/ml) and various concentrations of rapamycin (1–100 ng/ml). After incubation for 24 h, the cells on the lower surface of the membrane were stained and counted under a light microscope in at least five different fields (original magnification, ×200). All assays were performed in triplicate.

2.5. Growth assay

Cell growth was assessed using a WST-1 Cell Counting Kit (Wako Pure Chemical Ind., Ltd., Osaka, Japan). Briefly, NUGC4 cells (7.5×10^3) suspended n RPMI (100 μl) containing 0.1% BSA were seeded in each well of a 96-well plate. Various concentrations of recombinant CXCL12 and rapamycin (100 ng/ml) were added to the wells and the cultures were incubated for a further 72 h. WST-1 solution (10 μl) was added to each well and the cultures were incubated at 37 °C for 90 min. Absorbance at 450 nm was measured using an immunoreader.

2.6. Transfection and detection of autophagic cell death

NUGC4 cells were placed on 35 mm PLL-coated dish at 2×10^5 cells/well. Cells were then transfected with Green Fluorescent Protein-microtubule-associated protein 1 light chain 3 protein (GFP-LC3) adenovirus vector (5×10^9 pfu/ml), which was kindly provided by Dr. Tamotsu Yoshimori (Department of Cell Regulation, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan)[23] and incubated in RPMI containing 0.1% BSA with lysosomal protease inhibitors such as E64d (10 ng/ml; Peptide Institute Osaka, Japan) and pepstatin A (10 ng/ml; Peptide Institute) and with rapamycin (200 ng/ml) or vehicle (DMSO).

After incubating for 6 h, cells were fixed with 4% paraformaldehyde (PFA)-PBS for 15 min at room temperature, and then autophagy was evaluated by fluorescence microscopy and confocal microscopy. The incidence rate of autophagy was analysed to quantify the amount of GFP-LC3 dots of GFP-LC3-positive cells by manual counting.

2.7. Statistical analysis

The mean and SD were calculated for all variables. Between-group statistical significance was determined using unpaired Student's *t* test when appropriate. $P < 0.05$ was considered as statistically significant.

3. Results

3.1. Activation of mTOR signalling by chemokine CXCL12 in peritoneal disseminated gastric cancer cells

We first compared the phosphorylation of Akt by both representative cytokines (EGF, HGF, VEGF and TNF-α), known triggers of peritoneal carcinomatosis, and chemokine CXCL12 using Western blotting.

Fig. 1A shows that CXCL12 and EGF induced rapid and intense phosphorylation of Akt in disseminated gastric cancer cells, NUGC4 cells, which peaked at 2 min after the stimulation. In contrast, deleted phosphorylations of Akt was observed by stimulation of HGF, VEGF and TNF-α at 10 min. To identify the downstream pathway of Akt in NUGC4, we examined the activation of mTOR signaling pathway components (S6K and 4E-BP1), the molecules involved in cell survival.

As shown in Fig. 2A, Akt activation preceded the subsequent activation of downstream S6K and 4E-BP1. The peaks of enhanced phosphorylation of S6K and 4E-BP1 were seen at 10 and 5 min, respectively, after stimulation with CXCL12. We also observed that CXCL12-induced activation of S6K and 4E-BP1 in NUGC4 cells was inhibited selectively by an mTOR inhibitor, rapamycin (Fig. 2B).

These results suggest that peritoneal carcinomatosis of gastric cancer developed by CXCL12 is mediated through the mTOR signalling pathway.

3.2. Role of mTOR in CXCL12-promoted metastatic cell properties and cell growth

CXCL12 is known to trigger metastatic properties of several cancer cells .[24] We further investigated whether CXCL12 could enhance the production of MMP, and the migration and growth of peritoneal disseminated gastric cancer cells through the mTOR pathway.

Previous studies have shown the significant expression of MMP-2, -7, -9 and MT1-MMP in gastric cancer tissue.[25–29] We first examined the expression of MMPs in NUGC4 cells stimulated with or without CXCL12 by using RT-PCR. The results are shown in Fig. 3. First, NUGC4 cells or those treated with CXCL12 hardly expressed MMP9. Second, NUGC4 cells constitutively expressed MMP-7 and MT1-MMP. Third, NUGC4 cells hardly expressed MMP2 and slightly expressed MMP7 but progressively upregulated their expressions at 2 h after stimulation with CXCL12. Moreover, CXCL12-promoted MMP-2 and MMP-7 expressions were clearly inhibited by treatment with the mTOR inhibitor, rapamycin.

We previously reported that NUGC4 cells showed significant chemotactic response after 24 h incubation with CXCL12.[15] The chemotactic responses of NUGC4 cells to CXCL12 were significantly blocked by rapamycin in a concentration-dependent manner (Fig. 4). Incubation for 24 h with rapamycin (1–100 ng/ml) did not affect cell damage even in the presence of CXCL12 (data not shown).

We also examined the *in vitro* effect of rapamcyin on the CXCL12-induced growth of NUGC4 cells. Interestingly, CXCL12 significantly promoted NUGC4 cell proliferation and the promoted proliferation was completely blocked by rapamycin (Fig. 5).

3.3. Induction of autophagic cell death in gastric cancer by blocking the mTOR pathway

We finally investigated which mechanism was involved in the complete inhibition of NUGC4 cells growth by rapamycin. Microtubule-associated protein 1 light chain 3 (LC3), a homologue of yeast Atg8 (Aut7/Apg8), localises to autophagosomal membranes after post-translational modification. The C-terminal fragment of LC3 is cleaved immediately following

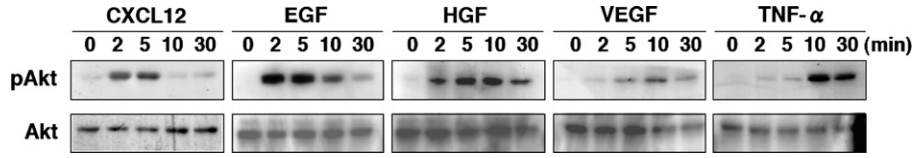

Fig. 1 – Western blot analysis for phosphorylation of Akt. Serum-deprived NUGC4 cells were seeded in a 6.0 cm dish (1×10^6 per dish), and stimulated with several growth factors and chemokine (EGF, HGF, VEGF, TNF-α and CXCL12) for the indicated time periods. Whole cell lysates were elecrophoretically fractionated, blotted onto a filter membrane, and probed with primary antibodies against phospho-Akt, Akt and PCNA. The bound primary antibodies were detected by HRP-conjugated anti-rabbit IgG and visualised with the ECL system. A representative of three separate experiments is shown. The accumulation of p-Akt was induced by several growth factors, and strongly by CXCL12.

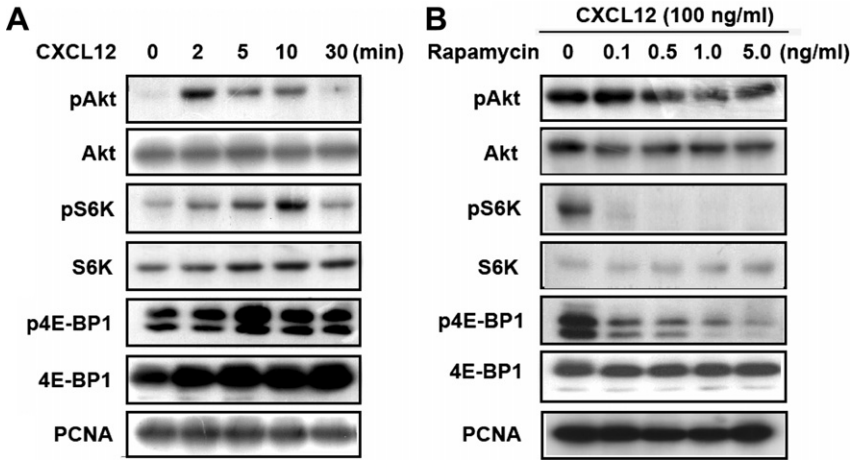

Fig. 2 – (A) Western blot (WB) analysis of Akt, S6, 4E-BP1 and eIF4E protein expression and time course of the induction of Akt, S6K, 4EBP-1 activity by CXCL12 in NUGC4. Serum-deprived NUGC4 cells were treated with CXCL12 (100 ng/ml, 0–30 min.), as indicated. Total Akt, S6 and 4E-BP1 protein, as well as their phosphorylated forms including Akt phosphorylated in serine (p^{Ser473}-Akt) and phosphorylated S6 (p^{Thr389}-S6) and phosphorylated 4E-BP1 ($p^{Thr37-46}$-4E), were detected in whole cell lysates. The accumulation of p-Akt, p-S6 and p4E-BP1 were strongly induced by CXCL12. No changes were observed in the total protein levels. (B) Inhibitors of mTOR (Rapamycin; 0–5 ng/ml) were added 30 min prior to initiation of chemokine treatments. Akt, S6K and 4EBP-1 phosphorylation in response to chemokine at 5 min were assessed in the absence (0) or presence of these inhibitors. Constitutively phosphorylated S6 and phosphorylated 4E-BP1 in NUGC4 cells are inhibited even at low concentrations of rapamycin, whereas no changes were observed in their total protein expression.

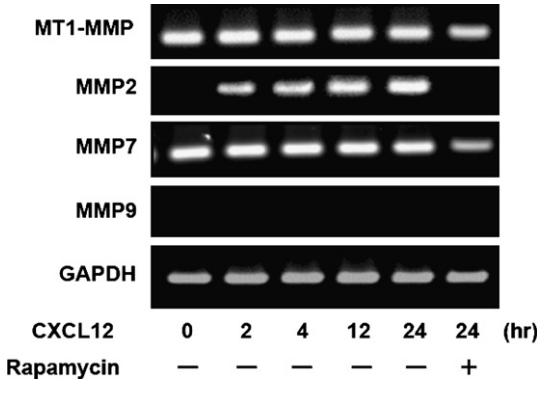

Fig. 3 – Expression of MMPs in NUGC4 cells stimulated with or without CXCL12 by using reverse transcription polymerase chain reaction (RT-PCR). CXCL12-promoted MMP-2 and MMP-7 expressions were clearly inhibited by the treatment of an mTOR inhibitor rapamycin.

synthesis to yield a cytosolic form called LC3-I (18 kDa). A subpopulation of LC3-I is further converted to an autophagosome-associating form, LC3-II (16 kDa).

Western blot analysis showed a drastic increase of the conversion in NUGC4 (Fig. 6A).

To visually observe the occurrence of autophagy, the green fluorescent protein (GFP)-tagged LC3 were overexpressed in NUGC4 cells.[15] The diffuse distribution of GFP-LC3 was observed in the absence of rapamycin (Fig. 7A), whereas a punctate pattern consisting of GFP-LC3 expression was increased in number and fluorescence intensity by autophagy in the presence of rapamycin. GFP-LC3 fusion protein was observed as coarse dots in the cytoplasm of NUGC4 (Fig. 7B and C). According to Western blotting data, mTOR inhibitor treatment enhanced dot formation.

These results suggest that blocking on the mTOR pathway induced autophagic cell death in gastric cancer equipped with metastatic properties by CXCL12.

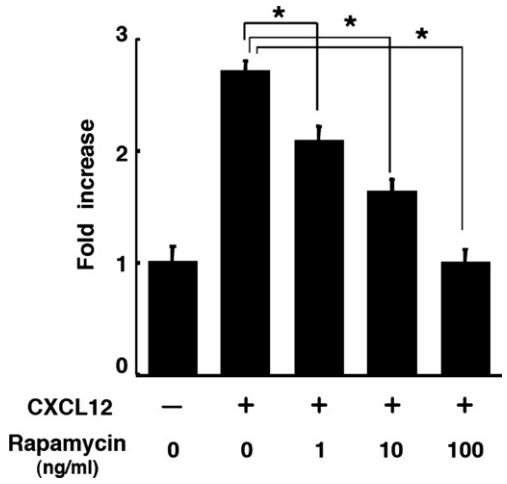

Fig. 4 – Induction of cell migration in CXCR4-expressing NUGC4 cells by CXCL12. NUGC4 cells expressing CXCR4 mRNA at high levels were examined for cell migration to CXCL12 using Transwell plates. Dose-dependent chemotactic responses of NUGC4 cells to CXCL12 and effect of rapamycin on CXCL12-mediated chemotaxis in NUGC4 cells. Columns, mean number of migrated cells per field from a representative experiment performed in triplicate. Bars, SD. *, $P < 0.05$.

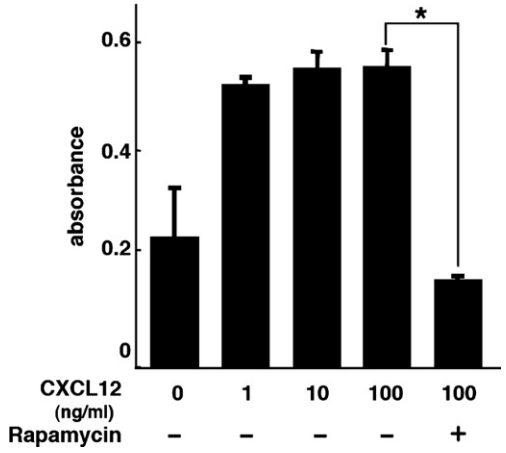

Fig. 5 – NUGC4 cells were grown in serum-free medium with or without the indicated doses of CXCL12. CXCL12 significantly increased the number of NUGC4 cells. NUGC4 cells were grown as above without or with rapamycin. Rapamycin significantly blocked CXCL12-induced cell proliferation. Results are representative of three independent experiments. Bars, SD. *, $P < 0.05$.

4. Discussion

Peritoneal dissemination of tumour cells is not only the most frequent pattern of gastric cancer recurrence but also a major cause of death among advanced gastric-cancer patients.[30] In the development of peritoneal carcinomatosis of gastric cancer, several cytokines, such as EGF, HGF, VEGF and TNF-α, directly promote cancer cell growth, and the permeability

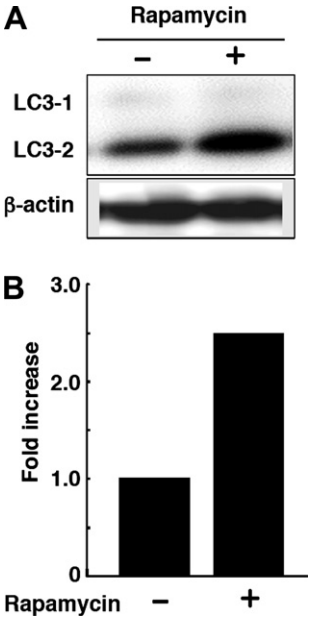

Fig. 6 – Cells treated with 200 ng/l rapamycin or the vehicle were harvested 6 h after treatment. LC3-I and LC3-II were detected on Western blotting, as described in Materials and Methods. Rapamycin induced the accumulation of autophagosome-incorporated LC3-II in NUGC4 cells (A). The relative expression level of LC3-II to β-actin is shown in (B). The LC3-II value in cells treated with rapamycin is two times or more higher than the vehicle.

property of VEGF was enhanced in the accumulation of malignant ascitic fluid.[17–20]

It is now considered that chemokines play a significant role in organ-selective cancer metastasis, because cancer cell migration and metastasis share many similarities with leukocyte trafficking.[31] Several chemokine receptors are regarded as molecules related to cancer metastasis, and chemokines added metastatic properties, such as migration, production of MMPs and growth, to cancer cells.[32–35] In fact, we also observed that representative cancer metastasis-inducible chemokine, CXCL12, prompted the metastatic proprieties of peritoneal disseminated gastric cancer NUGC4 cells (Figs. 3–5).

We recently reported that AMD3100 (a specific CXCR4 antagonist) effectively reduced tumour growth and ascitic fluid formation, with the result that chemokine CXCL12, secreted from peritoneal mesothelial cells, archived peritoneal carcinomatosis of gastric cancer.[15]

However, the signalling pathway involved in the peritoneal carcinomatosis induced by chemokine CXCL12 remained unclear in our recent report. We therefore first investigated which signalling pathways activated by cytokines and chemokines are associated with peritoneal carcinomatosis.

As shown Fig. 1, Akt was rapidly and strongly phosphorylated by chemokine CXCL12 as well as other cytokines (EGF, HGF, VEGF and TNF-α), leading us to the hypothesis that mTOR, which is located downstream of Akt, is a relay station of the signalling pathways involved in peritoneal disseminated gastric cancer cell NUGC4.

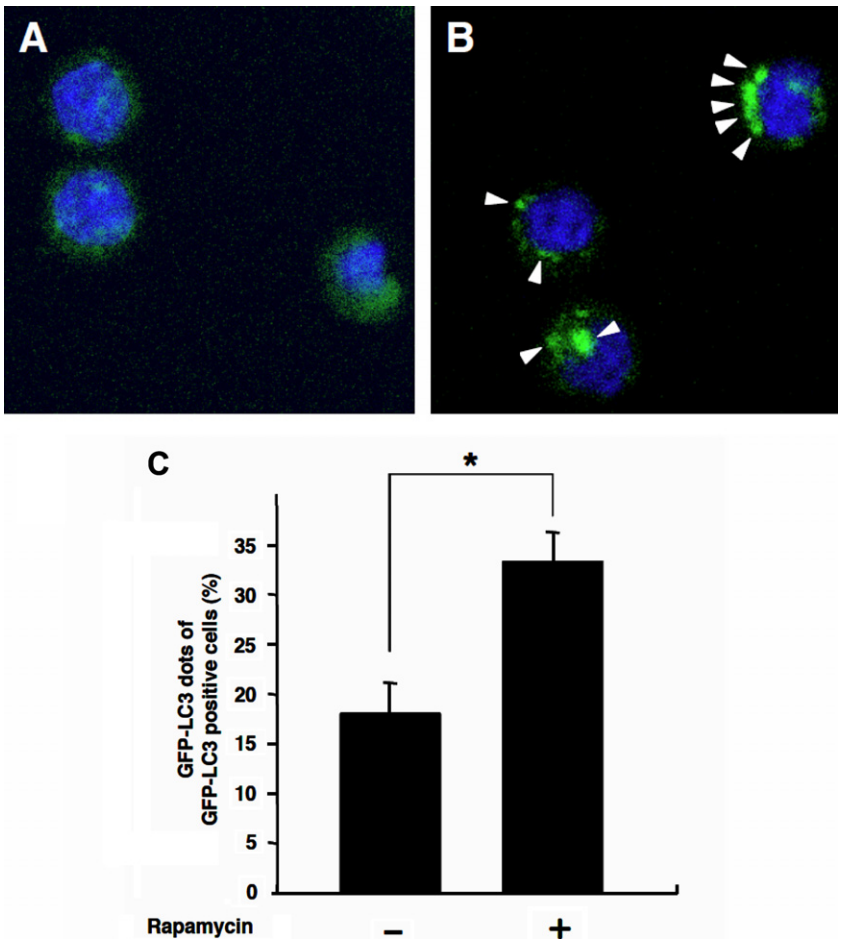

Fig. 7 – Tumour cells were transfected with the GFP-LC3 (A) expression vector in the absence of rapamycin. After 6 h culture with rapamycin, cells were fixed with 4% paraformaldehyde (PFA)-PBS for 15 min at room temperature, and then autophagy was evaluated by fluorescence microscopy and conforcal microscopy (B). The incidence rate of autophagy was analysed to quantify the amount of GFP-LC3 dots of GFP-LC3-positive cells by manual counting. Arrow heads indicate GFP-LC3 dots. Bars, SD. *, $P < 0.05$.

The coordinated action of cell cycle progression and cell growth (an increase in cell size and cell mass) is critical for sustained cellular proliferation. Fingar et al. reported that cell growth and cell cycle progression are separable processes in mammalian cells and that growth to the appropriate cell size requires mTOR- and PI3K-dependent signals.[36] Rapamycin acts as a specific inhibitor of mTOR, a serine/threonine kinase, that regulates the phosphorylation and activation of its two major downstream components, p70S6K and eIF4E-binding protein 1 (4E-BP1).[37,38] Phosphorylation of p70S6K allows the translation of ribosomal proteins.[39] Phosphorylation of 4E-BP1 regulates cap-dependent translation by enabling the formation of an active eIF4E complex.[40] As a result of access downstream of Akt, CXCL12 induced the activation of p70S6K and 4E-BP1 included in mTOR pathways, and this activation was completely blocked by rapamycin.

In this study, rapamycin also drastically inhibited metastatic cell properties, migration (Fig. 3) and MMP production (Fig. 4), but complete inhibition of CXCL12-induced cell growth in peritoneal disseminated gastric cancer cells should be noted (Fig. 5). Rapamycin and its derivatives, CCI-779, RAD001 and AP23573, effectively induce cell apoptosis, inducing the growth inhibition of several cancer cells .[41–43]

We therefore investigated surface exposure of phospholipid phosphatidylserine (PS) by apoptotic cells measured by Annexin V using FACS analysis. In the case of our study, however, rapamycin could not induce apoptosis and cell death in NUGC4 cells for a short time (6 h) (data not shown). While apoptosis is classified with type I programmed cell death, type II programmed cell death, autophagic cell death, exhibits extensive autophagic degradation of Golgi apparatus, polyribosomes and endoplasmatic reticulum, which precedes nuclear destruction.[44] We therefore extended our investigation to autophagic cell death. Interestingly, Figs. 6 and 7 show the induction of autophagy in gastric cancer by rapamycin. For the first time, we revealed the blocking on the mTOR signalling pathway by induced autophagic cell death in CXCL12 activated in peritoneal disseminated gastric cancer cells.

Currently, the mTOR inhibitor rapamycin and its derivatives, CCI-779, RAD001, and AP23573, are being evaluated in cancer clinical trials, including renal cancer, breast cancer, lymphoma, glioblastoma, sarcoma and several solid cancers. To date, the clinical results have shown good tolerability of

treatment with mTOR inhibitors in most reports and varying effectiveness of mTOR inhibitors in a variety of tumours.[45]

Nevertheless, there have been no reports about clinical trials with rapamycin in peritoneal disseminated gastric cancer cases.

In this study, we have focused on the association of peritoneal dissemination induced by chemokine CXCL12 only and the mTOR signalling pathway. However, we also observed that mTOR activation was induced not only by CXCL12 but also cytokines (EGF, HGF, VEGF and TNF-α) related to peritoneal carcinomatosis (data not shown).

Therefore, the theraputic strategy for blocking on the mTOR signalling pathway may commonly apply for the treatment of peritoneal disseminated gastric cancer, but is not limited to CXCR4 positive gastric cancer.

In conclusion, we have shown that rapamycin also suppressed migration and MMP production, which is the driving force of cancer cell invasion, and triggered cell death by autophagy. Therefore, mTOR may be a novel and efficient targeting molecule for gastric cancer involved in peritoneal dissemination.

Conflict of interest statement

None declared.

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