Anti-tumor Effects of Cyclolinopeptide on Giant-cell Tumor of the Bone

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Abstract. Aim: This study aimed to evaluate the antitumor effects of cyclolinopeptide (CL), which suppresses receptor activator of nuclear factor- κ B ligand (RANKL) signalling on giant-cell tumours of the bone (GCTB) cells. Materials and Methods: GCTB cell lines were established, and the inhibition of cell growth by CL was evaluated using the water-soluble tetrazolium salt-8 cell proliferation assay, cell cycle assay, and 5-ethynyl-2'-deoxyuridine (EdU) cell proliferation assay. RANKL and runt-related transcription factor 2 (RUNX2) expression levels were evaluated using real-time polymerase chain reaction before and after CL administration. Results: The dose-dependent inhibition of GCTB cells was significantly pronounced in the CL-administered group compared to the non-CL-administered group ($p < 0.05$). In the CL-administered group, the ratio of cells in the G₀/G₁ phase was increased, but the ratio of EdU-positive cells was decreased ($p < 0.05$). RANKL and RUNX2 levels were decreased in the CL-administered group ($p < 0.05$). Conclusion: CL has antitumor effects on GCTB in vitro.

The incidence of giant-cell tumor of the bone (GCTB), which is classified as an intermediate malignancy in the 2013 WHO classification of tumors (1), is 4%-5% of the total cases of bone tumors (2-5). Approximately 2% of GCTB

cases result in lung metastasis, which is fatal in some instances (6, 7). Curettage along with adjuvant therapy is the most common treatment method used for this tumor; nonetheless, the local recurrence rate is high, at approximately 15%-50%. Although the en bloc resection of the tumor can reduce the recurrence rate, loss of function due to resection is a major issue. Furthermore, it is difficult to perform the radical resection of tumors in anatomically difficult locations, such as the spine or pelvis, because an appropriate surgical margin cannot be secured (1).

GCTB is composed of two kinds of cells: osteoclast-like giant cells or its precursor expressing receptor activator of nuclear factor- κ B (RANK) and receptor activator of nuclear factor- κ B ligand (RANKL) (8). Mesenchymal stromal cells are thought to have proliferative abilities and are important for tumour growth (1).

Giant cells are induced by RANKL produced by proliferating stromal cells and cause bone absorption (8, 9). Furthermore, stromal cells express osteoblast markers such as alkaline phosphatase and runt-related transcription factor 2 (RUNX2) (10).

Flax (scientific name: *Linum usitatissimum*) is an annual grass of Linaceae that has originated in Central Asia and is cultivated all over the world. The dried mature seed is called linseed (flaxseed). Linseed oil, which is obtained by compressing or crushing seeds and extracting them with a solvent, is rich in unsaturated fatty acids, such as α -linolenic acid (a ω -3 fatty acid) (11-13). Linseed oil is sold as food or a supplement. Cyclolinopeptide A (CL-A), which is a cyclic peptide extracted from linseed, was first introduced in 1959 by Kaufmann *et al.* (14). Several studies have reported the cytoprotective and immunosuppressive effects of CL-A (15-17). Recently, 10 kinds of CLs, namely, B, C, D, E, F, G, H, I, J, and K, and 5 reductants of CL-F and G have been introduced

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Figure 1. Immunofluorescence for Histone H3.3 G34W. All three established cell lines showed staining with the anti-histone H3.3 G34W antibody.

as new cyclic peptides extracted from linseed (18-25). They have been found to inhibit RANKL signaling and the differentiation of myeloid cells into osteoclasts in mice (26, 27). Considering that CL is derived from natural substances and has low toxicity, new drugs that comprise CL are now being designed as safe drugs for long-term administration (25).

Several studies have shown that RANKL signalling is an important factor for tumour progression in GCTB. Thus, in this study, we evaluate the antitumor effect of CLs, which inhibit RANKL signal, on GCTB.

Materials and Methods

GCTB sample collection. Following the approval of the ethics committee of our hospital, we obtained consent from three GCTB patients for enrolment in this study. The patients underwent surgical resection and were diagnosed with GCTB. The resected specimens were partially used in the study.

Primary cell lines and cultures. The cell lines of GCTB stromal cells were produced from the collected tissue, as described by Goldring *et al.* (28). The GCTB tissue was minced with a clean scalpel, soaked in 0.2% collagenase type 1 (Wako®, Osaka, Japan),

and incubated for 1 h at 37°C in a 5% CO₂ incubator. The minced cells were then filtered with a cell strainer (100 µm) and centrifuged at 300 × g for 4 min. The supernatant was removed and suspended in Dulbecco's Modified Eagle Medium (DMEM) (High Glucose; Wako®) containing 10% fetal bovine serum and penicillin-streptomycin solution (×100; Wako®). Thereafter, the cells were seeded in a 75 cm² tissue culture flask (TPP®, Trasadingen, Switzerland). Confluent GCTB cells were treated with 0.05 w/v % Trypsin-0.53 mmol/l EDTA-4 Na Solution with Phenol Red (Wako®) and then subcultured to produce cell lines. The cell lines were maintained in a humidified 5% CO₂ incubator at 37°C.

Immunofluorescence. The presence of the H3F3A mutation in the established cell lines was evaluated using the anti-histone H3.3 G34W rabbit monoclonal antibody (RevMAb Biosciences®, San Francisco, CA, USA) (29). RANKL expression was evaluated using anti-RANKL antibody (ab9957; Abcam®, Cambridge, UK). The GCTB cells were treated with trypsin. DMEM was added to adjust the cell concentration to 5×10⁴ cells/ml, and 1 ml of cell suspension was spread onto the Nunc™ Lab-Tek™ Chamber Slide System (Thermo Scientific®, Waltham, MA, USA). The cells were cultured for 24 h. The medium was removed, and the cells were washed with phosphate buffer saline (PBS). The cells were then fixed by treatment with 4% formalin at room temperature for 10 min, washed several times with PBS, and

Figure 2. Immunofluorescence for RANKL. All three established cell lines showed staining with the anti-RANKL antibody.

treated with 0.2% Triton X-100 solution (Wako®) for 30 min at room temperature to permeabilize the cell membrane. After washing several times with PBS and treating them with 2% BSA solution for 30 min, the primary antibody diluent (anti-histone H3.3 G34W, 1:400; RANKL, 1:500) was administered, and the cells were incubated for 30 min at room temperature. After several washes with PBS, the secondary antibody (FITC goat antirabbit IgG; 1:200, Becton, Dickinson and Company®, Franklin Lakes, New Jersey, USA) was administered, and the cells were incubated at room temperature for 30 min. After several washes with PBS, two drops/well of NucBlue Live Cell Stain Ready Probes reagent (Invitrogen®, Waltham, MA, USA) were added to the cells and allowed to stay at room temperature for 5 min. The staining of the cells was observed under a fluorescence microscope (BZ-9000E KEYENCE®, Osaka, Japan).

Extraction of CL from linseed oil. Linseed oil was separated using silica gel column chromatography (Hexane/EtOH), and fractions containing CL were combined. The average CL content at this stage was ~40% according to HPLC.

Water-soluble tetrazolium salt-8 (WST-8) assay. GCTB cells were treated with trypsin, and the cell suspension was seeded in 96-well tissue culture plates (TPP®) at a concentration of 5×10^3 cells/well. After administering 0, 1.25, 2.5, 5, 10, and 20 nM of CL, the cells

were incubated for 72 h at 37°C in a humidified, 5% CO₂ atmosphere incubator. To measure the absorbance, 10 µl/well of Cell Counting Kit-8 reagent (DOJINDO®, Kumamoto, Japan) were, and cell viability was evaluated using the iMark™ Microplate Absorbance Reader (Bio-Rad®, Hercules, CA, USA). As a control, denatured CL (created by heating for 30 min at 70°C) was administered to the cells at concentrations of 0, 1.25, 2.5, 5, 10, and 20 nM. The cells were cultured for 72 h and were evaluated in the same manner as described above.

Cell cycle assay. The GCTB cells were treated with trypsin, and the cell suspension was seeded (1×10^5 cells/ml) in a 60.1 cm² tissue culture dish (TPP®). After the administration of 10 nM CL to each dish, the cells were incubated for 72 h. Cells without CL were incubated for 72 h and were used as controls. The incubated GCTB cells were treated with trypsin. Thereafter, approximately 1×10^6 cells were transferred to 1.5 ml microcentrifuge tubes (Watson®, Kobe, Japan) and centrifuged at $300 \times g$ for 5 min. The cells were then washed once with PBS, and 1 ml of ice-cold 70% ethanol was slowly added, and the cell concentration was adjusted to 5×10^5 – 1×10^6 cells/ml. The cells were fixed at –20°C for 3 h. Thereafter, 200 µl were transferred to new microcentrifuge tubes, centrifuged at $300 \times g$ for 5 min, and washed with PBS. Muse® Cell Cycle reagent (200 µl; Propidium Iodide, Ribonuclease A, Luminex®, Austin, TX, USA) was added to each microcentrifuge tube and incubated at

room temperature in the dark for 30 min. Measurements were obtained using the Muse® Cell Analyzer (Luminex®).

EdU assay. GCTB cells were treated with trypsin, and the cell suspension was seeded in a 60.1 cm² tissue culture dish (1×10⁵ cells/ml) and incubated with 10 nM CL for 48 h. The controls consisted of cells incubated without CL. GCTB cells were treated with trypsin, and the cell suspension was seeded in the Nunc™ Lab-Tek™ Chamber Slide System (Thermo Scientific®, Waltham, MA, USA) at a concentration of 5×10³ cells/well. After the administration of 1 µl of 10 mM EdU stock solution to each well, the cells were incubated for 3 h. The cells were fixed with 3.7% formaldehyde in PBS and then incubated at room temperature for 15 min. After the removal of the fixative, the cells were washed twice with 3% BSA in PBS. The washing solution was discarded, 1 ml of 0.5% Triton® X-100 in PBS was added to each well, and the cells were incubated at room temperature for 20 min. After washing twice with 3% BSA in PBS, 0.5 ml of Click-iT® reaction cocktail (1×Click-iT® reaction buffer, CuSO₄, and Alexa Fluor® azide, reaction buffer additive) was added to each well. The cells were shielded and incubated at room temperature for 30 min. The reaction cocktail was discarded, and the cells were washed once with 3% BSA in PBS. The nucleic acid of the cells was stained with NucBlue Live Cell Stain Ready Probes reagent (Invitrogen®). The cells were observed using a fluorescence microscope (BZ - 9000E KEYENCE®). The total number of cells in five visual fields and the number of EdU-positive cells were counted using ImageJ® (30, 31). The ratio of EdU-positive cells was calculated and compared statistically between the CL-administered group and the control group (Mann–Whitney *U*-test).

Real-time polymerase chain reaction (PCR). GCTB cells were treated with trypsin, and cell suspensions (approximately 5×10⁵) were seeded in six-well tissue culture test plates (TPP®). The cells were incubated with 10 nM CL for 48 h; control cells were incubated for 48 h without CL. After the removal of DMEM and washing with PBS, TRIZOL® Reagent (1 ml/ well; Invitrogen®) was added, and the cells were detached using a cell lifter (Corning®, Corning, NY, USA). The cell suspension was transferred to 1.5 ml microcentrifuge tubes and incubated at 30°C for 5 min. Subsequently, 0.2 ml chloroform (Wako®) was added to each tube, and the mixture was vortexed for 15 s and incubated at 30°C for 3 min. The mix was centrifuged at 4°C and 12000 × *g* for 15 min, and the aqueous layer was transferred to new microcentrifuge tubes. Isopropyl alcohol (0.4 ml; Wako®) was added to the tube, and the mixture was incubated at 30°C for 10 min. Thereafter, it was centrifuged at 12000 × *g* (4°C) for 10 min. The supernatant was removed and the RNA pellet was vortexed once with 70% ethanol. After centrifugation at 4°C and 7500 × *g* for 5 min, the supernatant was discarded, and the RNA pellet was air dried for 10 min. The pellet was then dissolved in RNase free water, and cDNA was prepared using the Affinity Script QPCR cDNA Synthesis Kit (Agilent®, Santa Clara, CA, USA). A mix containing 10 µl first-strand master mix, 3 µl oligo (dT) primer, and 1 µl Affinity Script RT/RNase Block enzyme was added to each 100 ng of RNA. The mixture was incubated as follows: 25°C for 5 min using a T100™ Thermal Cycler (Bio-Rad®) to allow for the annealing of the primer, 42°C for 45 min to allow for cDNA synthesis, and 95°C for 5 min to terminate the cDNA synthesis reaction. Thereafter, 3 µl cDNA was mixed with 30 µl of 2×QuantiTect SYBR Green 2 PCR Master

Figure 3. The effect of cyclolinopeptide (CL) on the cell viability of giant-cell tumor of the bone (GCTB) stromal cells. a) GCT-A, b) GCT-N, c) GCT-M. Cell viability was decreased in the CL-administered group in a concentration-dependent manner, whereas no decrement was observed in the denaturated CL-administered group (**p*<0.05, ***p*<0.01).

Mix (HotStarTaq® DNA Polymerase QuantiTect SYBR Green PCR Buffer dNTP mix, including dUTP SYBR Green I, ROX™ passive reference dye, 5 mM MgCl₂) and 0.5 µM of the primer. The primers used were as follows: glyceraldehyde 3-phosphate dehydrogenase (GAPDH) forward 5'TGCACCACCAACTGCTTAGC 3' and

Figure 4. The effect of cyclolinopeptide (CL) on the cell cycle of giant-cell tumor of the bone (GCTB) stromal cells. a) GCT-A, b) GCT-N, c) GCT-M. The ratio of cells in the G₀/G₁ phase was increased in the CL-administered group compared with the control group.

reverse 5' GGCATGGACTGTGGTCATGAG 3', RANKL forward 5' GCCTTTCAAGGAGCTGTGCAA 3' and reverse 5' ATCTAAC CATGAGCCATCCACCAT 3', and RUNX2 forward 5' TCTGGCC TTCCACTCTCACT 3' and reverse 5' AAGGTGGCTGG ATAGTGCAT 3' using the StepOne Real-Time PCR system (Applied Biosystems®, Waltham, MA, USA). Real-time PCR was performed as follows: initial activation at 95°C for 10 min, 40 cycles of incubation at 95°C for 30 sec (denaturation), annealing at 55°C for 1 min, and final extension at 72°C for 1 min.

Cycle threshold (Ct) values were calculated in each group. GAPDH expression was used as an internal control. The relative change in expression was determined according to the $2^{-\Delta\Delta C_t}$ method. The amount of mRNA expression in the CL-administered and control groups were statistically analyzed using the Wilcoxon signed-rank test.

Statistical analysis. All statistical analyses were performed with EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan),

which is a graphical user interface for R 2.13.0 (R Foundation for Statistical Computing, Vienna, Austria). EZR is a modified version of R commander (version 1.6-3) that is designed to add statistical functions that are used frequently in biostatistics (32).

Results

Confirmation of established GCTB cell lines. To confirm the established cell lines as GCTB stromal cells, the cells were stained with anti-histone H3.3 G34W, a driver gene mutation in GCTB stromal cells, and anti-RANKL antibody, which is highly expressed in GCTB stromal cells. All three established cell lines showed staining for anti-histone H3.3 G34W (Figure 1) and the anti-RANKL antibody (Figure 2). Therefore, all cell lines were confirmed as GCTB stromal cells.

Figure 5. The effect of cyclolinopeptide (CL) on the DNA synthesis of giant-cell tumor of the bone (GCTB) stromal cells. (a) Fluorescence microscope. (b) Ratio of EdU-positive cells. The ratio of EdU-positive cells was decreased in the CL-administered group compared with the control group (* $p < 0.05$).

Effect on the cell proliferation of GCTB stromal cells. To assess the effect of CL on the cell viability of GCTB stromal cells, WST-8 assay was performed. The cell viability was decreased in the CL-administered group concentration-dependently, but not in the denatured CL-administered group. Cell viability was significantly different between the two groups (Figure 3), thus indicating that CL decreased the cell viability of GCTB stromal cells.

To investigate the mechanism by which CL inhibits GCTB viability, cell cycle assay using flow cytometry and EdU assay were performed. In cell cycle assay, the ratio of cells in the G_0/G_1 phase was increased in the CL-administered group compared with the control group (Figure 4). In EdU assay, the ratio of EdU-positive cells was decreased in the CL-administered group compared with the control group (Figure 5a) and the difference was statistically significant (Figure 5b). These findings indicated that CL inhibited DNA synthesis of GCTB stromal cells.

Effect on the molecular biology of GCTB stromal cells. To investigate the effect of CL on the molecular biology of GCTB stromal cells, real-time PCR was performed. The

expression of *RANKL* mRNA was significantly decreased in the CL-administered group compared with the control group (Figure 6a). Similarly, *RUNX2*, which is upstream of *RANKL* and is highly expressed in GCTB stromal cells, was significantly decreased in the CL-administered group compared with the control group (Figure 6b). In contrast, the expression of *c-fos* mRNA in the CL-administered group was not significantly different from that in the control group (Figure 6c). These findings indicated that CL decreased the mRNA expression levels of *RANKL* and *RUNX2* but not *c-fos* in GCTB stromal cells.

Discussion

In this study, we established GCTB cell lines from clinical specimens because they are not commercially available. The cell lines were established according to the report by Goldring *et al.* (28) and were confirmed by examining the presence of the H3F3A mutation, which is a driver gene mutation in GCTB stromal cells, and the expression of *RANKL*, which is highly expressed in GCTB stromal cells, via immunofluorescence (8, 9, 29). In the WST-8 assay, which was used to examine the

Figure 6. Effect on the expression of RANKL, RUNX2 and c-fos of giant-cell tumor of the bone (GCTB) stromal cells. (a) RANKL: The expression levels of RANKL mRNA were decreased in the CL-administered group compared with the control group (* $p < 0.05$). (b) RUNX2: The expression levels of RUNX2 mRNA were decreased in the CL-administered group compared with the control group (* $p < 0.05$, ** $p < 0.01$). (c) c-fos: The expression levels of c-fos mRNA were not significantly different from that in the control group.

antitumor effect of CL, the number of live cells decreased in a concentration-dependent manner in all cell lines treated with CL. Thereafter, the mechanism by which this antitumor effect was exerted was examined using the cell cycle assay and flow cytometry. The number of cells in the G_0/G_1 phase was found to be increased in cells treated with CL. To assess the effect on

DNA synthesis, the EdU assay was performed, which showed a decrease in the number of EdU-positive cells. These results indicated that the antitumor effect of CL is based on the inhibition of cell proliferation *via* the suppression of DNA synthesis.

It is known that GCTB stromal cells strongly express RANKL. Activation of RANK-RANKL signalling induces bone destruction *via* the development of the giant cells (8, 9). The effect of CL on RANKL expression was evaluated by real-time PCR, which showed a decrease in the expression of this gene in all cell lines in the CL-administrated group. This finding suggested the possibility that CL prevents the bone destruction in GCTB. RUNX2, which is upstream of RANKL (33), is known to be associated with cell proliferation (34, 35). In the current study, CL suppressed the expression of RUNX2, which may be the key mechanism responsible for the suppression of RANKL expression and cell proliferation in the GCTB cells.

Previous studies have shown that CL-J, which is a cyclolinopeptide, selectively suppresses the MEK-ERK-c-fos pathway because of RANKL stimulation during the process of differentiation into osteoclasts (27). In the present study, we investigated the expression of c-fos in the GCTB cells and found that CL did not affect c-fos expression. Further studies are required to evaluate the effects of CL on other factors such as the upstream factors of RUNX 2 or other signalling pathways.

Drug therapy using anti-RANKL antibody (denosumab) has been reported to be effective for GCTB. By inhibiting the RANK-RANKL signalling in GCTB, denosumab suppresses bone destruction by preventing the development of giant cells (36, 37). However, denosumab cannot eliminate stromal cells, and the proliferation of stromal cells is maintained after denosumab treatment (38). Furthermore, the long-term use of denosumab presents problems such as abnormal bone metabolism and malignant transformation of the tumour (39-42). By contrast, flax is already widely consumed as a health food supplement (11). It has been reported that CL, which is an extract from flax, inhibits osteoclast differentiation in a dose-dependent manner without affecting normal cell viability (26). Therefore, CL might be a safer therapeutic agent than denosumab.

Conclusion

The findings of this study suggested that CL has antitumor effects on GCTB *in vitro*. In the future, drugs developed on the basis of CL are expected to be new therapeutic agents for GCTB.

Conflicts of Interest

The Authors declare no conflicts of interest associated with this manuscript.

Authors' Contributions

Yuta Taniguchi, Norio Yamamoto and Toshio Kaneda designed the study. Yuta Taniguchi wrote the initial draft of the manuscript. Norio Yamamoto, Akihiko Takeuchi and Toshio Kaneda contributed to analysis and interpretation of data, and assisted in the preparation of the manuscript. Junzo Kamei, Ailfarius Eko Nugroho, Toshio Kaneda and Hiroshi Morita provided the cyclolino peptide for the experiments. Hiroyuki Tsuchiya supervised the project as the head of department. All other authors have contributed to data collection and interpretation, and critically reviewed the manuscript. All Authors approved the final version of the manuscript, and agreed to be accountable for all aspects of the work, ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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