Xanthohumol inhibits angiogenesis by suppressing nuclear factor-κB activation in pancreatic cancer

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INTRODUCTION

Pancreatic cancer is the third leading cause of cancer-related death in the USA. In 2016, there were an estimated 53,070 new cases of pancreatic cancer and 41,780 pancreatic cancer-related deaths. The death rates for patients with pancreatic cancer are increasing, and pancreatic cancer is predicted to be the second leading cause of cancer-related death in the USA by 2030. Since 1997, gemcitabine therapy has become the standard treatment for pancreatic cancer. Compared with single-agent gemcitabine, FORFIRI-NOX and gemcitabine with nab-paclitaxel have recently been shown to be associated with improved survival; however, the effects of this drug
combination are largely palliative, and these regimens show increased toxicity. The 5-year relative survival rate for pancreatic cancer in the USA is only 8%. Therefore, there is an urgent need for the development of new therapeutic compounds with low toxicity.

Natural compounds are generally considered to be safe and have been shown to have anti-inflammatory and anticancer effects. Xanthohumol, a prenylated chalcone, is the principal flavonoid found in the hop plant (Humulus lupulus L.). Xanthohumol has been shown to inhibit the growth of different types of human cancer cells, including breast, colon, hepatocellular, ovarian, pancreatic and prostate cancer cells as well as leukemia cells. In addition, xanthohumol has been shown to induce both caspase-dependent and caspase-independent apoptosis and to inhibit invasion and angiogenesis. However, the mechanisms through which xanthohumol mediates these effects are not fully understood. Xanthohumol has been shown to inhibit nuclear factor-κB (NF-κB) activation. In addition, xanthohumol has been shown to have a low toxicity profile and high bioavailability, and orally administered xanthohumol does not affect major organ function in vivo.

Nuclear factor-κB is a transcription factor that is associated with cell proliferation, invasion, angiogenesis and metastasis in multiple types of cancer. In pancreatic cancer, NF-κB has also been shown to play a major role in angiogenesis, which is essential for tumor growth and metastasis. In our previous studies, we found that angiogenesis is essential for metastasis of pancreatic cancer to the liver. Moreover, NF-κB has been shown to be constitutively activated in pancreatic cancer cells. These findings suggest that agents inhibiting NF-κB activation could reduce angiogenesis in pancreatic cancer. In previous studies, vascular endothelial growth factor (VEGF) and interleukin-8 (IL-8) have been identified as key mediators of angiogenesis in pancreatic tumors. Indeed, we previously demonstrated that suppression of NF-κB signaling reduces the production of both VEGF and IL-8, which are major angiogenic factors, in pancreatic cancer. However, the effects of xanthohumol in pancreatic cancer have not been clearly elucidated.

In this study, we initially confirmed that low-dose xanthohumol inhibited NF-κB signaling. We next investigated whether xanthohumol suppressed angiogenesis in pancreatic cancer. We found that xanthohumol blocked angiogenesis in pancreatic cancer by reducing both NF-κB activity and consequent production of angiogenic factors, in vitro and in vivo. This is the first report demonstrating the effects of xanthohumol on pancreatic cancer angiogenesis. In addition, our results suggest that xanthohumol may be useful in treating pancreatic cancer.

2 | MATERIALS AND METHODS

2.1 | Reagents

Xanthohumol and DMSO were purchased from Sigma-Aldrich (St. Louis, MO, USA). Xanthohumol was dissolved in DMSO into stock concentrations of 50 mmol/L. Rabbit monoclonal antibodies against Ki67, NF-κB p65 and VEGF and rabbit polyclonal antibodies against CD31 and IL-8 were purchased from Abcam plc (Cambridge, UK). Neutralizing monoclonal anti human VEGF and IL-8 antibody were provided by R&D Systems (Abingdon, UK).

2.2 | Cell lines and cell culture

The human pancreatic adenocarcinoma cell lines BxPC-3, MIA PaCa-2 and AsPC-1 were obtained from ATCC (Rockville, MD, USA), BxPC-3 and AsPC-1 cells were cultured in Roswell Park Memorial Institute (RPMI-1640) medium (Sigma Aldrich), and MIA PaCa-2 cells were cultured in DMEM supplemented with 10% FBS, 10 000 U/mL penicillin, 10 mg/mL streptomycin and 25 μg/mL amphotericin B (Sigma Aldrich) in a 37°C humidified incubator with 5% CO₂.

2.3 | Cell proliferation assay

The proliferation assay was performed using a Premix WST-1 Cell Proliferation Assay System (Takara Bio, Japan) according to the manufacturer's instructions. BxPC-3 (3×10⁵), MIA PaCa-2 (2×10⁵) and AsPC-1 (1×10⁵) cells were seeded into each well of a 96-well plate in a total volume of 100 μL and cultured for 1 day. The cells were then treated with various concentrations (0-50 μmol/L) of xanthohumol. After 72 hours of incubation, absorbance was measured at 450 nm using a SpectraMax 340 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA).

2.4 | Real-time quantitative RT-PCR

Total RNA was extracted from cell pellets using an RNaseasy Plus Mini Kit (Qiagen, Tokyo, Japan), and total RNA (1 μg) was reverse transcribed using Super Script III First-Strand Synthesis SuperMix for quantitative RT-PCR (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Quantitative RT-PCR was carried out using TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) and TaqMan Gene Expression Assays for VEGF (Hs00900055_m1), IL-8 (Hs01553824_g1), and β-actin (Hs99999903_m1; Applied Biosystems, Foster City, CA, USA) with an Applied Biosystems 7900HT Fast Real-Time PCR System (Applied Biosystems). PCR conditions were as follows: fast mode at 95°C for 20 seconds, followed by 40 cycles at 95°C for 1 second and 60°C for 20 seconds. The relative expression levels of VEGF and IL-8 were normalized to the expression of β-actin in each sample.

2.5 | Analysis of vascular endothelial growth factor and interleukin-8 levels by ELISA

BxPC-3 (5×10⁵), AsPC-1 (5×10⁵) and MIA PaCa-2 (2×10⁵) cells were seeded into 6-well plates containing medium with 10% FBS overnight. The medium was then changed, and the cells were cultured for an additional 72 hours in the presence of different
concentrations of xanthohumol (0-5 μmol/L) with 5% FBS. After incubation, the culture medium was collected and centrifuged at 400 g for 5 minutes to remove particles. The supernatants were frozen at −80°C until use. The concentrations of VEGF and IL-8 were determined using ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s instructions.

2.6 | Protein extraction and nuclear factor-kB p65 activity assays

BxPC-3, AsPC-1 and Mia PaCa-2 cells were seeded in 100-mm dishes with 10% FBS and cultured to approximately 70% confluence. The cells were then treated with different concentrations of xanthohumol (0-25 μmol/L) with 2% FBS for 24 hours and the cells were stimulated with or without tumor necrosis factor (TNF)-α (10 ng/mL) for 30 minutes before the end of the incubation. After the indicated treatment, nuclear extracts were obtained from cells using a Nuclear Extraction kit (Active Motif, Carlsbad, CA, USA). The concentrations of proteins in the nuclear extracts were measured using a Pierce Bradford Protein Assay Kit (Thermo Fisher). The nuclear extracts were stored at −80°C until use. The activity of NF-κB was measured using a Trans AM NF-κB p65/p50 Transcription Factor Assay Kit (Active Motif) according to the manufacturer’s instructions. Five micrograms of nuclear extracts were used in the NF-κB activity assay.

2.7 | Angiogenesis assay

The influence of VEGF and IL-8 on tube formation by HUVEC in the presence of pancreatic cancer cells was investigated in triplicate using an Angiogenesis Kit (Kurabo, Osaka, Japan) according to the manufacturer’s instructions. We cocultured pancreatic cancer cells (BxPC-3), HUVEC and fibroblasts using a double-chamber method in 24-well plates. BxPC-3 cells (2 × 10^5 cells) were seeded into transwell chambers and allowed to adhere overnight. The chambers were then placed into the HUVEC/fibroblast coculture system. The basal medium only (control) or basal medium containing anti-VEGF antibody (10 μg/mL) or anti-IL-8 antibody (10 μg/mL) was added and changed every 3 days. Cells were cultured for a total of 11 days. HUVEC were then stained with anti-CD31 antibody according to the manufacturer’s protocols. The tube formation area was measured quantitatively over 15 different fields for each condition using an image analyzer (Kurabo). The influence of xanthohumol on pancreatic cancer-induced tube formation was examined in triplicate using an Angiogenesis Kit (Kurabo) according to the manufacturer’s instructions. Pancreatic cancer cells (BxPC-3) were cocultured with HUVEC and fibroblasts using a double chamber method in 24-well plates. BxPC-3 cells (2 × 10^5 cells) were seeded into transwell chambers and cultured for 1 day; the chambers were then placed into the HUVEC/fibroblast coculture system. The optimized medium was added with various concentrations of xanthohumol (0-1 μmol/L), and medium was refreshed every 3 days. Cells were cultured for 11 days, and the HUVEC tube formation was evaluated as described above.

2.8 | Animals

Female BALB/c nu-nu mice (6 weeks old) were purchased from Charles River Laboratories (Sulzbach, Germany). The animals were housed in standard Plexiglas cages (5 per cage) in a room maintained at constant temperature and humidity with a 12-hour light/dark cycle. Mice were fed regular autoclaved chow and provided with water ad libitum. All experiments were conducted according to the Guidelines for Animal Experiments of the Nagoya City University Graduate School of Medical Sciences and approved by the Animal Care and Use Committee of the Nagoya City University Graduate School of Medical Sciences.

2.9 | Experimental protocol

BxPC-3 human pancreatic cancer cells (5 × 10^6 cells in 100 μL Hank’s balanced salt solution) were injected subcutaneously into the right flank of each mouse. Tumor volume was measured once a week with a caliper. When the tumor size reached a volume of approximately 100 mm³, the mice were randomly divided into 2 groups (5 mice per group); mice in group I were not treated, whereas mice in group II were given xanthohumol (10 mg/kg/wk). We calculated the tumor volume according to the following formula: tumor volume (mm³) = (A × B²)/2, where A and B represented the longest and the shortest diameters in millimeters, respectively. The mice were injected intraperitoneally with DMSO or xanthohumol once a week for 5 weeks and then sacrificed 1 week later. Finally, the tumors were harvested from mice and fixed in formaldehyde for further analysis.

2.10 | Histopathological examination

Subcutaneous xenograft tumors were fixed with paraformaldehyde and embedded in paraffin. The sections were mounted on 3-amino-propyltriethoxysilane-coated slides. Dewaxed paraffin sections were placed in a microwave (10 minutes, 600 watts) to recover antigens before staining. Antibodies used were as follows: rabbit monoclonal anti-Ki67 antibodies (ab92742; 1:500), rabbit polyclonal anti-CD31 antibodies (ab124432; 1:1000), rabbit monoclonal anti-NF-κB p65 antibodies (ab32536; 1:100), rabbit monoclonal anti-VEGFA antibodies (ab52917; 1:100) and rabbit polyclonal anti-IL-8 antibodies (ab7747; 1:100; all from Abcam), followed by biotin-conjugated secondary antibodies. Peroxidase-conjugated streptavidin was used with 3,3-diaminobenzidine tetrahydrochloride (DAB; Biocare Medical, Concord, CA, USA) as the chromogen for detection. Hematoxylin was used for nuclear counterstaining.

Proliferation was quantified by determining the percentage of Ki67-positive cells. Results were expressed as the mean number of Ki67-positive cells ± the SD per high-power field. Ten fields were examined and counted from 3 tumors for each of the 2 treatment groups. For quantification of microvessel density (MVD) in sections stained for CD31, any distinct area of positive staining for CD31 was counted as a single vessel. Results were expressed as the mean
number of vessels ± SD per high-power field. Ten high-power fields were examined and counted from 3 tumors for each of the 2 treatment groups. For quantification of activation of NF-κB p65, the numbers of nuclear-positive cells were counted. Nuclear translocation of p65 was considered a marker of NF-κB activation. The numbers of NF-κB p65-positive (in nuclear), VEGF-positive and IL-8-positive cells were expressed as percentages of the total number of cells that were randomly counted in 10 high-power fields from 3 tumors of each of the 2 treatment groups for quantitative analysis.

2.11 | Statistical analysis

All experimental data were presented as the mean ± SD. Multiple group comparisons were performed using 1-way ANOVA with post-hoc Dunnett tests for subsequent individual group comparisons. Comparisons between 2 groups were performed using Student’s t-tests. Results with P-values of <.05 were considered significant.

3 | RESULTS

3.1 | Xanthohumol inhibited proliferation in pancreatic cancer cells

The effects of xanthohumol on the proliferation of human pancreatic cancer cells (BxPC-3, AsPC-1 and Mia PaCa-2 cells) were examined using WST-1 assays after incubation with various concentrations of xanthohumol (0-25 μmol/L) for 72 hours. As shown in Figure 1A, xanthohumol (10 μmol/L) significantly inhibited the proliferation of all 3 pancreatic cancer cell lines in a concentration-dependent manner.

3.2 | Xanthohumol inhibited nuclear factor-kB activation in pancreatic cancer cells

To examine the effects of xanthohumol on the activity of NF-κB, we performed Trans AM NF-κB p65/p50 Transcription Factor Assays. As shown in Figure 1B, treatment with various concentrations of xanthohumol for 24 hours significantly inhibited NF-κB activation under treatment with or without TNF-α in all 3 pancreatic cancer cell lines in a concentration-dependent manner. Interestingly, xanthohumol decreased the activity of NF-κB when used at a low concentration that did not affect the proliferation of the pancreatic cancer cells.

3.3 | Xanthohumol suppressed VEGF and IL-8 mRNA expression in pancreatic cancer cells

To determine whether xanthohumol inhibited VEGF and IL-8 mRNA expression, we performed real-time qPCR. As shown in Figure 2, VEGF and IL-8 mRNA levels were significantly suppressed by xanthohumol treatment in a concentration-dependent manner.

3.4 | Xanthohumol suppressed the secretion of vascular endothelial growth factor and interleukin-8 in pancreatic cancer cells

To determine whether the reduction in mRNA expression led to a reduction in protein expression, we measured the concentrations of secreted VEGF and IL-8 after treatment with xanthohumol using ELISA. As shown in Figure 3, xanthohumol significantly inhibited the secretion of VEGF and IL-8 in a concentration-dependent manner (>1 μmol/L).

3.5 | Xanthohumol inhibited tube formation by HUVEC in coculture with BxPC-3 cells

To examine the effects of BxPC-3-derived VEGF and IL-8 on tube formation by HUVEC, we performed in vitro angiogenesis assays using neutralizing antibody against VEGF and IL-8. As shown in Figure 4, anti-VEGF and anti-IL-8 antibody suppressed tube formation induced by BxPC-3 cells. To examine the effects of xanthohumol on tube formation by HUVEC, we performed in vitro angiogenesis assays. As shown in Figure 4, coculture with BxPC-3 cells significantly enhanced tube formation in HUVEC, and this enhancement was significantly inhibited by treatment with xanthohumol in a concentration-dependent manner (>0.5 μmol/L).

3.6 | Xanthohumol suppressed tumor growth and angiogenesis in a subcutaneous xenograft model

To determine the antitumor effects of xanthohumol in vivo, a BxPC-3 subcutaneous xenograft model was developed in nude mice. Once the tumors were developed, xanthohumol (10 mg/kg) or vehicle was administered weekly by intraperitoneal injection for 5 weeks. As shown in Figure 5, xanthohumol significantly inhibited tumor growth 21 days after treatment (Figure 5B,C). Moreover, loss of body weight was not observed, and body weights were similar between the 2 groups (Figure 5D).

3.7 | Xanthohumol inhibited proliferation and angiogenesis and reduced the expression of nuclear factor-κB p65, vascular endothelial growth factor and interleukin-8

To determine the effects of xanthohumol on proliferation and angiogenesis, we examined the expression of Ki-67, the cell proliferation marker, and CD31, an MVD marker, in tumor tissue. As shown in Figure 6, xanthohumol significantly downregulated the expression of Ki-67 and CD31. We next examined both the activation of NF-κB p65 and the expression of angiogenic factor (VEGF and IL-8). As shown in Figures 6 and 7, xanthohumol significantly decreased both the activation of NF-κB p65 and the expression of VEGF and IL-8 in tumor tissues. As shown in Figure 7, xanthohumol significantly suppressed VEGF and IL-8 mRNA expression in pancreatic cancer tumors.
The aim of this study was to determine whether xanthohumol, a flavonoid compound isolated from the hop plant (*Humulus lupulus* L.), could inhibit angiogenesis by blocking NF-κB activation in pancreatic cancer. Our data demonstrated that xanthohumol inhibited both proliferation and NF-κB activation and suppressed the expression of VEGF and IL-8 in various human pancreatic cancer cell lines. In addition, we confirmed that xanthohumol suppressed tube formation, which was induced by pancreatic cancer, in an in vitro angiogenesis model. Finally, xanthohumol inhibited tumor growth and angiogenesis in a subcutaneous xenograft model in vivo.

Previous studies have shown that most pancreatic cancer cells (approximately 70%) exhibit constitutive activation of NF-κB. Moreover, NF-κB is thought to have an important role in the regulation of apoptosis, oncogenesis, and angiogenesis. Indeed, we previously reported that blockade of NF-κB activity by the proteasome inhibitor MG132 inhibited the expression of the pro-angiogenic molecules VEGF and IL-8 and suppressed cancer-induced angiogenesis. These reports were consistent with our results demonstrating that xanthohumol inhibited the expression of VEGF and IL-8 by blocking NF-κB activation in pancreatic cancer.

The aggressive growth and metastasis of solid tumors, including pancreatic cancer, depend on angiogenesis, which is mediated by...
Various pro-angiogenic molecules secreted by pancreatic cells, including VEGF and IL-8, have been identified as key mediators of pancreatic cancer angiogenesis. Thus, it is reasonable to assume that inhibiting NF-κB activation, which leads to suppression of VEGF and IL-8 expression, would suppress angiogenesis in pancreatic cancer. In our study, we demonstrated that xanthohumol suppressed cancer-induced tube formation in HUVEC in an in vitro angiogenesis assay.

Interestingly, this is the first report showing that xanthohumol inhibited angiogenesis by blocking NF-κB activation in pancreatic cancer cells. We hypothesized that xanthohumol may not only have direct antiproliferative effects on pancreatic cancer cells at higher concentrations but may also have anti-angiogenic effects through suppression of VEGF and IL-8 expression in pancreatic cancer cells at lower concentrations. Xanthohumol, at concentrations >10 μmol/L, inhibited proliferation in pancreatic cancer cells in our in vitro analysis.

However, when used at concentrations of <5 μmol/L, xanthohumol inhibited NF-κB-dependent angiogenic activities in pancreatic cancer cells. At this concentration, we did not observe any cytotoxicity in WST-1 assays in pancreatic cancer cells. Thus, we concluded that xanthohumol affected pancreatic cancer-induced angiogenesis through downregulation of VEGF and IL-8 production, which was specific and mediated through NF-κB inactivation.

In addition to our in vitro results, our findings showed that xanthohumol significantly inhibited tumor growth in a subcutaneous xenograft model. Our immunohistochemistry analysis showed that xanthohumol suppressed 2 major markers: the proliferation marker Ki-67 and the MVD marker CD31. We also demonstrated that xanthohumol downregulated both the activation of NF-κB p65 and the expression of angiogenic factor (VEGF and IL-8). Thus, our results suggested that xanthohumol inhibited angiogenesis in pancreatic cancer by downregulating these angiogenic proteins in vivo.
the focus of this study is to examine the efficacy of xanthohumol on pancreatic cancer growth and tumorigenesis from the standpoint of angiogenesis, we used a subcutaneous injection model and successfully demonstrated that xanthohumol could inhibit pancreatic cancer growth by blocking NF-κB and its downstream angiogenic targets (VEGF and IL-8) in vitro and in vivo. Because our next focus is the efficacy of xanthohumol on pancreatic cancer metastasis, in the near future, we intend to perform an intra-splenic liver metastasis assay using 2 cancer cell lines.

In this study, we focused on the effects of natural products, which are generally regarded as safe and nontoxic. Indeed, our findings demonstrated that xanthohumol did not affect the body weights of nude mice, consistent with the results of previous studies. In a previous report, orally administered xanthohumol was shown to have no adverse effects on the functions of major organs and did not affect female fertility or the development of offspring of Sprague-Dawley rats. Chemicals that inhibit NF-κB activation, such as bortezomib (a protease inhibitor), have various side effects (eg, acute...
lung injury, peripheral neuropathy, tumor lysis syndrome and myelosuppression). Therefore, it is very difficult to use these drugs in combination with chemotherapies for treating pancreatic cancer.\textsuperscript{35,36} However, because we showed that xanthohumol has few adverse effects, xanthohumol may not be harmful to humans.

Overall, our results showed, for the first time, that xanthohumol inhibited angiogenesis by blocking NF-κB and its downstream targets, leading to the inhibition of angiogenesis, even at low concentrations. Because low-dose xanthohumol did not block normal physiological function, use of the natural product xanthohumol may be safer and may show reduced toxicity compared with other available treatments. Therefore, we concluded that xanthohumol may have significant potential as an effective therapy for pancreatic cancer.

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**CONFLICT OF INTEREST**

The authors declare no potential conflicts of interest.

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