Xanthohumol inhibits cellular proliferation in a breast cancer cell line (MDA-MB231) through an intrinsic mitochondrial-dependent pathway

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Abstract

Aims: Xanthohumol isolated from hops has been reported to exhibit anticancer effects in diverse human cancers. However, its effect on breast cancer has not yet been clearly defined. The purpose of this study was to investigate the effects of xanthohumol on breast cancer cell proliferation. Materials and Methods: After treatment with 5 μM, 10 μM, and 20 μM xanthohumol for 48 h, cells from the human breast cancer cell line MDA-MB-231 were studied using colony assay, flow cytometry, and western blotting. Results: The survival rate of the MDA-MB231 cells treated with 10 μM and 20 μM xanthohumol for 48 h decreased significantly by 64.7 ± 1.8% and 40.1 ± 1.8%, respectively. The numbers of SubG0/G1 cells in the group treated with 10 μM and 20 μM xanthohumol increased significantly to 11.3 ± 0.2 and 18.4 ± 0.1, respectively. A ladder pattern of DNA fragmentation was also observed. Xanthohumol increased the expression of Bax in the mitochondria, which correspondingly decreased in the cytoplasm. The activity of caspase-3 and caspase-9 was shown to increase significantly in the treated groups but not in the control group. Conclusions: Xanthohumol inhibited the proliferation of MDA-MB-231 cells through a mitochondria- and caspase-dependent apoptotic pathway. This result suggests that xanthohumol might serve as a novel therapeutic drug for breast cancer.

Key Words: Apoptosis, breast cancer, caspase, mitochondria, xanthohumol

Introduction

In recent years, there has been a growing interest in phytochemical compounds with anticancer potential, which show lower toxicities than conventional chemotherapeutic agents.[1–3] A number of chemicals originating from medicinal herb plants have been reported to exhibit therapeutic activities as anticancer drugs.[4–7] The induction of apoptosis or debilitation of cancer cells by natural compounds, without causing excessive damage to normal cells, is a key factor in the identification of methods for cancer prevention and therapy.[8,9]

Xanthohumol is a prenylated chalcone found naturally in hops (Humulus lupulus L.), an essential raw material for beer brewing and a rich source of prenylated flavonoids. Recent studies have reported a promising role for xanthohumol as a chemopreventive agent, because it can modulate the metabolism of carcinogens and act through cytotoxic/static mechanisms on cancer cells.[10] This prenylated flavonoid belongs to a subclass of the flavonoids, and it has been demonstrated to inhibit proliferation of human breast cancer cell line (MCF-7), colon cancer cell line (HT-29), and ovarian cancer (A2780) cell line in vitro.[11,16]

Cancer chemopreventive properties of xanthohumol have also been identified by cell- and enzyme-based in vitro bioassays using markers relevant for the inhibition of carcinogenesis during the initiation, promotion, and progression phases.[11]

Despite recent studies focused on xanthohumol, few data are available about the anticancer effects of xanthohumol from hops in the aggressive breast cancer cell line MDA-MB-231.

In this study, we evaluated the antiproliferative activities and the mechanisms underlying these activities of xanthohumol in MDA-MB-231.

Materials and Methods

Xanthohumol was purchased from Sigma Chemicals (St. Louis, MO). MDA-MB231 cell lines from American type cell collection were maintained in D-MEM (Gibco BRL, New York, USA), supplemented with 10% fetal bovine serum (Hyclone Laboratories, Logan, UT, USA), at 37°C and with 5% CO2.

The experiments were carried out 4 times independently. To determine the cell survival rate after treatment with xanthohumol, absorption of the dye 3-(4, 5-dimethylthiazol-2-yl)-2.5-diphenyl tetrazolium bromide was determined through a colorimetric assay. Visible absorption at 540 nm was measured using an ELISA reader (THERMO max, USA). After propidium iodide (PI) staining, the DNA content was analyzed using FACScan flow cytometry (Becton-Dickinson, NJ, USA) according to the manufacturer’s instructions. The percent of SubG0/G1 cells in the DNA histogram was calculated relative to the total number of cells present.

Genomic DNA was isolated from treated and untreated cancer cells by using a Wizard Genomic DNA purification kit (Promega Co., WiWconsin Medicine, and WI) following the manufacturer’s protocol. Five micrograms of genomic DNA was run on a 1.5% agarose gel for examining DNA fragmentation.

After treating the cultured cells with cisplatin, the cells were washed twice with PBS. The obtained cells were then reacted with Buffer A (250 mM sucrose, 20 mM Heps, pH 7.5, 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 17 μg/mL PMSF, 8 μg/mL aprotinin, 2 μg/mL leupeptin) for 20 min, and then crushed with a homogenizer 10 times and centrifuged at 4°C, 750 × g, for 10 min. The supernatant was transferred to a new tube, subjected to repeated centrifugation at 4°C, 20,000 × g, for 10 min, and then used for cytosol electrophoresis; the remaining pellet was retained for mitochondria electrophoresis. For the subsequent analyses, western blotting was performed.

After treating the cultured cells with various concentrations of xanthohumol, the cells were captured and cleaned twice with cold Hank’s buffered salt solution. The obtained cells
were dissolved on ice with RIPA solution (50 mM Hepes, pH 7.4, 150 mM NaCl, 1% deoxycholate, 1 mM EDTA, 1 mM PMSE, 1 μg/ml aprotinin, 2 mM Na3VO4, and 100 μM phenylarsine oxide). After mixing with double the volume of sample buffer (5 mM EDTA, 4% SDS, 20% glycerol, 200 mM Tris, pH 6.8, and 0.06% bromophenol blue) the dissolved cell suspension was boiled at 100°C for 3 min and subjected to 12.5% SDS-PAGE. Upon completion of electrophoresis, the protein from the gel was transferred to a nitrocellulose membrane at 4°C and 30 V for 16 h. The membrane was then blocked with a blocking buffer (5% skim milk) at room temperature for 1 h. The protein on the membrane was probed with the primary antibody (antiprocaspsases 3, 8, and 9; diluted 1:1000 in TBST), at room temperature for 2 h. The protein was then probed with a secondary antibody-IgG-conjugated horseradish peroxidase (diluted 1:1000 in TBST, Amersham Co., Buckinghamshire, England) at room temperature for 1 h. Western blotting was subsequently conducted. The cells were dissolved in a lysis buffer (1% Triton X-100, 0.32 M sucrose, 5 mM EDTA, 1 mM PMSE, 1 μg/ml aprotinin, 1 μg/mL leupeptin, 2 mM DTT, 10 mM Tris-HCl, pH 8.0) at 4°C for 15 min and then centrifuged at 14,000 rpm for 15 min. The protein content in the upper layer of the liquid obtained after centrifugation was quantified via UV/visible absorption (OD540) following 30 min submersion in the BCA solution. An equivalent amount of protein was diluted in the caspase assay buffer (100 mM Hepes, 10% sucrose, 0.1% Chaps, 1 mM PMSE, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 2 mM DTT, pH 7.5) and allowed to respond to a fluorescent substrate (AMC-DEVD) at 37°C for 30 min. Fluorescence was then measured with a fluorometer (Molecular Devices Co., Sunnyvale, USA). Proteolytic cleavage of the fluorogenic substrates 50 μM AMC-DEVD, 50 μM Ac-LEAD-AFc (Ac-Leu-Glu-His-Asp-AFc), and 50 μM Z-IETD-AFc (Z-Ile-Glu-Thr-Asp-AFc) for caspase-3, caspase-9, and caspase-8 proteases, respectively, was measured for assessing caspase activity. The results were statistically processed using student’s t-test, where P < 0.05 were considered to be significant.

**Results**

To observe the effect of xanthohumol on cellular growth in breast cancer, MDA-MB-231 cells were treated with 5 μM, 10 μM, and 20 μM xanthohumol for 48 h. Survival in the groups treated with xanthohumol decreased in proportion to the treatment dose, and the survival rate in the group treated with 10 and 20 μM xanthohumol for 48 h decreased significantly by 64.7 ± 1.8% and 40.1 ± 1.8%, respectively [Figure 1a]. To observe the effect of xanthohumol on the cell death of breast cancer cells, the number of SubG0/G1 cells was analyzed through PI staining. Xanthohumol increased the number of SubG0/G1 cells depending on the treatment dose. In particular, the number of Sub G0/G1 cells in the groups treated with 10 μM and 20 μM xanthohumol increased significantly (to 11.3 ± 0.2 and 18.4 ± 0.1, respectively), unlike in the control group [Figure 1b].

First, the apoptotic effect of xanthohumol was shown in a DNA fragmentation assay [Figure 2a]. The ladder patterns were detected by electrophoresis of the DNA from the xanthohumol-treated group. To observe the effect of the concentration of xanthohumol on the cleavage of PARP, MDA-MB231 cells were exposed to xanthohumol at concentrations of 5 μM, 10 μM, and 20 μM for 48 h, and the degree of PARP cleavage was measured. As a result, in the group treated with xanthohumol, the PARP decreased to 116 kDa in a concentration-dependent manner [Figure 2b]. The expression of ICARD also decreased according to the concentration after treatment with xanthohumol.

To investigate whether the mitochondria were involved in the xanthohumol-mediated killing of the MDA-MB231 cells, we exposed the cells to xanthohumol at concentrations of 5 μM, 10 μM, and 20 μM for 24 h. The mitochondria and cytoplasm were then separated by electrophoresis, and then the expressions of cytochrome c and Bax – apoptosis-related proteins – were investigated using western blotting. From the results, the expression levels of cytochrome c in the mitochondria of the group treated with xanthohumol

![Figure 1: Cellular growth of xanthohumol treated breast cancer cells (MDA-MB 231). The survival rate in the MDA-MB231 cells that was treated with 10-μM and 20-μM xanthohumol for 48 h decreased significantly 64.7 ± 1.8% and 40.1 ± 1.8%, respectively, unlike in the control group. The numbers of SubG0/ G1 cells in the group with treatment with 10 and 20 μM xanthohumol increased significantly (11.3 ± 0.2 and 18.4 ± 0.1, respectively), unlike in the control group. Each value is the mean value ± S.E.M. of four independent experiments. (*P < 0.05, †P < 0.01 vs. control)](519)
decreased in a concentration-dependent manner, and the expression of cytochrome c in the cytoplasm increased correspondingly [Figure 2c]. Furthermore, xanthohumol increased the expression of Bax in the mitochondria, which correspondingly decreased in the cytoplasm [Figure 2c].

To examine, whether the increase in caspase-3, caspase-8, and caspase-9 activity was involved in the process of the xanthohumol-mediated killing of MDA-MB231 cells, we exposed the cells to various concentrations of xanthohumol for 48 h, and then the caspase activities were measured using the fluorescent dyes Ac-DEVD-AFC, Ac-IETD-AFC, and Ac-LEHD-AFC for caspase-3, caspase-8, and caspase-9, respectively. The results showed that caspase-3 activity increased significantly in all the treated groups, but not in the control group [Figure 3a]. This result was consistent with the result for cleavage of procaspase-3 [Figure 3b]. Because increase in caspase-3 activity is known to be activated by procaspases 8 and 9, the activities of these procaspases were also measured. The activity and cleavage of procaspase-8 was not changed by xanthohumol treatment; however, the activity of caspase-9 increased in a dose-dependent manner. This result was also verified by the cleavage of procaspase-9 [Figure 3a and b].

To verify the apoptosis mechanism induced by xanthohumol, we observed the number of Sub G0/G1 cells by using PI staining after pretreating the caspase-3, caspase-8, and caspase-9 depressants with xanthohumol. In the pre-treatment cases (caspase-3 with Ac-DEVD-CHO; caspase-9 with Z-LEHD-FMK, and pan-caspase with Z-VAD-FMK), the number of SubG0/G1 cells due to xanthohumol treatment were reduced significantly, but not in the group that had been treated with the caspase-8 depressant, Z-IETD-FMK [Figure 4].

**Discussion**

Recently, xanthohumol has been reported to induce apoptosis in diverse human cell lines, which can potentially be exploited for the treatment of cancer. In addition, its anti-breast cancer effects have been reported in conjunction with other mechanisms. The combination of these effects suggests a promising potential for the development of xanthohumol-based therapies.

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**Figure 2**: DNA fragmentation and apoptosis related protein expression by xanthohumol in MDA-MB231 cells. (a) Agarose gel (1.5%) electrophoresis demonstrating apoptotic DNA fragmentation; M indicates marker for DNA size. (b) Western blotting with PARP and cleaved PARP. (c) The cytochrome c of the mitochondria in the group with xanthohumol decreased in a dose dependent manner, and the expression of cytochrome c in the cytoplasm correspondingly increased. Besides, xanthohumol increased the expression of Bax in the mitochondria and decreased that in the cytoplasm.
Selective apoptosis of cancer cells has become a new mechanism thereof were investigated in MDA-MB231 cells. In this study, the effects of xanthohumol on cell growth inhibition, its apoptotic effects, and the underlying mechanism thereof were investigated in MDA-MB231 cells. Selective apoptosis of cancer cells has become a new focus in the development of anticancer medicines, and many studies are currently being conducted to discover natural anticancer substances extracted from plants or chemical substances that selectively induce the apoptosis of cancer cells. The general process of apoptosis involves characteristic phenomena such as cell contraction by fast cell dehydration, pneumatization of the cell membrane, increased intra-cytoplasmic calcium density, chromatin condensation, and the formation of ladder-pattern DNA fragmentation (200 base pairs) through endonuclease activity. Our study also demonstrated apoptosis by xanthohumol through DNA fragmentation analysis. In addition, the Bcl-2 and caspase family proteins are regarded as basic regulators of apoptotic cell death. Kitanaka et al., found that overexpression of Bax induced apoptotic cell death in COS-7 cells through the activation of caspase-3. They suggested that caspase activation is essential for BAX-induced apoptosis. We confirmed the apoptotic effect of xanthohumol by analyzing intracellular signaling molecules such as Bax and the caspases, which are related to the apoptotic pathway. The caspase protease family, whose apoptosis-inducing mechanism was recently reported, plays an essential role in inducing inflammatory responses and apoptosis. The caspases have been found to exist normally in a non-activated form in cells until they are activated by an intracellular environmental change by an exterior stimulus. Fourteen kinds of caspases have been identified to date, and among these, caspase-3, caspase-8, and caspase-9 play important roles in apoptosis. The inductive mechanism of apoptosis consists of an extrinsic pathway, which takes place through a death receptor in the cell membrane, and an intrinsic pathway, which occurs in the absence of a death receptor, by means of intracellular mitochondria. Caspase-9 participates in the intrinsic pathway, and caspase-8 belongs to the typical caspase protease family that participates in the extrinsic pathway. However, the caspase-3 protease, which acts during the final stage of apoptosis, has a role in both the extrinsic and intrinsic pathways, existing in a pro-enzyme form in the cytoplasm as the mammalian ced-3 homologue. In its pro-enzyme form, the procaspase-3 protease is known to be activated through the cleavage of the pro-form by the initiator caspases such as activated caspase-8 and caspase-9.

In this study, the activity and cleavage of the pro-forms of caspase-3, caspase-8, and caspase-9, which play essential roles in the apoptosis mechanism in the caspase protease family, were investigated. Xanthohumol increased the activity of the caspase-3 and caspase-9 proteases in MDA-MB231 cells according to the treatment time and dose. Xanthohumol treatment was found to increase the isolation of cytochrome c in the cytoplasm rather than in the mitochondria. Conversely, this increase was found to occur in the cytoplasm by facilitating the movement of the Bax protein into the mitochondria. According to the results of a recent study, cytochrome c is released from the
mitochondria into the cytoplasm through disruption of the mitochondria membrane potential. The released cytochrome c is known to activate the caspase-3 protease by combining with apoptotic protease activating factor-1 in the cytoplasm to form an apoptosome.[11-16]

Caspase-8 protease, one of the initiator caspases that activates caspase-3, is known to be activated by the Fas/FasL system.[25,26] Fas, also known as “APO-1” or “CD95,” and its ligand, FasL, are known as mediators of activation-induced cell death and transmit the signals of apoptosis. As a member of the FAS TNF-receptor family, it also transmits the apoptosis activity signals to the Fas-associated death domain (FADD) by combining FAS-L and FADD, whereas, FADD converts caspase-8, an initiator caspase in a non-activated form, into an activated form.[27,28] In this study, we investigated whether increase in the activity of the procaspase-8 protease caused by Xanthohumol was involved in the apoptosis induction mechanism in MDA-MB231 cells. However, the activity of the procaspase-8 protease did not increase, and there was no cleavage of the pro-form of procaspase-8 either. These results suggest that the Fas/FasL/caspase-8 system (i.e., the extrinsic pathway) is not involved in the mechanism underlying the apoptotic effect of xanthohumol.

Considering the above 2 results, we determined that mitochondrial Bax and caspase-3 and caspase-9 activities increased after treatment with xanthohumol in MDA-MB231 cells in our experiment. The above results suggest that the mechanism of xanthohumol-mediated apoptosis in MDA-MB231 cells involves an increase in Bax protein levels in the mitochondria, the resulting release of cytochrome c into the cytoplasm, procaspase-9 protease activity, and caspase-3 protease activity, thereby implying that an intrinsic pathway is involved. Caspase-3, caspase-8, and caspase-9 are involved in xanthohumol-mediated induction of MDA-MB231 cell death. From these results, Ac-DEVD-CHO and Z-LEHD-FMK significantly reduced the number of SubG0/G1 cells, thereby verifying that caspase-3 and caspase-9 were involved in the xanthohumol-mediated induction of apoptosis. However, Z-IETD-FMK could not suppress the increase in the number of SubG0/G1 cells, thereby indicating that the caspase-8 protease is not involved in the xanthohumol-mediated induction of apoptosis.

**Conclusion**

Xanthohumol induced growth inhibition and had an apoptotic effect on MDA-MB231 cells through a mitochondria- and caspase-dependent apoptotic pathway. Our results suggest that xanthohumol may provide a future novel therapeutic drug option for breast cancer.

**Acknowledgment**

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**References**


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Oral cancer, the sixth most common cancer in the world, remains challenging due to its late detection and high mortality rate.

**Visualization Techniques**

Visualization of epithelium, including colposcopy and direct oral microscopy, has been gaining popularity as better results are achieved. These techniques have been recruited to the CD95 (Fas/APO-1) death-inducing signaling complex, helping in the early detection of invasive carcinomas. Chemiluminescence examination of suspicious lesions appears to give reliable results, aiding in applying it as a diagnostic aid in early detection.

**Cytology and Adjuvant Techniques**

Various techniques like cytology, brush biopsies, toluidine blue, and lugol's iodine have been helpful. Direct oral microscopy has been a gold standard for a histopathological diagnosis. But, the early malignant change is not easy to identify, and the malignant potential cannot be based solely on clinical findings.

**Biopsy Selection**

The selection of right biopsy site is crucial for accurate diagnosis. Here is the need of adjuvant diagnostic tools in selection of right biopsy site to pave a right path toward early malignant change. Biopsy has always remained the golden standard for a histopathological diagnosis. The advantages of visualization techniques over cytology have been noted in screening of cervical neoplasia, cervical epithelium, and in screening of cervical neoplasia.

**Chemiluminescence Examination**

Chemiluminescence examination of reactive lesions can show similar autofluorescence, which requires careful interpretation. Innovations in application of this technology are being carried out. Based on the principle of autofluorescence, fluorescence spectroscopy is gaining a foothold as an effective diagnostic tool.

**Raman Spectroscopy**

Few studies based on Raman spectroscopic methodology are being carried out. Based on the principle of autofluorescence, fluorescence spectroscopy in biomedical application, and laser-induced fluorescence has been gaining importance.

**Autofluorescence Imaging**

Autofluorescence imaging of potentially malignant mucosa lesions is being explored. It is being recruited to the CD95 (Fas/APO-1) death-inducing signaling complex. Cell 1996;85:817-27.

**Characterization of Fas (Apo-1, CD95)-Fas ligand interaction**

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