Connexin 43-dependent tumor-suppressing effect of the Bowman-Birk protease inhibitor on M5076 ovarian sarcoma-bearing mice

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Abstract. The present study was designed to confirm whether the Bowman-Birk inhibitor (BBI) induces an increase in p27 accumulation without S phase kinase-associated protein 2 (skp2) degradation by means of the expression of connexin (Cx) 43 as a gap junctional intercellular communication (GJIC)-dependent pathway in mice with M5076 ovarian sarcoma. M5076 ovarian sarcomas (1×10^5 cells/animal) were subcutaneously transplanted onto the backs of BDF_1 mice receiving 10, 20 or 40 mg/kg of purified BBI intraperitoneally. Relative tumor weight ($p < 0.01$, $r = 0.503$) was negatively correlated with the dose of BBI. In contrast, the relative density of Cx43 mRNA ($p < 0.01$, $r = 0.570$) and Cx43 ($p < 0.01$, $r = 0.718$) was positively correlated with the dose of BBI, as were p21 ($p < 0.01$, $r = 0.633$), p27 ($p < 0.01$, $r = 0.561$) and skp2 ($p < 0.01$, $r = 0.733$). We therefore suggest that the anti-carcinogenic effects of BBI induce negative growth control by means of an increase in p27 accumulation caused by the expression of Cx43 as a GJIC pathway.

Introduction

The Bowman-Birk inhibitor (BBI) is a soybean-derived serine protease inhibitor with both trypsin and chymotrypsin inhibitory activites (1). BBI was identified by Bowman in 1946 (2) and later purified by Birk in 1961 (3). This molecule consists of an 8-kDa polypeptide (71 amino acids) and has two separate protease inhibitor sites: sub-domain 1 (NH_2-terminal) and sub-domain 2 (COOH-terminal) for trypsin- and chymotrypsin-like serine proteases, respectively (4). Of the two sites, sub-domain 2 is the more important for chemoprevention, suggesting the importance of chymotrypsin inhibition in BBI chemopreventative activity (4-6). The anti-carcinogenic activity of BBI has been detected at nanomolar concentrations (7), and its ability to suppress carcinogenesis in animals exceeds that of the other potential chemopreventive agents identified in soybeans (8-10).

As cell-cell interaction structures, gap junctions (GJs) are membrane channels that permit the transfer of small water-soluble molecules (≤ 1 kDa), including inositol triphosphate, from the cytoplasm of one cell to that of surrounding cells (11). This process is called gap junctional intercellular communication (GJIC), and plays an important role in the maintenance of homeostatic control in multi-cellular organisms for the regulation of cell proliferation, differentiation, apoptosis, wound-healing and the adaptive responses of differentiated cells (12,13). Most tumor cells have a reduced ability to communicate between themselves and/or with surrounding normal cells, confirming the importance of functional GJIC in growth control (11). Therefore, the inhibition of GJIC between adjacent cells has been postulated to be one of the important events occurring during the promotional stage of cancer (14,15). GJs are composed of proteins known as connexins (Cxs), which consist of a multi-gene family of highly related proteins with molecular weights between 26 and 70 kDa. More recently, direct evidence of the role of GJIC in tumor suppression has been obtained by transfecting Cx genes into non-communicating tumor cells (14,16-19). On the other hand, there is increasing evidence of GJIC-independent roles for Cxs in the control of cell growth and the suppression of carcinogenicity (20). Based on these reports, Cxs are considered to act as tumor suppressor genes in a GJIC-dependent and -independent manner.

A previous study of ours demonstrated that the anti-carcinogenic effects of BBI induced negative growth caused by the restoration of the expression of Cx43 genes in M5076 ovarian sarcoma-bearing mice (21). Zhang *et al* (22) reported that Cx43 suppresses the G1-S phase of cell cycle transition by increasing p27 levels. It has recently been reported that a

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novel route by which Cx43 inhibits cell proliferation is the suppressed expression of S phase kinase-associated protein 2 (skp2), the human F-box protein that regulates p27 ubiquitination via a GJIC-independent pathway (13,22,23). The present study was therefore designed to confirm whether BBI induces increases in p27 accumulation without skp2 degradation due to the expression of Cx43 as a GJIC-dependent pathway.

Materials and methods

Chemicals and antibodies. Purified BBI (>99% pure) was obtained from Dr K. Takamatsu (Fujioil Co., Osaka, Japan). Primary antibodies to mouse Cx43 (Chemicon International Inc., Temecula, CA, USA), mouse p21 (Upstate Cell Signaling Technology, NY, USA), mouse p27 (BD Sciences, Tokyo, Japan), mouse skp2 (Zymed Laboratories Inc., CA, USA), and mouse ß-actin (Sigma-Aldrich Co., St. Louis, MO, USA), and all the respective secondary anti-mouse IgG-conjugated antibodies (Beckman Coulter Co., Cedex, France), were purchased from the companies indicated. M5076 ovarian sarcoma cells were kindly provided by Dr T. Tashiro (Japanese Foundation for Cancer Research, Tokyo, Japan).

Animals. Male C57BL/6 and BDF_1 (F_1 from C57BL/6 female and DBA/2 male) mice, 5 weeks of age and 20-25 g in weight, were obtained from Clea Japan Inc. (Tokyo, Japan) and housed in a room maintained at $22\pm1°C$ and 55.5% relative humidity. The mice were kept in groups of four per cage and given free access to regular chow pellets (CE-2, Clea Japan Inc.) and fresh water. They were acclimatized for 1 week prior to use, and kept throughout under standard conditions.

In vivo tumor experiment. C57BL/6 mice were used for the animal passage of M5076 cells, and BDF_1 mice were used for *in vivo* experiments. For animal passage, M5076 ovarian sarcomas ($1x10^6$ cells/animal) were transplanted intraperitoneally (i.p.) into C57BL/6 mice. Ascitic cells were collected on the 14th day after transplantation (24).

M5076 ovarian sarcomas ($1x10^5$ cells/animal) were subcutaneously transplanted onto the backs of BDF_1 mice. After 15 days, the mice were randomly assigned to the control (injection of sterile saline), low (10 mg BBI/kg), middle (20 mg BBI/kg), or high (40 mg BBI/kg) dosage groups. Each treatment group comprised 7 animals. Purified BBI was intraperitoneally injected on day 15, 18, 20, 22, 25, 27, 29, 32 and 34 after transplantation. The mice were sacrificed on the 35th day after inoculation by cervical dislocation, and the solid tumors were immediately removed and weighed.

Real-time polymerase chain reaction analysis. Total RNA was extracted with a lysis reagent (Qiazol, Qiagen Inc., Valencia, CA, USA) according to the manufacturer's instructions, and the RNA extract was stored at -80°C until use. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The following primers (each at a final concentration of 1 pM) were used: for Cx43 (Accession no. NM 010288), 5'-CTT GAA CAT CAA GCT GCC AATC-3' and 5'-TCC ACG GGA ACG AAA TGA AC-3'; for GAPDH (Accession no. BC 083149), 5'-TGC ACC ACC AAC TGC TTA GC-3'
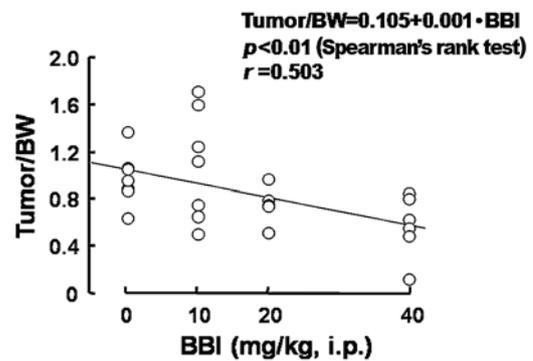
and 5'-TGG CAG TGA TGG CAT GGA-3'. All primer pairs were designed to encompass at least one intron of the genomic sequence to allow for the discrimination of any sequences amplified from contaminating genomic DNA. Additionally, all were synthesized by Operon Biotechnologies, Inc. (Huntsville, AL, USA) and were of HPSF quality.

Aliquots of the cDNAs were incubated with Takara Premix Taq (Takara Bio Inc., Tokyo, Japan). The polymerase was activated (1 min at 95°C) and then up to 40 cycles (5 sec at 95°C, 1 min at 60°C) were performed on an iCycler (Bio-Rad Laboratories Inc., Hercules, CA, USA). Real-time PCR was conducted with an Applied Biosystem TaqMan 7500 (Applera GmbH, Germany) in 96-well plates using SYBR®-Premix ExTaq (Applera GmbH) in 25 μl reaction mixtures. Results were analyzed by absolute quantification (standard curve) using the ABI PRISM 7500 Sequence Detection System V1.2.3 (Applera GmbH).

Western blot analysis. Tumor tissues were minced and homogenized with a homogenizer in ice-cold lysis buffer. Supernatants were collected and used to examine the expression of different proteins by Western blotting. Protein concentrations were determined by the Bradford method (Biowave S2100, Funakoshi Co., Tokyo, Japan).

Total protein extract (20 μg) from the tumors of mice treated with various doses of BBI was loaded onto 10% SDS-polyacrylamide gel for analysis of the expression of Cx43, p21, p27 and skp2. ß-actin was used as an internal standard. After electrophoresis, the proteins were transferred onto nitrocellulose membranes. Protein expression levels were detected using enhanced chemiluminescence detection systems (ChemDoc, Bio-Rad Laboratories, Tokyo, Japan). The relative density of each protein band on a blot was measured using computer software Quantity One Version 4.5 (Bio-Rad). Molecular sizing was conducted using the Rainbow MW marker (Amersham Health, Buckinghamshire, UK).

Statistical analysis. Data were expressed as the means ± standard deviation. The relationship between the doses of BBI and each parameter was evaluated by Spearman's rank test. To test changes in relation to BBI, data for each group were analyzed by repeated-measures ANOVA followed by the Bonferroni test. The level of significance was determined to be $p<0.05$.

Figure 1. Relationship between Bowman-Birk inhibitor (BBI) dosage and tumor/body weight (BW) ratio in M5076-bearing mice.

Table I. Relationship between Bowman-Birk inhibitor dosage and study parameters.

	Control	Bowman-Birk inhibitor		
		10 mg/kg	20 mg/kg	40 mg/kg
Cx43 mRNA (density/GAPDH)	0.509±0.062	0.572±0.152	0.675±0.127	0.970±0.160[a]
Cx43 (density/ß-actin)	0.667±0.076	0.990±0.238[a]	1.091±0.209[a]	1.259±0.214[a]
p21 (density/ß-actin)	0.675±0.089	0.925±0.247	1.194±0.247[a]	1.212±0.289[a]
p27 (density/ß-actin)	0.717±0.173	0.939±0.223	1.199±0.273[a]	1.168±0.261[a]
skp2 (density/ß-actin)	0.625±0.109	0.874±0.249	1.167±0.290[a]	1.358±0.334[a,b]

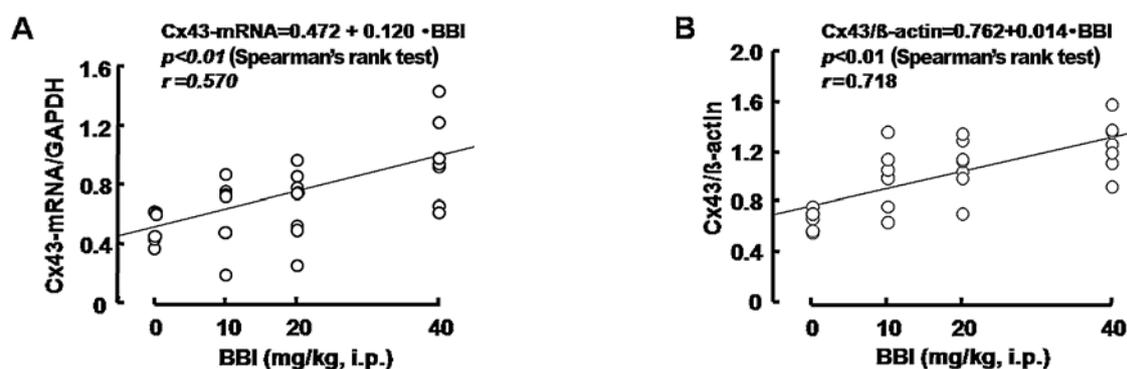[a]$p<0.05$ versus control; [b]$p<0.05$ versus 10 mg/kg (Bonferroni test) ($p<0.0083$).

Figure 2. Relationship between Bowman-Birk inhibitor (BBI) dosage and connexin 43 levels in M5076-bearing mice. (A) Real-time PCR analysis of Cx43 mRNA expression and (B) Western blot analysis of Cx43 density per ß-actin in M5076-bearing mice.

Results

Fig. 1 shows the relationship between BBI dosage and relative tumor weight (tumor/body weight) in M5076 ovarian sarcoma-bearing mice. Relative tumor weight was negatively correlated with the dose of BBI ($p<0.01$, $r=0.503$). High doses of BBI significantly reduced relative tumor weight by 34.0% compared to control levels ($p<0.05$).

The relative densities of Cx43 mRNA per GAPDH mRNA and Cx43, p21, p27 and skp2 per ß-actin are summarized in Table I. Fig. 2 shows the relationship between BBI dosage and the relative density of Cx43 mRNA (Cx43 mRNA/GAPDH mRNA) and Cx43 (Cx43/ß-actin) in M5076 ovarian sarcoma-bearing mice. The relative density of Cx43 mRNA ($p<0.01$, $r=0.570$) and Cx43 ($p<0.01$, $r=0.718$) was positively correlated with the dose of BBI. The relative density of Cx43 mRNA in mice that received 40 mg/kg BBI (0.970±0.128) was significantly higher than in the control mice (0.509±0.076, $p<0.05$). In addition, the relative density of Cx43 in mice that received 10 mg/kg (0.990±0.238), 20 mg/kg (1.091±0.209), and 40 mg/kg BBI (1.259±0.214) was significantly higher than in the control mice (0.667±0.076, $p<0.05$).

Fig. 3 shows the relationship between BBI dosage and the relative densities of proteins, including p21, p27 and skp2, in M5076 ovarian sarcoma-bearing mice. The relative densities of p21 ($p<0.01$, $r=0.633$), p27 ($p<0.01$, $r=0.561$) and skp2 ($p<0.01$, $r=0.733$) were positively correlated with the dose of BBI. At 20 and 40 mg/kg, BBI significantly increased the relative densities of p21, p27 and skp2 compared to control levels ($p<0.05$, respectively).

Discussion

The present study demonstrates that BBI induces increases in p27 accumulation without skp2 degradation through the expression of Cx43 as a GJIC-dependent pathway. This suggests that the anti-carcinogenic effects of BBI induced negative growth control due to an increase in p27 accumulation caused by the expression of Cx43 as a GJIC-dependent pathway.

Epidemiological evidence indicates that diets containing high amounts of soybean products are associated with a lower incidence of cancer and lower mortality rates (25,26). In an effort to explain this phenomenon, epidemiologists have proposed various hypotheses, including differences in diet and environmental exposure to carcinogens (25,26). Dietary comparisons of the Asian with a typical Western diet reveal many differences, among them the fact that Asian populations, in particular those of China and Japan, consume more soybean products than those of Western countries. Consequently, several of the components of soybeans have been isolated and investigated as cancer preventive agents (27). BBI is a soybean-derived serine protease inhibitor with both trypsin and chymotrypsin inhibitory activities (1). This polypeptide has been shown to be a very potent 'pure' chemopreventive agent, and is the only protease inhibitor from soybeans which possesses isolated chymotrypsin inhibition activity (27).
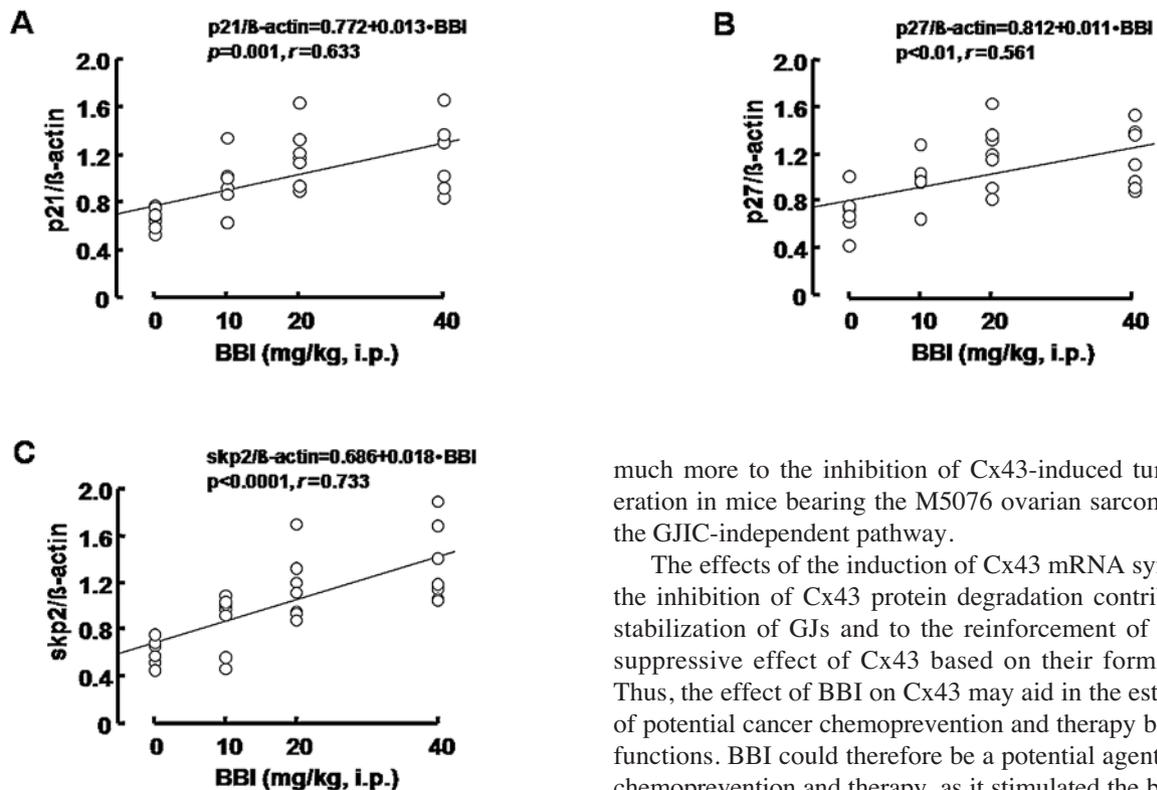
Figure 3. Effects of Bowman-Birk inhibitor (BBI) treatment on p21, p27 and skp2 expression levels per ß-actin in M5076-bearing mice.

This study demonstrates that Cx43 mRNA and Cx43 protein levels are positively correlated with BBI dosage. Zhang *et al* (13,22,23) suggested that Cx genes inhibit tumor cell growth, most probably via two pathways. One is linked to the Cx-mediated GJIC function, in which putative growth inhibitory factors are assumed to diffuse through GJ channels and subsequently to induce growth inhibition, while in the other it is likely that Cx proteins themselves play GJIC-independent biological roles in inhibiting tumor cell growth (23). Enforced Cx43 expression markedly increased p27 levels and inhibited the activities of cyclin-dependent kinases 2 and 4. Thus, we confirmed that the anti-carcinogenic effect of BBI induced negative growth control by means of the accumulation of p27 in mice bearing M5076 ovarian sarcoma. This accumulation was the result of the increased synthesis and reduced degradation of p27 proteins. The N-terminal domain of Cx43, which forms the GJ, is sufficient for the promotion of the synthesis of p27 protein, in which cyclic AMP is involved (23,24). In contrast, the C-terminal domain of Cx43, which does not form a GJ, was sufficient for the inhibition of the expression of skp2. Although the down-regulation of skp2 was responsible for the increase in the levels of p27 protein by the GJIC-independent pathway, we did not observe a down-regulation of skp2 expression in mice bearing M5076 ovarian sarcoma. In this study, BBI induced an increase in skp2 levels, an effect which was strongly dose-dependent. This increase might be an attempt at compensating for the increase in p27, rather than a direct action of BBI. These findings reveal a GJIC-dependent pathway through which Cx43 inhibits tumor growth by causing an increase in the synthesis of p27 protein. We suggest that the GJIC-dependent pathway contributed much more to the inhibition of Cx43-induced tumor proliferation in mice bearing the M5076 ovarian sarcoma than did the GJIC-independent pathway.

The effects of the induction of Cx43 mRNA synthesis and the inhibition of Cx43 protein degradation contribute to the stabilization of GJs and to the reinforcement of the tumor-suppressive effect of Cx43 based on their formation (28). Thus, the effect of BBI on Cx43 may aid in the establishment of potential cancer chemoprevention and therapy based on Cx functions. BBI could therefore be a potential agent for cancer chemoprevention and therapy, as it stimulated the biosynthesis of Cx43 and suppressed the degradation of the Cx43 protein (29,30). Destruction of the Cx43 protein mainly depends on the ubiquitin-proteasome system (29,30), and chymotrypsin-like activity involved in the proteasome is specifically required for its degradation (30,31).

In conclusion, the present study suggests that the anti-carcinogenic effects of BBI are due to the induction of negative growth control by means of an increase in p27 accumulation caused by the restoration of the expression of Cx43 as a GJIC pathway, an effect which was strongly dose-dependent.

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