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Isoflavone-deprived soy peptide suppresses mammary tumorigenesis by inducing apoptosis

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Abbreviations: COX-2, cyclooxygenase 2; DMBA, 7,12-dimethylbenz[α]anthracene; FITC, fluorescein isothiocyanate; HSP90, heat shock protein 90; IKK, IκB kinases; MMP-9, matrix metalloproteinase-9

Abstract

During carcinogenesis, NF-κB mediates processes associated with deregulation of the normal control of proliferation, angiogenesis, and metastasis. Thus, suppression of NF-κB has been linked with chemoprevention of cancer. Accumulating findings reveal that heat shock protein 90 (HSP90) is a molecular chaperone and a component of the IκB kinase (IKK) complex that plays a central role in NF-κB activation. HSP90 also stabilizes key proteins involved in cell cycle control and apoptosis signaling. We have determined whether the exogenous administration of isoflavone-deprived soy peptide prevents 7,12-dimethylbenz[α]anthracene (DMBA)-induced rat mammary tumorigenesis and investigated the mechanism of action. Dietary administration of soy peptide (3.3 g/rat/day) significantly reduced the incidence of ductal carcinomas (50%), the number of tumors per multiple tumor-bearing rats (49%; $P < 0.05$), and extended the latency period of tumor development (8.07 ± 0.92 weeks) compared to control diet animals (10.80 ± 1.30; $P < 0.05$). Our results have further demonstrated that soy peptide (1) dramatically inhibits the expression of HSP90, thereby suppressing signaling pathway leading to NF-κB activation; (2) induces expression of p21, p53, and caspase-3 proteins; and (3) inhibits expression of VEGF. In agreement with our *in vivo* data, soy peptide treatment inhibited the growth of human breast MCF-7 tumor cells in a dose-dependent manner and induced apoptosis. Taken together, our *in vivo* and *in vitro* results suggest chemopreventive and tumor suppressive functions of isoflavone-deprived soy peptide by inducing growth arrest and apoptosis.

Keywords: apoptosis; breast neoplasms; chemoprevention; HSP90 heat-shock proteins; isoflavones; NF-κB; soybean proteins

Introduction

Epidemiologic studies suggest that dietary factors play an important role in the etiology of different types of cancer. Accumulating evidence suggests that high consumption of soybean and soybean-related products contributes to a reduced risk of breast cancer (Messina and Barnes, 1991; Messina *et al*., 1994). However, most studies with soy products have focused on an isoflavone (genistein). Genistein has been shown to inhibit protein tyrosine kinase and topoisomerase II activities, as well as inhibit the activation of NF-κB and Akt signaling pathways, which are important for maintaining homeostasis between cell survival and death (Banerjee *et al*., 2008). In addition, genistein has been reported to have an antioxidant property and to be a potent inhibitor of angiogenesis and metastasis (Sarkar and Li, 2002, 2003). Although the chemopreventive effects and mechanism of genistein activity have been thoroughly studied, the effects of defined soy peptide on mammary tumorigenesis have not been examined and require further investigation. Previous results have suggested anticancer activity of hydrophobic peptides extracted from soy proteins (Kim *et al*., 2000), but

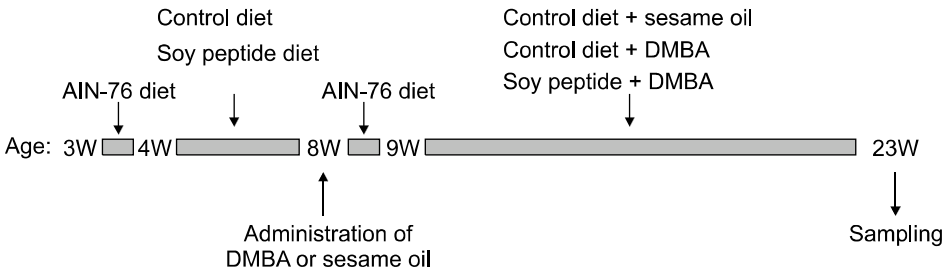

Figure 1. Experimental design of rat mammary tumorigenesis induced by DMBA. Three groups of female Sprague Dawley rats at 4 weeks of age were fed either a control diet or a soy peptide diet for 4 weeks before DMBA administration. Each diet was continued from week 9 until the end of the experiment. Each group was composed of 12 rats. Control diet + sesame-oil administration; Control diet + DMBA administration; Soy peptide diet + DMBA administration.

the tumor preventive function remains to be explored.

Transcription factor NF-κB binds to consensus elements within the promoter regions of a variety of target genes, including immunoreceptors, *c-myc*, p53, cell adhesion molecules, and enzymes involved in tumor metastasis. Under normal conditions, NF-κB is retained in the cytoplasm of cells, where it is bound by IκBs (Ghosh and Karin, 2002). During carcinogenesis, NF-κB mediates several of the events associated with multistep processes, including promotion of cell survival and deregulation of normal control of proliferation, metastasis, and angiogenesis (Karin, 2006). Previous reports have demonstrated that many chemopreventive reagents downregulate NF-κB expression *in vitro* and *in vivo* (Bharti and Aggarwal, 2002; Surh, 2003). Among others, heat shock proteins (HSPs) are molecular chaperones and have been reported to regulate apoptosis and cell death (Whitesell and Lindquist, 2005). HSPs regulate the apoptotic machinery through chaperone function by affecting protein assembly and folding, the ubiquitin degradation pathway, and protein translocation (Takayama *et al*., 2003). During NF-κB signaling, HSP90 forms a complex with Cdc37, plays an important role in TNF-dependent translocation, and activation of the IκB kinases (IKK; Chen *et al*., 2002). Furthermore, HSP90 activity is also important for IKK biosynthesis and for constitutive and inducible IKK and NF-κB activation (Broemer *et al*., 2004).

In this study, we have investigated the role of isoflavone-deprived soy peptide in breast cancer carcinogenesis and prevention using a well-established mouse model and a human breast cancer cell culture system. Our data showed that isoflavone-deprived soy peptide is capable of inhibiting breast carcinogenesis through downregulation of HSP90 expression, thereby suppressing the NF-κB signaling pathway *in vitro* and *in vivo*. This is the first report to suggest that isoflavone-deprived soy peptide can prevent breast tumorigenesis.

Results

Soy peptide suppressed DMBA-induced rat mammary tumorigenesis

To examine whether isoflavone-deprived soy peptide has any chemopreventive effect, soy peptide was administered beginning at 4 weeks of age before carcinogen administration and continued until the termination of the experiment (Figure 1). No adverse effect on body weight was detected during the experimental period (Table 1). There was no evidence of spontaneous tumor development in the animals in the control diet + sesame oil group during the entire period of the study (Table 1). Administration of soy peptide did not reveal any

Table 1. Effects of soy peptide diet on DMBA-induced mammary tumorigenesis.

	Body weight at sacrifice (g)	Mean diet intake (g/day)	Tumor weight (g)	Latent period (weeks)
Control diet + Sesame oil (n = 12)	282.50 ± 9.08^{ns}	12.15 ± 3.14^{ns}	-	-
Control diet + DMBA (n = 12)	275.25 ± 12.30	11.95 ± 2.86	9.96 ± 2.49	8.07 ± 0.92^{ns}
Soy peptide diet + DMBA (n = 12)	259.24 ± 13.46	12.34 ± 4.29	3.38 ± 0.72*	10.80 ± 1.30

*Values are significantly different at $P < 0.05$ using independent t-test. ^{ns} not significant.

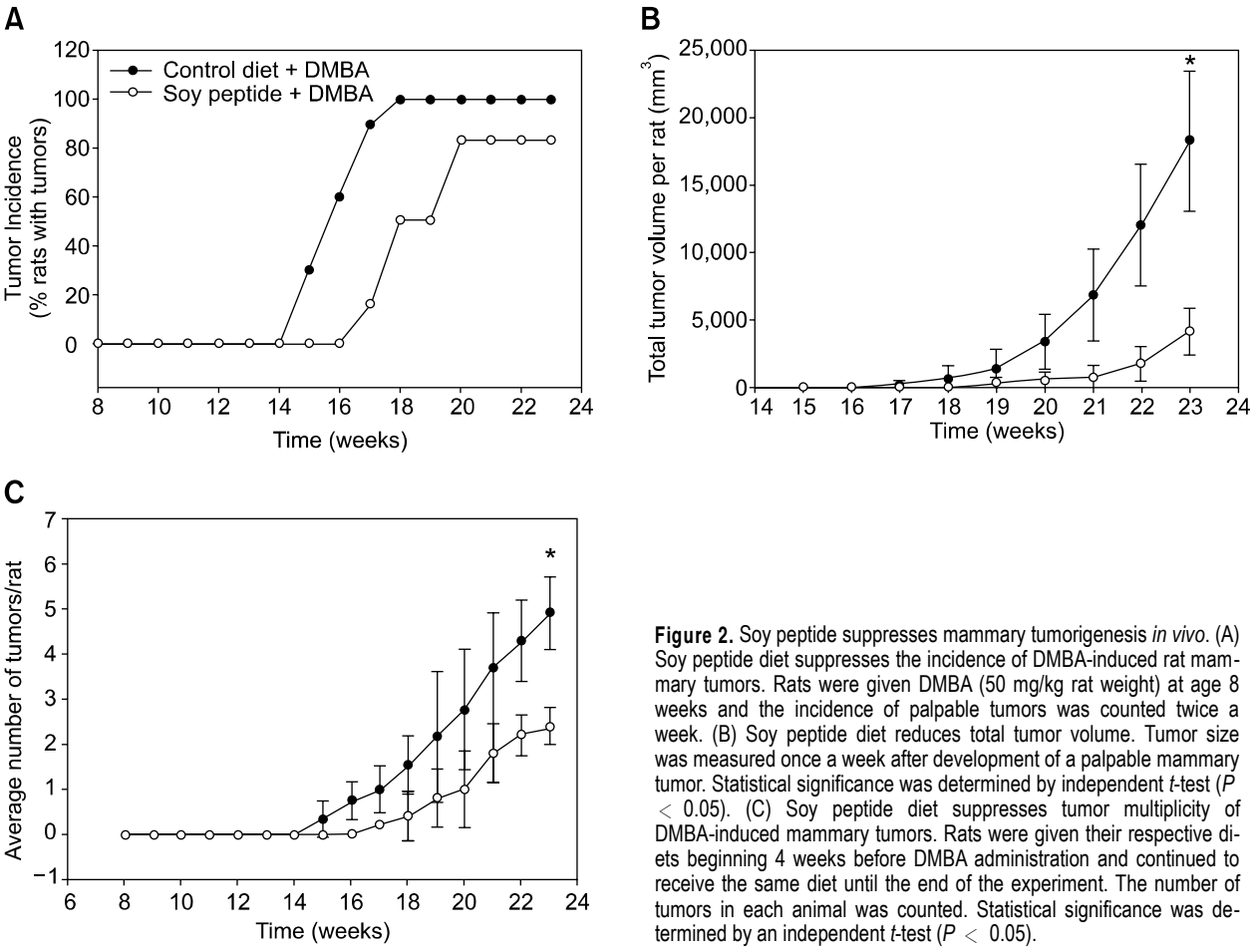

Figure 2. Soy peptide suppresses mammary tumorigenesis *in vivo*. (A) Soy peptide diet suppresses the incidence of DMBA-induced rat mammary tumors. Rats were given DMBA (50 mg/kg rat weight) at age 8 weeks and the incidence of palpable tumors was counted twice a week. (B) Soy peptide diet reduces total tumor volume. Tumor size was measured once a week after development of a palpable mammary tumor. Statistical significance was determined by independent *t*-test ($P < 0.05$). (C) Soy peptide diet suppresses tumor multiplicity of DMBA-induced mammary tumors. Rats were given their respective diets beginning 4 weeks before DMBA administration and continued to receive the same diet until the end of the experiment. The number of tumors in each animal was counted. Statistical significance was determined by an independent *t*-test ($P < 0.05$).

gross changes in the livers, lungs, kidneys, or gastrointestinal tracts of the animals (data not shown). In the control diet + DMBA group, all of the rats treated with DMBA developed palpable breast tumors within 8.07 ± 0.92 weeks after DMBA treatment (Table 1 and Figure 2A), and the average volume and number of tumors per tumor-bearing animal were 18,297.39 ± 5,231.24 mm^3 and 4.90 ± 0.81, respectively (Figure 2B and C). In contrast, the dietary administration of soy peptide reduced the incidence of palpable tumors by 17% (Table 2 and Figure 2A) and the average volume and number of tumors per tumor-bearing animal were 4,122.64 ± 1,719.07 mm^3 and 2.40 ± 0.39, respectively ($P < 0.05$, Figure 2B and C). Furthermore, a significant difference in tumor weight was observed. In the control diet + DMBA group, the tumor weight was 9.96 ± 2.49 g compared to 3.38 ± 0.72 g for the soy peptide + DMBA group (Table 1). In addition, histopathologic analysis of tumor samples revealed that tumors obtained from the control diet + DMBA group had 100% ductal carcinomas, whereas tumors excised from the soy peptide + DMBA group had ductal carcinomas (50%), papillomas (16%), or fibroadenomas (16%; Table 2). The dietary administration of soy peptide significantly reduced the incidence of ductal carcinomas (50%). In contrast, normal mammary epithelium from the control diet + sesame oil group showed no pathologic abnormalities (Table 2).

Table 2. Summary of histopathologic examination of soy peptide diet on DMBA-induced mammary tumorigenesis.

	Group	
	Control diet + DMBA (*n* = 12)	Soy peptide diet + DMBA (*n* = 12)
Total number of first palpable tumors examined	12 (100%)	10 (83%)
Number of ductal carcinomas (%)	12 (100%)	6 (50%)
Number of papillomas (%)	0 (0%)	2 (16%)
Number of fibroadenomas (%)	0 (0%)	2 (16%)

Soy peptide suppressed expression of HSP90 and NF-κB proteins *in vivo*

As an initial step to identify the target genes involved in the tumor preventive effect of soy peptide, we performed rat cDNA microarray analysis. Our cDNA microarray profiling analysis revealed a dramatic suppression of HSP90, cyclin dependent kinase 4 (cdk4), VEGF thioredoxin reductase 2, and glutathione S-transferase theta 2 in tissues of rats fed with the soy peptide diet. In contrast, the genes for protein tyrosine phosphatase epsilon polypeptide, p450 (cytochrome) oxidoreductase, glycosylation-dependent cell adhesion molecule 1, and solute carrier family 10 (member 1) were downregulated (Table 3). Among the differentially expressed genes, HSP90 was chosen for further study because its expression was decreased $>$ 10-fold in the soy peptide-treated group and known to be a key gene involved in the signaling pathway involved in tumorigenesis (Maloney and Workman, 2002). Immunohistochemistry demonstrated that HSP90 was expressed at a basal level in the soy peptide + DMBA group, but at a much higher level in the control diet + DMBA group (Figure 3A, top panel). Since HSP90 is known to regulate the NF-κB signaling pathway, we also examined the expression of NF-κB protein (p65) by immunohistochemistry. The soy peptide diet significantly reduced the level of NF-κB compared to the strong cytoplasmic and nuclear NF-κB expression in tumor tissues of the control diet (Figure 3A, bottom panel). To further confirm the effect of soy peptide on DMBA-induced NF-κB and HSP90 expression, we examined protein expression using immunoblot analysis. Consistent with our immunohistochemical analysis data, the control diet + DMBA group expressed high levels of NF-κB (p65) and HSP90 protein, whereas all animals from the soy peptide + DMBA group showed a very low expression of NF-κB and HSP90 proteins (Figure 3B). We also examined the expression of key proteins involved in growth arrest, apoptosis, and angiogenesis. The expression of p53, p21, and cleaved caspase-3 proteins was dramatically increased, whereas the expression of VEGF was suppressed in the soy peptide + DMBA group (Figure 3B). Consistent with an induction of cleaved caspase-3 protein, we observed a 2-fold increase of caspase-3 activity in the soy peptide + DMBA group (Figure 3C).

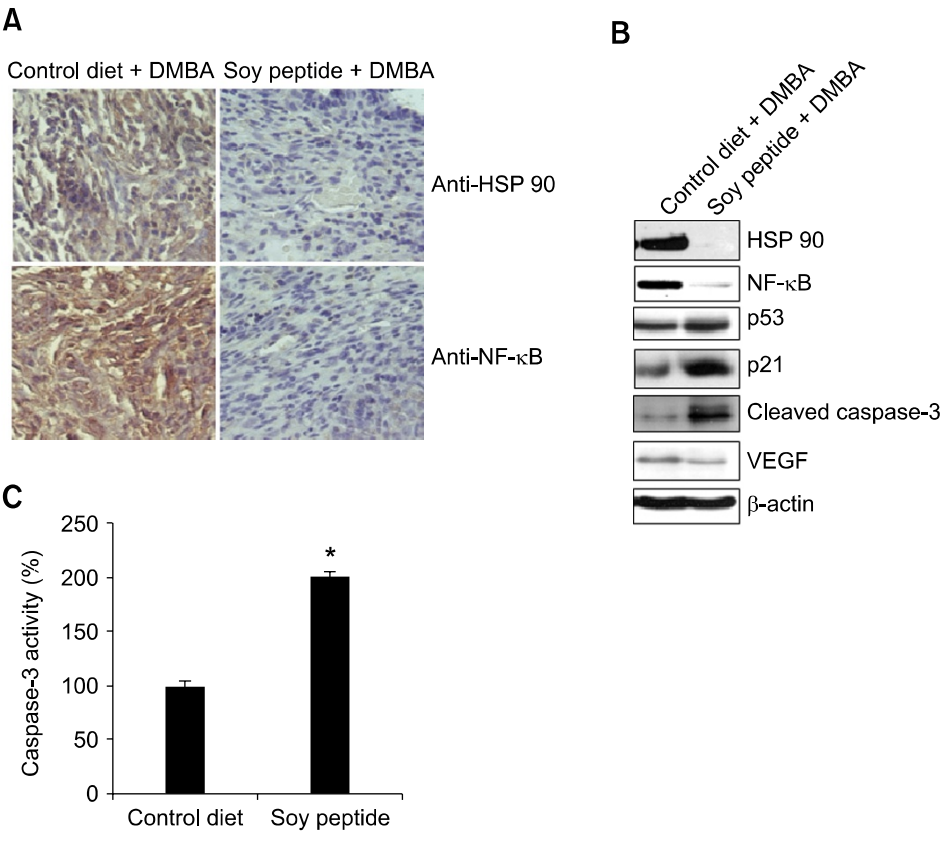

Figure 3. Soy peptide suppresses expression of NF-κB and HSP90 proteins *in vivo*. (A) Representative immunohistochemical staining of breast tumor tissue from control diet + DMBA and soy peptide + DMBA group. Immunohistochemical staining for HSP90 and NF-κB (p65) protein demonstrated strong positive staining in ductal carcinoma from control diet + DMBA group (left panel), whereas very weak staining of each protein in the soy peptide+ DMBA group was observed (right panel). Original magnification, 400×. (B) The effect of soy peptide on the apoptosis associated protein expression. Whole tissue extract prepared from mammary tissue derived from animals of each group was analyzed by Western blot analysis with an indicated antibody as described in the Methods. β-actin was used as a loading control. (C) Soy peptide + DMBA group increases the activity of caspase-3. The activity of caspase-3 was assayed using DEVD substrate with whole tissue lysates prepared from the control diet + DMBA or soy peptide + DMBA tumors.

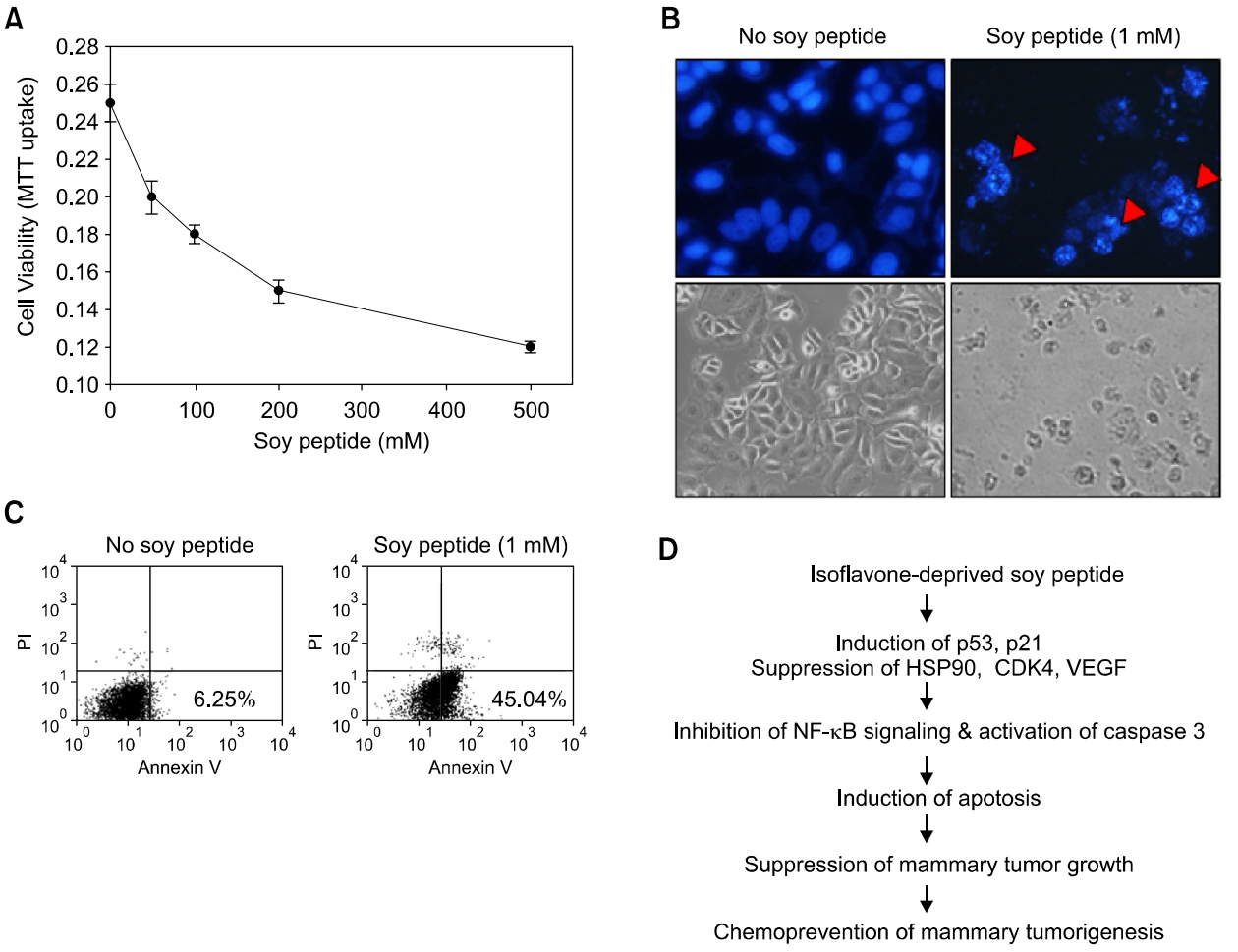

Figure 4. Suppression of NF-κB and HSP90 by soy peptide induces apoptosis in human breast MCF-7 cells. (A) MCF-7 cells were plated onto 96-well plate at 5×10^3 cells and incubated at 37°C. After overnight incubation, cells were grown in either fresh control medium or fresh medium containing indicated concentration of soy peptide for 72 h. Cell growth was determined by the mean absorbance. Each experiment was done in triplicate. (B) Soy peptide induces nuclear fragmentation. MCF-7 cells plated in 4-chamber slide were treated with 1 mM soy peptide for 24 h and then fixed in ethanol followed by staining with DAPI. Cell morphology was observed by light microscopy, and nuclei were examined by fluorescence microscopy. (C) Induction of apoptotic cell death by soy peptide. MCF-7 cells treated with medium or 1 mM soy peptide for 24 h were incubated with FITC-labeled Annexin V and PI and then analyzed by FACS. (D) Summary for suppression of mammary tumorigenesis by isoflavone-deprived soy peptide.

Soy peptide-induced apoptosis in human breast cancer cells

To investigate whether soy peptide suppressed the growth of human breast cancer cells, MCF-7 cells were incubated for 72 h in the presence of different concentrations of soy peptide and cell growth was assessed by MTT assay. Soy peptide inhibited the growth of MCF-7 cells in a dose-dependent manner, with almost 50% suppression of cell viability at 500 μM concentration (Figure 4A). Treatment of soy peptide at 1 mM for 24 h induced the prominent nuclei fragmentation, as evidenced by DAPI nuclear staining, as well as morphologic changes of cells (Figure 4B). Induction of apoptosis was further confirmed by Annexin V- PI double staining (Figure 4C). We observed a $>$ 7-fold increase of apoptosis in soy peptide-treated cells.

Discussion

Soy peptide is the most abundant component in soy product, but has not been thoroughly studied compared to isoflavones. In our study, we initiated investigations into whether isoflavone-deprived soy peptides have any effect on the chemoprevention and suppression of breast cancer development. Our findings demonstrated the chemopreventive and tumor suppressive effects of isoflavone-deprived soy peptide in a well-established animal

model of breast cancer. Since DMBA-induced rat mammary gland carcinomas are reported to be similar to human breast cancers in several aspects, including histopathology, the origin of the cancers from ductal epithelial cells, and the dependency on ovarian hormones for tumor development (Russo *et al*., 1990), it is anticipated to have comparable effects of soy peptide on the suppression of human breast cancer development. Using various molecular and cellular analysis methods, we have demonstrated that the dietary administration of isoflavone-deprived soy peptide significantly reduced the volume, number, and weight of ductal carcinomas per multiple tumor-bearing rats, and extended

Table 3. Gene expression profile of DMBA-induced rat mammary tumors fed with soy peptide diet.

Upregulated genes (> 2-fold)	Downregulated genes (> 2-fold)
1. Biological process	1. Biological process
Signal tranduction	*Cell communication*
Protein tyrosine phosphatase, receptor type, epsilon polypeptide	Cell division cycle 2 homolog A (*S. pombe*) (Cdc2)
2. Cellular component	Heat shock protein 86 (Hsp90α)
Intracellular	Insulin-like growth factor binding protein 3 (IGFBP3)
p450 (cytochrome) oxidoreductase	Cadherin EGF LAG seven-pass G-type receptor 3
Contactin associated protein 1	Importin 13
Cytosolic cysteine dioxygenase 1	*Signal tranduction*
3. Molecular function	S100 calcium-binding protein A9 (calgranulin B)
Cancer	Serine/threonine kinase 10
v-maf musculoaponeurotic fibrosarcoma oncogene homolog	Farnesyl diphosphate farnesyl transferase 1
Enzyme	2. Cellular component
CDP-diacylglycerol synthase 1	*Extracellular*
5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP	Matrix metalloproteinase 23
Carnitine palmitoyltransferase 2	Fibrinogen, gamma polypeptide
Catechol-O-methyltransferase	Serine (or cysteine) proteinase inhibitor, clade A, member 1
Isocitrate dehydrogenase 3, gamma	Calpain, small subunit 1
Other groups	*Intracellular*
Glycosylation dependent cell adhesion molecule 1 (GLYCAM1)	Cytochrome P450, subfamily 4B, polypeptide 1
Ubiquitin conjugating enzyme E2I	Myosin heavy chain 11
Transport	Nuclear pore glycoprotein 62
Solute carrier family 38, member 2	3. Molecular function
Solute carrier family 10, member 1	*Cancer*
Na^+/Pi-cotransporter type IIc	MAS1 oncogene
	Cell cycle regulator
	Cyclin-dependent kinase 4 (CDK4)
	Enzyme
	Cysteine-sulfinate decarboxylase
	Thioredoxin reductase 2
	Glutathione S-transferase, theta 2
	NAD^+-specific isocitrate dehydrogenase b subunit
	Dihydrofolate reductase
	Serine/threonine kinase 10
	Kinesin light chain 1
	Heterogeneous nuclear ribonucleoprotein H1
	Transcription factors
	General transcription factor IIa, 2 (12 kD subunit)
	Transcription elongation factor B (SIII), polypeptide 3
	Myc box dependent interacting protein 1 (Bin 1)
	Max dimerization protein 3 (Mad 3)
	Signal transducer
	Vascular endothelial growth factor (VEGF)
	Cathepsin E
	Transport
	Solute carrier family 7, member 7
	Solute carrier family 16, member 1
	Solute carrier family 2, member 1

the latency period of tumor development compared to the control diet + DMBA group and exerted its tumor suppressive effect by inducing apoptosis *in vivo*. Our data suggest that inhibition of NF-κB signaling by downregulation of HSP90 expression might be one of the mechanisms that contribute to the chemopreventive and tumor suppressive effects of isoflavone-deprived soy peptide. This is the first report to show the tumor suppressive effect of isoflavone-deprived soy peptide *in vivo*.

It has been demonstrated that the natural dietary intake of many phytochemicals can block or suppress multistage carcinogenesis and also confer cancer chemoprevention. For example, resveratrol suppresses DMBA-induced rat mammary carcinogenesis by inhibiting NF-κB activation and expression of COX-2 and MMP-9 proteins (Banerjee *et al*., 2002). Furthermore, accumulating evidence suggests the involvement of common molecular targets for various chemopreventive phytochemicals (Surh, 2003). Numerous intracellular signal transduction pathways converge with the activation of the transcription factors. Among target genes, NF-κB is a ubiquitous eukaryotic transcription factor that mediates the expression of genes involved in tumor promotion, angiogenesis, and metastasis. Activation of NF-κB blocks apoptosis and promotes cell proliferation, as well as increases cellular resistance to chemotherapeutic drugs and radiation treatment (Beg and Baltimore, 1996; Wang *et al*., 1998). Thus, NF-κB is a prime target of diverse classes of chemopreventive phytochemicals (Garg and Aggarwal, 2002). Consistent with this observation, our findings demonstrated that the administration of a soy peptide diet suppresses expression of NF-κB protein (p65), as well as induces both the activation of caspase-3 and the suppression of VEGF protein expression *in vivo* (Figure 3B). Taken together, isoflavone-deprived soy peptide confers its tumor suppressive effect by targeting the NF-κB pathway.

To further explore the mechanism of chemopreventive and tumor suppressive effect of soy peptide, we searched for its target molecules and found HSP90 as one of the upregulated genes. In addition, our cDNA microarray revealed a dramatic suppression of cyclin-dependent kinase 4 (cdk4) and VEGF mRNAs in tissues of rats fed with a soy peptide diet (Table 3). Taken together, soy peptide may exert its chemopreventive and tumor suppressive effect by inhibition of NF-κB-mediated signaling.

HSP90 is a molecular chaperone required for the stability and function of a number of signaling proteins involved in promoting cancer cell growth or survival or both. Important proteins, such as Akt, Raf, Cdk4, Her2, and HIF-1α, which are frequently involved in overlapping pathways of mediating cancer cell survival, were identified as HSP clients (Maloney and Workman, 2002; Neckers and Ivy 2003; Takayama *et al*., 2003). Also, HSP90 members are required for inducible and constitutive activity of the IKK complex and of NF-κB (Broemer *et al*., 2004). Our cDNA microarray analysis also suggests the potential involvement of soy peptide in other important processes, such as tumor invasion and angiogenesis, by controlling key gene expression and activity. Taken together, soy peptide can inhibit many important biological processes involved in apoptosis, cell cycle progression, angiogenesis, and tumor invasion, resulting in its chemopreventive and tumor suppressive effect on mammary tumorigenesis *in vivo*.

In summary, one of the major findings of this study was that soy peptide deprived of isoflavone can exert its chemopreventive and tumor suppressive effects by inhibiting the NF-κB signaling pathway implicated in many aspects of tumorigenesis. Suppression of the major angiogenic factor, VEGF, by soy peptide also suggests its potential anti-angiogenic effect mediated via suppression of NF-κB in breast tumorigenesis. Studies that explore the effects of soy peptide on other processes involved in angiogenesis and metastasis, as well as drug resistance, will provide further insight into understanding the mechanism of isoflavone-deprived soy peptide in prevention and/or suppression of breast cancer, along with minimal side effects.

Methods

Animals

Female Sprague-Dawley rats were purchased from Daehan Biolink Co., LTD (Chungbuk, Korea). At 3 weeks of age, the rats were fed AIN-76 (American Institute of Nutrition 76) growth diet for 1 week. The animals were housed in a room maintained at a constant temperature (22 ± 2°C) and humidity (55 ± 5%) under 12 h of light and 12 h of darkness per day. At 4 weeks of age, the rats were assigned to one of three groups: 1) the control diet + sesame oil group (n = 12) received a control diet and served as a negative control; 2) the control diet + DMBA group (n = 12) was designated as a positive control and received a control diet; and 3) the soy peptide + DMBA group (n = 12) received an experimental diet containing soy peptide (*vide infra*). Diets were provided in a powdered form and tap water was provided *ad libitum* for all three groups.

Diets and soy peptide powder preparation

The AIN-76 diet was a modified American Institute of

Nutrition 76 diet composed of 18% protein, 10% fat, 15% cornstarch, 5% cellulose, 3.5% AIN-76 mineral mixture, 1% AIN-vitamin mixture, 0.2% choline bitartrate, and 47.3% sucrose. Control diet provided protein from casein and was isocaloric and isoprotein. The soy peptide diet also provided ~18% protein from soy peptide. The isoflavone-deprived soy peptide was prepared as follows: defatted soybeans were dispersed in distilled water and heated for 15 min at 121°C, treated with endopeptidase at pH 8.0 and 60°C for 2 h, exopeptidase at pH 5.0 and 55°C for 4 h, then hydrolyzed with amylase and exopeptidase for 12 h. The hydrolysates were ultrafiltered, followed by concentration using spray-drying at 55°C. The solvent was substituted with water and the extracts were lyophilized. Soy peptide consists of 4.91% water, 44.44% crude protein, 5.34% crude fat, 30.57% carbohydrate, 14.74% ash, and 0.11% fiber, as previously reported (Kim *et al*., 2000; Shin *et al*., 2001). To purify the soy peptide, XAD-2 adsorption chromatography, Sephadex G-25 gel chromatography, and reverse phase HPLC were utilized. The ethanol extracts of thermoase hydrolyzate soy proteins were separated by Sephadex G-25 chromatography. This procedure separates the isoflavone fraction from the soy peptide fraction completely. The soy peptide fraction was further fractionated by several reverse phase HPLCs, and then the sixth fraction was collected and the peptide sequence was performed using a Precise Protein Sequencing System (Applied Biosystems). The primary sequence of the peptide was identified as X-Met-Leu-Pro-Ser-Tyr-Ser-Pro-Tyr.

Mammary tumorigenesis model

At 8 weeks of age, the rats in the control diet + DMBA and soy peptide + DMBA groups were given a single dose (50 mg/kg body weight) of DMBA (Sigma Chemical Co., St. Louis, MO) solubilized in sesame oil by oral gavage to induce mammary tumors, whereas the rats in the control diet + sesame oil group were given sesame oil alone. After DMBA administration, the rats were fed with the AIN-76 diet for 1 week. At 9 weeks of age, the rats were assigned to the control diet + sesame oil, control diet + DMBA, and soy peptide + DMBA groups, as indicated in Figure 1. The body weights of the rats were measured weekly, the rats were examined for incidence of mammary tumors by palpation twice per week, and the rats were monitored daily for signs of toxicity. At the completion of the study, the rats were sacrificed by CO_2 asphyxiation. The tumor volume was calculated by the following formula: tumor volume (mm^3) = ($a \times b^2$) / 2, where a = length in mm and b = width in mm. Tissues from normal mammary glands and mammary tumors were weighed and fixed in 10% neutral buffered formalin for 12 h, then embedded in paraffin for immunohistochmical staining or snap-frozen in liquid nitrogen for subsequent RNA isolation and tissue lysate preparation. The incidence of different tumor types from each group is summarized in Table 2. This animal study was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Samsung Biomedical Research Institute (SBRI). SBRI is an Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International) accredited facility and abides by the Institute of Laboratory Animal Resources (ILAR) guide.

Microarray analysis

Total RNA from excised rat mammary tumors was isolated using the TRIzol extraction reagent (Gibco BRL, Rockville, MD), according to the manufacturer's recommendations. The integrity of mRNA was confirmed by electrophoresis in a denaturing 1% agarose gel. The rat cDNA microarray contained 5K cDNA clones selected from the Rat Gene Index of GenomicTree Inc. (Daejun, Korea).

Immunoblot analysis and colorimetric assay for caspase-3 activity

Normal rat mammary glands and mammary tumors (100 mg each) were homogenized in 800 μl of ice-cold homogenization buffer (20 mM HEPES [pH 7.4], 75 mM NaCl, 2.5 mM $MgCl_2$, 0.2 mM EDTA, 0.05% Triton X-100, 20 mM β-glycerophosphate, 1 mM Na_3VO_4, 0.5 mM DTT, 10 mM NaF, and protease inhibitor cocktail (Roche, Mannheim, Germany) as described previously (Lee *et al*., 2002). Equal amounts of total tissue lysates (50 μg) were resolved by SDS-PAGE and subjected to immunoblotting with indicated primary antibodies against NF-κB (p65,1:500), HSP90 (1:1,000), p53 (1:1,000), p21 (1:500), and caspase-3 (1:1,000), followed by incubation with respective HRP-conjugated secondary antibodies for 1 h, and then detected by ECL reagent (Amersham Pharmacia Biotech, Arlington Heights, IL), as described previously (Park *et al*., 2005). Equal protein loading was confirmed by incubation with an anti-β-actin antibody. To determine caspase-3 activity, 40 μg of each tissue lysate was incubated with colorimetric DEVD substrate (Calbiochem) at 30°C for 3 h and then absorbance at 405 nm (OD_{405}) was measured by an Xfluor 4 spectrometry reader (TECAN, Austria).

Immunohistochemistry

Immunoperoxidase staining was performed according to the protocols provided by the manufacturer (Dako LSAB kit; Dako, Carpinteria, CA), as described previously (Nam *et al*., 2001). In brief, the 5 μm thick sections mounted on slides were processed with the indicated primary antibodies (anti-NF-κB [p65], 1:500; Santa Cruz Biotechnology Inc., Santa Cruz, CA) or anti-HSP90 antibody (1:1,000; Stressgen Biotechnologies, Victoria, Canada), followed by incubation with biotinylated secondary antibodies. Bound peroxidase was visualized by the addition of substrate-DAB solution and slides were counterstained with Mayer's hematoxylin (DAKO) to stain the nuclei. Positively stained cells appeared brown, while negative cells were blue.

Cell culture and MTT assay

Human breast adenocarcinoma derived MCF-7 cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD). Cells were grown in RPMI 1640 supplemented with 10% FBS and 1% penicillin and streptomycin in an atmosphere of 5% CO_2 at 37°C. The MTT colorimetric assay was performed according to the

method previously described (Park *et al*., 2001). The growth inhibition was measured by the mean absorbance using a plate reader (Termomax, Molecular Device) at 540 nm. Each experiment was performed in triplicate.

Apoptosis assays

Apoptosis-mediated cell death was examined as described (Lee *et al*., 2008) with minor modifications. In brief, MCF-7 cells were seeded onto chamber slides at a density of 5×10^4 cells per well and then treated with 1 mM soy peptide dissolved in RPMI 1640 medium for 24 h. Cells were incubated with fluorescein isothiocyanate (FITC)-labeled Annexin V and propidium iodide (PI) for 15 min and then analyzed on FACS Vantage (Becton Dickinson, San Jose, CA). For evaluation of nuclear morphology, the cells plated onto a chamber slide, fixed in methanol, and stained with DAPI (1 μg/ml in methanol) for 15 min, washed with 1 × PBS three times, followed by treatment with VectaShield (Vector Laboratories, Burlingame, CA) and examined under a fluorescence microscope.

Statistical analysis

Body weight and diet intake were determined and the statistical significance of differences in the data were evaluated by one way ANOVA. Tumor weight, latent period, tumor volume, and tumor multiplicity were calculated by an independent *t*-test at each time point. A probability value < 0.05 was considered statistically significant.

Acknowledgements

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